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Oospore viability and variation in zoospore and hyphal tip derivatives of the hybrid alder Phytophthoras

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Summary

Oospore viability, oospore germinability, and phenotypic variation among zoospore and hyphal tip derivatives of the standard and natural variant types of the hybrid alder Phytophthoras were investigated. Oospore viability in the standard hybrid, estimated by the tetrazolium bromide method, was low (≈ 31–36%). No germination was observed in more than 4000 oospores, although germination did occur in the *Phytophthora cactorum, Phytophthora citricola* and *Phytophthora cambivora* controls. This is consistent with the known meiotic irregularities in this hybrid. Mean oospore viabilities in the natural variants were significantly different ($p < 0.001$), ranging from approximately 24% in the UK variant to approximately 75% in the Dutch variant. Again, no oospore germination was observed. Zoospore and hyphal tip derivatives of the standard hybrid and of the Swedish and Dutch variants resembled the 'parent' isolate in phenotype. The derivatives of the German and UK variants, however, often differed from the parent type. Those of the UK variant were extremely and continuously variable in colony patterns, growth rates, temperature–growth relationships and fertility levels. Although these results do not support the view that the natural variants arise as genetic breakdown products of the standard hybrid, this possibility cannot yet be excluded. The apparent inviability of the oospores suggests that the mycelium and zoospores are mainly responsible for the survival and spread of the alder Phytophthoras in the field.

1 Introduction

In 1993, a lethal disease of alder (*Alnus* spp.) was observed along rivers and in horticultural shelterbelts in Britain. It was caused by an unusual new *Phytophthora*, superficially resembling *Phytophthora cambivora,* a common pathogen of hardwood trees in Europe (Brasier et al. 1995). Since then, the disease has been shown to occur widely across Europe including the Netherlands, Belgium, Sweden, France, Germany, Austria and Hungary. Field surveys show it to be locally very damaging (e.g. Gibbs 1995; Hartmann 1995; Gibbs et al. 1999; Streito et al. 1999). The new *Phytophthora* represents a threat to both natural and managed alder stands in Europe and to the stability of riparian ecosystems. It may also represent a threat to alders outside Europe.

An initial study of the alder *Phytophthora* showed that it differed from *P. cambivora* in being self-fertile rather than outcrossing, in having a submerged rather than an aerial colony type, and in its markedly different optimum temperature for growth. It also exhibited an unusually high level of zygotic abortion. This combination of properties suggested that it might be a species hybrid involving *P. cambivora* as a parent (Brasier et al. 1995). Another study has shown that the alder *Phytophthora* is a highly aggressive pathogen of alder bark, whereas *P. cambivora* is not (Brasier and Kirk 2000).

The species hybrid hypothesis was recently investigated in detail. It was demonstrated that the alder *Phytophthora* comprises a range of species hybrids (Brasier et al. 1999). A common, 'standard' alder *Phytophthora* type occurs across much of Europe, from

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Scotland and Sweden to Austria and south-east France. This type is near-tetraploid (n = 18–22) and unable to complete meiosis beyond metaphase I. Standard isolates have dimorphic sites in the internal transcribed spacer (ITS) region of their rDNA genes (i.e. they have DNA sequences representative of more than one species), consistent with their being allopolyploids between *P. cambivora* and another *Phytophthora* related to *Phytophthora fragariae*. Other phenotypically and genetically distinct alder Phytophthoras, collectively termed natural 'variants', also occur in parts of Sweden, UK, Germany and the Netherlands. These have unique colony morphologies, gametangial morphologies and temperature–growth relationships and their colonies tend to be unstable. Their chromosome numbers are generally intermediate between diploid and tetraploid, and the lower chromosome number 'Swedish' and 'Dutch' variants are able to complete meiosis. The ITS profiles of the variants are different from those of the standard type, tending to be more like those of *P. cambivora* or of *P. fragariae*. These natural variants might be genetic breakdown products of the standard type, backcross products or products of further hybridization events. As a whole, the alder Phytophthoras appear to be a swarm of recent species hybrids that are still in process of evolution (Brasier et al. 1999).

During routine subculturing, a standard alder *Phytophthora* colony produced a morphologically unique sector with a different gametangial type and ITS profile, i.e. it resembled a natural variant (C.M. Brasier, unpublished). The main objective of the present work was to investigate the hypothesis that the natural variants might arise from the standard hybrid via germinating oospores or via somatic segregation (Brasier et al. 1999). Oospore viability, oospore germinability and the phenotypes of large numbers of zoospore and hyphal tip derivatives of standard alder Phytophthoras were examined. A range of natural variants were included both for comparison with the standard hybrid, and to examine the relationship between oospore viability and chromosome number. *Phytophthora cambivora* was included as a 'parent' species.

2 Materials and methods

2.1 Phytophthora isolates studied

The origins of the key *Phytophthora* isolates examined are given in Table 1. The properties of the standard alder *Phytophthora* and of the Dutch, German, UK and Swedish variants are summarized in Brasier et al. (1999). Isolate P818v is a unique sector that arose in a colony of standard isolate P818 (Table 1). Its properties (not previously published) and those of the standard alder *Phytophthora* are compared in Table 2.

2.2 Media, culture maintenance and growth rate tests

2.2.1 Media

Carrot agar (CA) was prepared as described by Brasier (1967). Soft carrot agar (soft CA) was prepared like CA but with 5 g instead of 15 g agar per litre. Pea broth was prepared as described by Trione (1974). Distilled water agar (DWA) was prepared by adding 15 g agar (Davis Gelatine, Christchurch, NZ) to 1000 ml of distilled water and autoclaved for 15 min at 121°C. For soft distilled water agar (soft DWA), only 10 g agar (Davis Gelatine) was added per 1000 ml of distilled water. For distilled water agar with rifamycin (DWA + Rif), 3 ml of 1% w/v solution of Rifamycin SV sodium salt (Sigma-Aldrich Co. Ltd, Poole, UK) was added in 1 litre of DWA. The P_{10} VPTH selective medium was prepared as described by Tsao and Guy (1977).

Table 1. **Key *Phytophthora* isolates studied**

Phytophthora species	Isolate no.	Location	Sampled by	Sampled from	Year of sampling
Alder Phytophthoras					
Standard form	P668, P766, P767, P670, P671	UK	J. Rose	abl[1]	1994
	P785	UK	J. Rose	abl	1995
	P791	UK	J.N. Gibbs	abl	1995
	P807	UK	M. Lipscombe	abl	1995
	P818	Germany	G. Hartman	abl	1995
	P840, P847, P849	UK	S. Gregory	abl	1996
	P846	France	J.C. Streito	abl	1996
	P884	Austria	T. Cech	abl	1996
	P891	UK	G. MacAskill	abl	1997
	P935, P936, P938, P945, P946, P948, P949, P950, P951, P952, P957, P959, P960	UK	J. Delcan	abl	1997
Cultural variant	P818v	(Laboratory variant)			1995
Dutch variant	P770	Holland	H. Van Kesteren	abl	1994
	P972	Holland	C. van Dyck	abl	
UK variant	P841	UK	S. Gregory	abl	1996
Swedish variant	P875, P876, P887, P888	Sweden	C. Olsen	abl	1997
German variant	P889, P890	Germany	T. Jung	abl	1997
P. cactorum	P923	UK	J. Delcan	strawberry, soil[2]	1997
P. citricola	P930	UK	J. Delcan	strawberry, soil[2]	1997
P. cambivora	BE10	UK	J. Rose	oak soil[3]	1998
	P1011	UK	J. Rose	oak soil[3]	1998

[1] abl: alder bark lesion; [2] soil beneath strawberries; [3] soil beneath oak.

Table 2. **Differences between cultural variant P818v and the standard alder *Phytophthora***

Alder *Phytophthora* type	Colony type[2]	Oogonial type	Antheridial type[3]	Growth temperatures		Chromosome number	Meiosis	ITS nucleotide sequence. Selected polymorphic site no[4]:											
				Optimum	Maximum			1	2	3	4	5	6	7	8	9	10	11	12
Cultural variant P818v	dense white woolly	markedly ornamented	(2 A) + 1 A	25	30	18 – 22	na	C	T	T	A	C	C	T	G	A	C	A	G[5]
Standard[1]	uniform appressed	ornamented	2 A + (1 A)	25	30	18 – 22	incomplete	T	A	C	T	C	T	C	A	T	T	G	A
								C	T	T	A		C	T	G	A	C	A	G

[1] Based on information in BRASIER et al. (1999). Genbank accession no. for standard hybrid type AF 139366.
[2] See Fig. 2.
[3] 2 A + (1 A), mainly large two-celled amphigynous, some single-celled amphigynous antheridia; (2 A) + 1 A, some two-celled amphigynous, predominantly single-celled amphigynous antheridia.
[4] Other ITS sequence sites are identical.
[5] COOKE, BRASIER and DUNCAN, previously unpublished.
na, not assessed.

2.2.2 Culture maintenance

Isolates used in these studies were routinely maintained on CA plates at 20°C in darkness and subcultured at 4 week intervals. Stock cultures of single zoospore and single hyphal tip derivatives were stored briefly on CA at 4°C in darkness before being examined.

2.2.3 Growth rate tests

Growth rate tests were carried out in 9 cm Petri dishes containing 20 ml of CA. A 5 mm diameter inoculum plug from a 5- to 10-day-old culture was placed in the centre of each plate. Plates were incubated in darkness at 20°C. Two colony diameters were measured after 24 and 96 h of incubation (BRASIER et al. 1993). The colony morphologies and oogonial production levels of the cultures were assessed after a further 10 days incubation in darkness at 20°C.

Differences in overall mean growth rates of standard versus variant isolates were analysed by *t*-tests.

2.3 Single hyphal tip and monozoospore cultures

For hyphal tip (ht) production, a small plug taken from a 'parent' colony on CA was placed on a DWA plate for 7 days at 20°C. Hyphal tips were obtained by making a cut 2–3 mm behind the growing hyphal tip with a fine tungsten needle. Several hundred hts per isolate were transferred individually to CA plates, incubated in the dark at 20°C for 2–3 days and stored at 4°C. To produce monozoospore cultures (mz), 'parent' isolates were grown individually in 9 cm diameter Petri dishes containing pea broth for 3 days in the dark at 20°C. Colonies were decanted and thoroughly washed with distilled water. Fresh, unsterile pond water was added to just cover the colonies. The dishes were incubated in the dark at 20°C for 1–2 days (BRASIER et al. 1993) and placed in a refrigerator at 4°C for 20 min to induce zoospore release. A 1 ml zoospore suspension was spread onto a plate of P_{10} VPTH selective medium to reduce the growth of bacteria, and incubated for 1 day at 20°C. Resulting monozoospore colonies were subcultured repeatedly on the selective medium until visually free of bacteria, transferred individually to CA plates and stored at 4°C.

Growth rates of approximately 200 newly derived ht and 200 mz derivatives from each 'parent' isolate were assessed using one CA plate and a 5 mm diameter inoculum plug per derivative. Ten replicate plates of the parent isolate were set up for comparison. The plates were then incubated for a further 10 days in darkness before being assessed for colony morphology and oogonial frequency. Because of the large number of plates involved, each parent alder *Phytophthora* isolate and its derivatives were examined in a separate experiment. The two *P. cambivora* control isolates and their derivatives were examined in a single experiment.

Mean growth rates of ht, mz and parental isolates were compared by Duncan's multiple range test (Table 3). *F*-tests were used to compare growth rate variability (as standard deviations or SDs), between parent isolates, mz derivatives and ht derivatives (Table 4). Colony diameters of hts derived from different sector types of UK variant P841 (Table 5) were compared by ANOVA using a least significance test (LSD).

2.4 Oospore germination and viability

Isolates were inoculated onto 20 ml soft CA in 9 cm Petri dishes, with four replicate plates per isolate. The plates were sealed with Parafilm and incubated in the dark at 20°C for 30 days to allow formation and maturation of oogonia. Concentrated oogonial suspensions were obtained by grinding large (approximately 25 cm^3) culture blocks containing oospores in a homogenizer with 10 ml of sterile distilled water. The suspensions were centrifuged five or six times at 4000 ***g*** for 1 min to remove the agar. The oogonia were then

Table 3. **Characteristics of monozoospore and single hyphal tip derivatives**

Alder *Phytophthora* type or *Phytophthora* species	'Parental' Isolate no.	Colony variability	'Parental' isolate characteristics[1]		Mean growth rate and SD (mm/day)		Colony variability mz and ht derivatives	Oogonial frequency range in mz and ht derivatives	Oogonial morphology of mz and ht derivatives
			Oogonial Frequency range	Growth rate (mm/day) and SD[2]	Monozoo-spore (mz) derivatives[2]	Single hyphal tip (ht) derivatives[2]			
Standard	P938	–	+ + +	1.39 ± 0.05 a	1.47 ± 0.07 b	1.48 ± 0.09 b	–	+ + +	P
Standard	P791	–	+ + +	1.13 ± 0.08 a	1.28 ± 0.08 b	1.10 ± 0.18 a	+	+ + +	P
Standard	P846	–	+ + +	1.31 ± 0.04 a	1.30 ± 0.04 a	1.25 ± 0.15 b	+/+ +	+ + +	P
UK variant	P841	+ + +	+/+ + +	1.09 ± 0.03 a	1.13 ± 0.09 b	1.01 ± 0.16 c	+ + +	–/+ + +	V
German variant	P889	+ +	+/+ + +	1.38 ± 0.07 a	1.11 ± 0.24 b	1.02 ± 0.27 c	+ + +	+/+ + +	P
Swedish variant	P876	+	+ + +	1.26 ± 0.03 ab	1.26 ± 0.07 b	1.29 ± 0.07 a	+	+ + +	P
Dutch variant	P972	+	+ +/+ + +	1.38 ± 0.06 a	1.32 ± 0.09 ab	1.30 ± 0.09 b	–	+ +/+ + +	P
P. cambivora	BE10	–	N/A	1.57 ± 0.09	1.56 ± 0.05	1.54 ± 0.06	–	N/A	N/A
P. cambivora	P1011	–	N/A	1.35 ± 0.06	1.25 ± 0.06	1.26 ± 0.04	–	N/A	N/A

[1] Ten 'mass' subcultures.
[2] a, b, c, different letters within a row indicate means are significantly different.
P, oogonial morphology parental; V, oogonial morphology highly variable.
Colony variability: –, nil; + , low; + + , sporadic; + + + , high.
Oogonial frequency: –, nil; + , rare; + + , occasional; + + + , frequent.
N/A, not applicable.

Table 4. **Statistical comparisons of growth-rate variation in parent isolates, monozoospore derivatives and single hyphal tip derivatives**

Alder *Phytophthora* type or *Phytophthora* species	Isolate no.	Comparative SD		
		p vs. mz	p vs. ht	mz vs. ht
Standard	P938	ns	<	>
Standard	P791	ns	<	>
Standard	P846	ns	<	>
UK variant	P841	<	<	>
German variant	P889	<	<	ns
Swedish variant	P876	<	<	ns
Dutch variant	P972	<	<	ns
P. cambivora control	BE10	<	ns	ns
P. cambivora control	P1011	ns	ns	<

p, growth rate SD of parent; mz, growth rate SD of zoospore derivatives; ht, growth rate SD of hyphal tip derivatives; ns, SDs not significantly different; < , first item has significantly lower SD ($p \leq 0.01$); > , first item has significantly higher SD ($p \leq 0.01$).

resuspended in 0.01 g Novozym 234 (Sigma-Aldrich Co. Ltd) to lyse any hyphal fragments and sporangia in the suspension, incubated for 2 h at 20°C and centrifuged six times at 4000 ***g*** for 1 min; then 0.5 ml aliquots of the oospore suspension were pipetted and spread onto plates of 9 ml soft DWA + Rif (to suppress any bacterial contamination). Ten oospore-spread plates per isolate were sealed with Parafilm and incubated at 20°C in darkness. Oospores were observed for germination periodically under the microscope at × 400 for 1 month (up to 8 months in selected tests).

Viability of oospores was tested using the tetrazolium bromide stain method (SUTHERLAND and COHEN 1983). Aliquots (0.5 ml) of oospore suspension (prepared as above) were mixed with 2 ml of 0.1% tetrazolium bromide (Sigma-Aldrich Co. Ltd) in 1 mM potassium phosphate buffer (pH 6.3) (Sigma-Aldrich Co. Ltd) and incubated at 36°C for 2 days. Oogonia were examined under the microscope at × 100. Only those containing a mature thick-walled oospore that stained pink were considered potentially viable. Those that were pink but with an aborted oospore, or were blue, black or unstained, were considered non-viable (EL-HAMALAWI and ERWIN 1986). Approximately 200 oogonia were scored per isolate (sometimes fewer, see Table 6). The test was repeated six times for each of the alder *Phytophthora* isolates and twice for the control isolates.

Oospore viability was analysed by ANOVA. Within each alder *Phytophthora* isolate, the means of the six individual oospore viability tests were unexpectedly variable, i.e. there was a degree of instability from test to test. To obtain a measure of the instability for each isolate, the expected (theoretical) standard error of the mean for the six tests $[\sqrt{(pq/n)}]$ was compared with the actual standard error observed. The ratio between the two indices was then calculated, the degree of divergence from unity indicating the level of instability.

2.5 Principal component analysis

Since growth rates tests for the derivatives of different isolates were set up separately, the overall variability of the different alder *Phytophthora* types was determined in a comparative analysis using six indices, with the parental data equal to 100. Indices were calculated for the relative mean growth rate and standard deviation of the hyphal

tip derivatives and of the zoospore derivatives, for the mean viability of the oospores, and for the ratio between the replicate and the theoretical standard error of oospore viability (see above). These were compared where appropriate with the parental indices.

3 Results

3.1 Preliminary examination of stored alder Phytophthora isolates

Alder *Phytophthora* isolates received into the Forestry Commission (FC) *Phytophthora* culture collection from 1993 onwards were routinely assigned to the standard type or to the UK, Swedish, Dutch or German variant types on the basis of their colony pattern, optimum temperature and upper temperature limit for growth, gametangial type and, for selected isolates, chromosome number and ITS type (BRASIER et al. 1995, 1999) (see Table 2). On this basis, of 67 isolates received, 58 were classified as standard and nine as natural variants. They were then stored under paraffin oil.

In a preliminary study, all 67 isolates were revived from storage, grown for 10 days on CA at 20°C in darkness and examined for their colony characteristics. Of the 58 isolates originally classified as standard, 30 had degenerated, their properties no longer conforming to the standard type (cf. Fig. 1). The changes included different levels and patterns of aerial mycelial production, unusually ragged, slow growth or unusually fast growth and total loss of ability to produce oogonia. Although of obvious interest because of their altered characteristics, these 30 degenerate cultures were omitted from the ensuing experiments. The remaining 28 cultures, which conformed to the standard type (Fig. 2a–d), were retained for further study, together with nine isolates comprising the Swedish, German and Dutch variants (Fig. 2e–j), and isolate P818v (Fig. 2l), which had all retained their original characteristics. UK variant P841 could not be assessed in this way because it is characteristically variable from one subculture to another.

The growth rates of the 28 standard isolates and nine variant isolates (including P818v) were compared on CA at 20°C (Fig. 1), with three replicate plates per isolate. The means of the two groups, at 13.5 ± 1.6 and 9.8 ± 2.9 mm/day, respectively, were significantly different (F-ratio test: $P < 0.01$; $t_{9,5} = 3.56$).

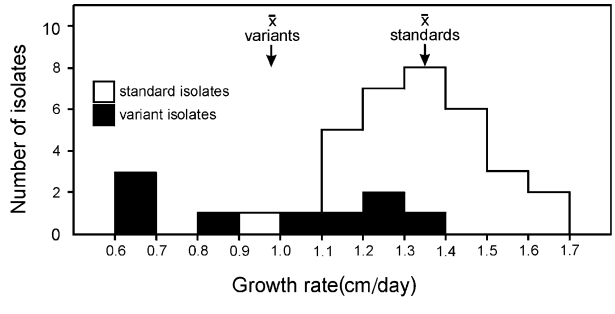

Fig. 1. Growth rate distributions for the standard isolate and variant groups on CA. Arrows represent the group means. □, standard isolates; ■, variant isolates

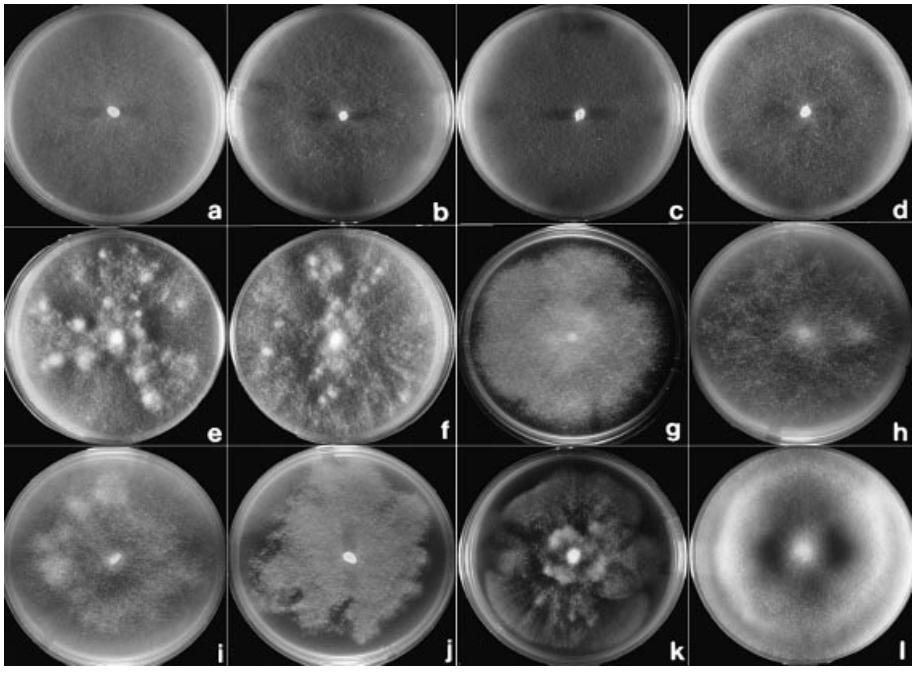

Fig. 2. Colony types of the alder *Phytophthora*s. (a–d), standard isolates from UK, France, Austria and Germany (Isolates P671, P834, P844 and P818, respectively.); (e, f), Swedish natural variant showing chimaeric fertile patches (P876); (g, h), German natural variant (P889 and P890); (i, j), Dutch variant (P770 and P972); (k), UK variant (P841); (l), cultural variant (P818v) that originated as a sector of standard isolate P818 [(d), above]

3.2 Genetic stability of the alder Phytophthoras

3.2.1 Variation in asexual derivatives

From the above pool of isolates, three standard isolates from different countries and representative German, Swedish, Dutch and UK variant isolates (Table 3) were assessed for the genetic stability of their asexual derivatives; together with two isolates of *P. cambivora* as controls. Approximately 200 hyphal tip (ht) and 200 monozoospore (mz) derivatives were examined for each alder *Phytophthora* isolate, and 100 from each *P. cambivora* isolate. These were then compared for growth rates and colony characteristics with the 'parent' isolate (Table 3).

Growth rates. In both the standard and variant types, the mean growth rate of the 200 mz or 200 ht derivatives was usually significantly different from that of 10 replicate subcultures taken from the parent isolate (four of seven cases with mz and five of seven cases with ht; Table 3); but there was no consistent pattern. Some patterns were observed in the levels of variability (as standard deviations or SDs) of ht and mz derivatives (Table 4). In both the standard and variant groups, the SDs of the ht derivatives were always greater than that of the parent ($p < 0.01$). With the mz derivatives, however, the SDs were always significantly greater than those of the parent in the variant isolates ($p < 0.01$), but were usually of the same order as the parents in the standard isolates. There were also significant differences between the SDs of the mz and ht derivatives in all three standard isolates examined ($p < 0.01$), whereas this difference was significant in only one variant type, the UK variant.

In the *P. cambivora* controls, no differences were observed between the growth rate means of the ht, mz and parent isolates (Table 3). The SDs were very small and in the same range as those of the various alder *Phytophthora* types (Table 4).

Colony patterns and oogonial morphologies. The colony patterns, oogonial frequencies and oogonial morphologies of mz and ht derivatives of the standard types and of the Swedish and Dutch variants did not differ markedly from those of the parent types (Table 3). In contrast, both the parent isolates and the mz and ht derivatives of the German and UK variants (P889 and P841) were highly variable in all these characters, those of P841 particularly so (Table 3; Fig. 3a–h). The derivatives of the *P. cambivora* controls were very stable, exhibiting almost no variation in colony patterns.

3.2.2 Unusual variability in hyphal tip derivatives of UK variant P841

When first isolated in 1996, P841 produced a fluffy irregular-looking colony, very distinct from the standard alder Phytophthoras (see Fig. 2). On subculturing it proved highly unstable, producing a range of novel, non-parental colony types and sectors. These ranged from slow-growing, dense-white aerial types (cf. Fig. 3f) to fast-growing, submerged, finely striate types (cf. Fig. 3b). It was evident that no single subculture could be considered representative of P841. To assess whether each colony type was continuously variable, or whether some colony types could be 'stabilized', 12 visually different sector/colony types were selected and designated 'sources s1-s12' (Table 5). Between five and 10 single ht subcultures were then made from these 12 sources by subculturing them to soft DWA. The hts were then transferred singly to CA plates and their cultural characteristics assessed. Some were further subcultured for tests on their upper temperature limit for growth.

Properties of the hts from six of the 12 sources, chosen to be representative, are shown in Table 5. The number of surviving hts per source ranged from 17 to 100%. The hts exhibited considerable variation in colony diameters (i.e. growth rates); in oogonial frequencies, which ranged from mainly sterile (no oogonia, e.g. hts of s12) to consistently highly fertile (abundant oogonia, e.g. hts of s8); and in upper temperature limits for growth,

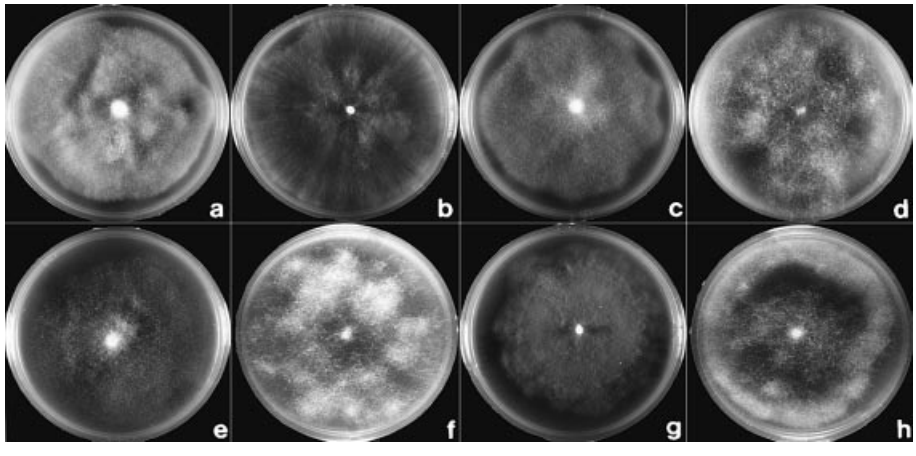

Fig. 3. Colony types of UK variant P841. (a), mass subculture of P841; (b–h), colonies of hyphal tip derivatives of P841 from different sector types (sources): s7/ht29; s3/ht10; s10/ht40; s3/ht11; s12/ht49; s7/ht28 and s5/ht21, respectively. Note the wide range of colony patterns, from submerged striate (b) to felty (c) and to dense cottony (f)

which ranged from 27.5 up to 32.5°C; and in colony patterns (Fig. 3). They also exhibited a remarkable range of oogonial and antheridial morphologies. These have been described elsewhere (BRASIER et al. 1999), as also has the fact that six phenotypically diverse hts exhibited an identical ITS sequence (see Table 5).

In some cases all the ht derivatives resembled their source in their overall properties (e.g. s8, Table 5). Other sets of hts were somewhat or highly phenotypically diverse (e.g. s3, s7, Table 5). There were also significant differences between the mean colony diameters of ht derivatives from different sources. Thus, the overall mean diameter of the hts from source s2, at 42.5 mm, was significantly smaller than the mean diameters of the hts from s7, s8 or s12, at 63.9, 65.1 and 63.9 mm, respectively ($p \leq 0.05$). Likewise, the mean diameter of the hts from s3 was significantly smaller than that of the hts from s8 or s12.

3.2.3 Oospore germination and viability

Oospore germination and viability was investigated using the same isolates as in the asexual variation tests. Control isolates of the homothallic *Phytophthora cactorum* and *Phytophthora citricola* and A1 × A2 pairings of the heterothallic *P. cambivora* were included for comparison.

As estimated by the tetrazolium method, the mean oospore viabilities of the control *P. cactorum* and *P. citricola* isolates over two tests were very high, at approximately 95–97%, whereas the SEs were low at 0.3–0.4 (Table 6). Those of four *P. cambivora* pairings, also over two tests, were somewhat lower at approximately 54–74% (average), with SEs of 0.6–4.3.

Mean oospore viability in the three standard alder *Phytophthora* isolates, assessed over five or six tests (Table 6), was consistently low at 31.3–36.4% and their mean values were not significantly different. In contrast, those of the variants ranged from only 24.2% in the UK variant to 75.7% in the Dutch variant and were significantly different ($p \leq 0.001$). With the exception of the Swedish variant, the SEs of the variants were significantly larger (range, 8.2–10.7) than those of the standard isolates (range, 3.1–4.8). The Swedish variant exhibited an SE of only 1.5, more similar to the SEs of the *P. cambivora* controls.

The SEs of the alder Phytophthoras over the six viability tests were larger than expected. They were therefore compared with the theoretically expected SEs, providing a ratio (Table 6). The observed SEs were shown to be up to five and a half times their expected size The UK, Dutch and German variants showed the highest ratios (range, 4.8–5.6), the standard isolates lower ratios (2.2–3.5), and the Swedish variant a low ratio (1.1). Instability across the six viability tests was therefore highest in the UK, Dutch and German variants, intermediate in the standard isolates and virtually absent in the Swedish variant.

All attempts to germinate the oospores of the alder Phytophthoras were unsuccessful (Table 6). None of the 4200 oospores of the standard isolates tested, or of the 1400 oospores of each of the variant isolates tested, produced a germ tube; although in a few oospores of all types a thinning of the inner oospore wall occurred, suggesting that germination had been initiated. In contrast, 54% of the control *P. cactorum* oospores and 5% of the *P. citricola* oospores germinated, which is consistent with the higher levels of oospore viability recorded in these species. In both these isolates, germination occurred 5–14 days from the beginning of incubation. Most of the oospores produced a germ sporangium. In addition, from 0.5 to 3% germination occurred in three of the four *P. cambivora* pairings tested. In this case germination was exclusively via a germ tube.

3.2.4 Principal component analysis

Figure 4 shows a two-dimensional biplot of a principal component analysis summarizing the relationships between the alder Phytophthoras examined. It is based on six indices:

Table 5. **Properties of hyphal tip derivatives of UK variant P841**

Source	Properties of source colony or sector: Morphology	Properties of source colony or sector: Oogonial frequency[1]	No. of growing hyphal tips/total	ht no.	Properties of hyphal tip derivatives: Mean colony diam. 7 days (mm)	Properties of hyphal tip derivatives: Oogonial frequency[1]	Properties of hyphal tip derivatives: Upper limit for growth (°C)	ITS type[2]	Colony types
s2	felty sector	+	5/9 (56%)	4	40	+	27.5	Dutch	five similar, to'parental'
				5	34	sterile			
				6	46	+ +			
				7	43	+			
				8	51	+ +			
s3	fine striate sector	+ +	4/5 (80%)	9	40	+ +	30.0	Dutch	three similar to 'parental'; one (ht 11) unique
				10[3]	53	+ +			
				11[3]	30	sterile	30.0	Dutch	
				12	52	+ + + +	30.0	Dutch	
s6	appressed dense white sector	nd	1/6 (17%)	26	47	+ + +	32.5	Dutch	as 'parental'
s7	fast sector	nd	6/6 (100%)	27	59	+ +	32.5	Dutch	six all different; one (29) as 'parental'
				28[3]	50	+ + +			
				29[3]	61	+ +			
				30	51	+ + +			
				31	59	+ + +			
				32	54	+ +			
s8	fast sector	nd	6/6 (100%)	33	57	+ + + +			all six similar, to 'parental'
				34	68	+ + + +			
				35	68	+ + + +			
				36	68	+ + + +			
				37	65	+ + + +			
				38	65	+ + + +			
s12	fast sector	nd	5/6 (88%)	48	70	sterile	32.5	Dutch	four similar, non-parental; one unique
				49[3]	70	sterile			
				50	61	sterile			
				51	46	+ +			
				52	73	sterile			

[1] oogonia: + , rare; + + , occasional; + + + , frequent; + + + + , abundant; nd, no data. [2] Dutch, same ITS sequence as the Dutch and German variants (see BRASIER et al. 1999). [3] Colony illustrated in Fig. 3.

Table 6. **Comparative viability of oospores of alder *Phytophthora* isolates**

Phytophthora species or type	Isolate no.	Percentage viable oospores[1] Test no.[2] I	II	III	IV	V	VI	Mean and SE	Ratio actual to theoretical standard error	Oospore germination No. of tests	Percentage germination observed
Alder *Phytophthora* type											
Standard	P938	14	41	41	48	37	39	36.4 ± 4.8	3.5	7	0
Standard	P791	19	34	25	36	36	38	31.3 ± 3.1	2.3	7	0
Standard	P846	–	42	25	30	25	36	31.9 ± 3.2	2.2	7	0
UK variant	P841	17	–	9[2a]	23	47[2b]	–	24.2 ± 8.3	4.8	7	0
German variant	P889	11	55[2c]	38[2d]	–	58	–	40.6 ± 10.7	5.5	7	0
Swedish variant	P876	48	52	59	53	55	53	53.3 ± 1.5	1.1	7	0
Dutch variant	P972	–	83	43	88	81	83[2e]	75.7 ± 8.2	5.6	7	0
Controls											
P. cactorum	P923	96	–	95				95.6 ± 0.3	0.2	1	54
P. citricola	P930	97	–	97				97.1 ± 0.4	0.5	1	5
P. cambivora pairing	P1010 × B12	75	76	–				75.4 ± 0.6	nt	1	0.5
P. cambivora pairing	P1011 × B12	53	57	–				54.8 ± 2.3	nt	1	3
P. cambivora pairing	P1011 × B10	68	59	–				63.3 ± 4.3	nt	1	0.5
P. cambivora pairing	P1010 × B10	70	75	–				72.4 ± 2.9	nt	1	0

[1] Viable oospore = pink staining and thick walled; all others were considered nonviable.
[2] Test of 200–220 oospores in each case except [2a] = 177, [2b] = 76, [2c] = 100, [2d] = 116, [2e] = 100.
–, not tested.

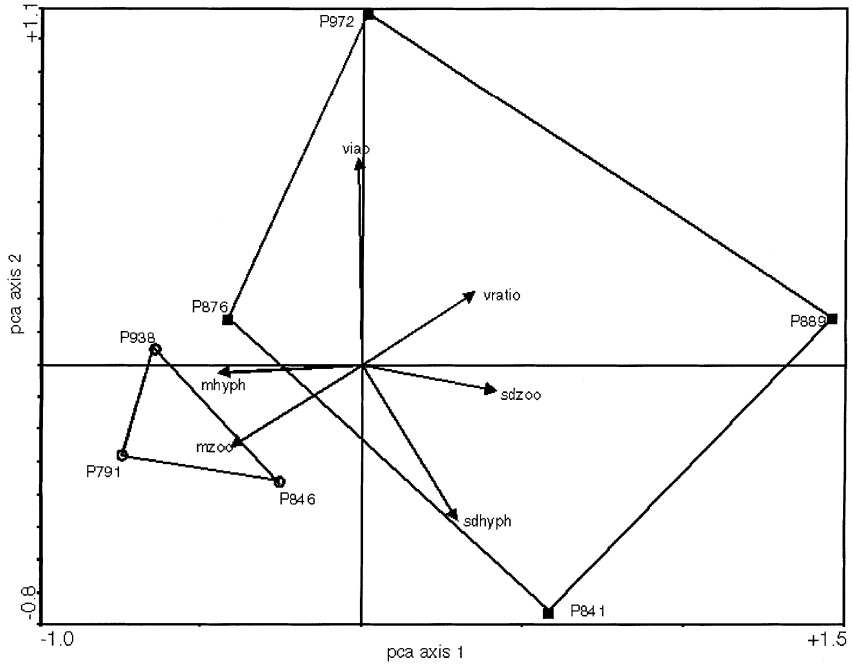

Fig. 4. Biplot of a principal component analysis involving standard (○), and variant (■), types of the alder *Phytophthora*s. Vectors: sdhyph, SD vector of growth rates of hyphal tip derivatives with respect to the parent isolate; sdzoo: SD vector of growth rate of zoospore derivatives with respect to the parent isolate; mhyph: hyphal tip growth rate vector with respect to the parent; mzoo: zoospore growth rate vector with respect to the parent; viab: oospore viability; vratio: ratio of replicate/theoretical SE of oospore viability

mean growth rate of zoospore derivatives, mean growth rate of hyphal tip derivatives, SD of zoospore derivatives, SD of hyphal tip derivatives, mean oospore viability and the observed versus expected oospore viability SE ratio. Some 80% of the overall variation was accounted for by the two axes shown in the plots (52.3% in the vertical and 28.9% in the horizontal axis). The directions of the eigenvectors are indicated in Fig. 4.

The three standard isolates (P791, P846 and P938) group relatively closely, indicating that they are similar to each other, and more consistent with regard to the different variables. The Swedish variant, P876, also aligned close to the standard isolates, indicating a comparable consistency. In contrast, the UK, German and Dutch variants represented by P841, P889 and P972, respectively, were widely separated, indicating that each has a relatively unique and inconsistent set of variables.

4 Discussion

A main aim of this study was to test the hypothesis that major phenotypic variants, equivalent to the naturally occurring Dutch, German, UK and Swedish variants, might be obtained from oospore, zoospore or hyphal tip derivatives of the standard allopolyploid alder *Phytophthora*. The hypothesis was based partly upon the observation that a colony of standard isolate P818 had given rise to a sector exhibiting many of the characteristics of a

natural variant (P818v, Tables 1 and 2); and partly upon the known phenotypic, cytological and molecular properties of the variants, which suggest they could be genetic breakdown products of the standard hybrid (BRASIER et al. 1999).

In terms of this primary objective, the results were inconclusive. First, no oospores could be germinated. Thus, using an established enzyme-based germination method, no germination was observed in more than 4000 oospores of the standard hybrid. In contrast, oospore germination did occur in *P. cactorum, P.citricola* and *P. cambivora* controls. This is consistent with previous evidence that meiotic failure and high levels of abortion occur in the standard hybrid (BRASIER et al. 1995, 1999). Attempts to germinate the oospores under different light regimes, in liquid cultures and after prolonged (8 months) incubation were also negative (J. DELCÁN, unpublished data). Second, several thousand zoospore and hyphal tip derivatives of standard isolates were examined. These exhibited little variation, and were essentially 'parental' in phenotype.

Nonetheless, the possibility that the natural variants arise via genetic breakdown or mitotic crossing over in the somatic nuclei of standard isolates cannot yet be eliminated. Such events might occur at too low a frequency to be demonstrated experimentally without some form of artificial selection. In addition, although no oospores could be germinated and approximately 65% were recorded as nonviable, approximately 35% were visually well-formed, thick-walled oospores that stained pink with tetrazolium salts. This indicates a degree of viability, if not of germinability. Meiotic non-disjunction events, giving rise to incompletely divided restitution nuclei and autopolyploidy, are known to occur with some frequency even in chromosomally 'normal' *Phytophthora* species (e.g, *Phytophthora infestans*, BRASIER and SANSOME 1975; JUDELSON and YANG 1998; CARTER et al. 1999). Meiosis in the standard hybrid tends to be highly aberrant, and is likely to result in many chromosomally unbalanced and hence non-viable nuclei. However, occasional balanced, recombinant nuclei might be produced. If such nuclei were incorporated into a germ tube (this might be produced from an oogonium or an antheridium as well as from an oospore), these could conceivably give rise to natural variants, provided that the new genet was environmentally fit (see BRASIER et al. 1995; BRASIER 1999).

As in the standard type, oospores of the natural variants could not be germinated. However, significantly higher oospore viability levels were exhibited by the Swedish and Dutch variants, at approximately 53 and 76%, than the UK and German variants at approximately 24 and 40%. This is consistent with evidence that the Swedish and Dutch variants have lower heteroploid chromosome numbers ($n \approx 12$ and 14, respectively) and can complete meiosis; whereas the UK and German variants have higher chromosome numbers ($n \approx 17$) approaching that of the standard type and, also like the standard type, exhibit frequent meiotic failures (BRASIER et al. 1999). The zoospore and hyphal tip derivatives of the Swedish and Dutch variants also exhibited little phenotypic variation, whereas those of the UK and German variants exhibited considerable variation (discussed below).

Despite the fact that only limited variation was exhibited by the propagules of the standard hybrid, further evidence was obtained of unusual genetic instability of the alder Phytophthoras as a whole. First, a majority of 58 standard alder *Phytophthora* isolates stored under paraffin oil were found to be no longer wild-type when re-subcultured. They exhibited markedly altered colony patterns and changes in fecundity levels, including total loss of fertility. In other *Phytophthora* spp. such changes in colony characteristics within only a year or so in storage are, in our experience, rare. Equivalent aged cultures of most species in the Forestry Commission *Phytophthora* culture collection (approximately 1000 isolates), such as those of *P. cambivora* or *P. cactorum*, tend to retain their colony patterns and fertility levels (although cultures that are greater than 20 years old do often show changes).

Second, the zoospore, hyphal tip and 'mass' subculture derivatives of UK variant, P841 (and to a lesser extent those of German variant P889) showed remarkable, sometimes continuous variability in colony patterns, fertility levels and temperature–growth relationships. They also produce an extraordinary range of oogonial and antheridial types (BRASIER et al. 1999). It is apparent (Table 5) that some P841 sector types are more stable than others. However, in many P841 ht derivatives an unpredictable relationship appears to exist between growth-rate, temperature–growth patterns and sexual fecundity. The phenomenon resembles the chimaeric sexually fertile and sterile patches reported previously in colonies of the Swedish and Dutch variants (BRASIER et al. 1999; see Fig. 3e, f). It also resembles the continuous phenotypic variation observed by CATEN and JINKS (1969) and HAMM and HANSEN (1982) among single zoospore lineages of *P. infestans* and *Phytophthora megasperma*, and by ZHENG and KO (1995) among zoospore derivatives of a previously 'selfed' isolate of *Phytophthora cinnamomi.*

The basis of the continuous variability in P841 is not clear. One possibility is that it reflects a high frequency of mitotic recombination in an unbalanced interspecific heteroploid. Mitotic recombination probably accounts for phenotypic variants among clones of *P. cinnamomi* in Australia (DOBROLOWSKI 2000). Another possibility is that it reflects cytoplasmic segregation (CATEN and JINKS 1969). However, the phenotypic variation observed in the P841 segregants seems too large for a cytoplasmic phenomenon. A further explanation could lie in the endogenous balance between sexual and asexual development in a new species hybrid. Even in 'normal' Phytophthoras, this balance is a complex one (BRASIER 1999). The alder Phytophthoras are probable hybrids between two developmentally rather different Phytophthoras: *P. cambivora*, which is a fast-growing, sexually outcrossing species with an optimum growth temperature of approximately 28°C that produces sex organs abundantly in pairings on CA; and a *Phytophthora* close to *P. fragariae*, the latter being a slow-growing, inbreeding and nutritionally fastidious species with a optimum growth temperature of approximately 20°C, that struggles to produce oogonia on artificial media such as CA and often fails to do so (WILCOX et al. 1993). The existence of such differing developmental controls within a single nucleus, as in the standard hybrid or in the higher chromosome number variants such as P841, may generate considerable internal conflict (cf. BRASIER et al. 1999; ENGLISH et al. 1999), resulting in unusual developmental instability. It might also account for the high levels of instability observed in the oospore viability tests. To some extent, it might be expressed epigenetically (cf. RAMSDALE 1999). To establish the underlying genetic basis of the phenomenon, detailed biometric and molecular analyses of many serial generations may be needed.

A high level of oospore germination was observed in *P. cactorum* (in agreement with other workers, e.g. (MACINTYRE and ELLIOT 1974) whereas a relatively low level occurred in *P. cambivora*. These differences could reflect differences in ecological strategy. *Phytophthora cactorum* is an inbreeding species that produces oospores rapidly and abundantly. Oospores may also have a prominent role in its day-to-day dissemination, and therefore tend to germinate promptly. *Phytophthora cambivora* is an outcrossing species. Its oospores may therefore function mainly as long-term resting spores and be genetically programmed to germinate over a longer timescale. Sporadic sexual reproduction in this species could also result in an accumulation of recessive lethal mutations. Such lethals would be expressed as homozygotes in some recombinants, resulting in postzygotic failures (BRASIER and SANSOME 1975; BRASIER 1992; GOODWIN 1997).

It has proved very difficult to isolate the standard alder *Phytophthora* from soil around the base of diseased alder trees in the field (J. DELCÁN, unpublished). Moreover, in a preliminary experiment in which mycelium and oospores of a standard alder *Phytophthora* isolate were incorporated into unsterile soil, the alder *Phytophthora* could

not be re-isolated after 1 month's incubation, even though other Phytophthoras such as *Phytophthora gonapodyides* were recovered (J. DELCÁN, unpublished). These observations, together with its low oospore viability described here, suggest the standard alder *Phytophthora* has poor survival ability in soil and that oospores are unlikely to contribute significantly to its survival and spread in nature. Survival in the absence of oospores is not unusual for a *Phytophthora*. Other root-infecting species such as *P. cambivora* and *P. gonapodyides* appear to accomplish their life-cycles successfully without regular production of oospores. Local spread of the standard alder *Phytophthora* along river systems may be mainly via zoospores and via dispersal of infected alder debris containing mycelium. Spread over longer distances, including international spread, may be mainly via distribution and planting of infested nursery stock.

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Résumé

Viabilité des oospores et variations chez les zoospores et l'extrémité des hyphes, chez les Phytophthoras hybrides de l'aune

La viabilité des oospores, leur aptitude à germer, et les variations phénotypiques des zoospores et de l'extrémité des hyphes ont été étudiées chez le type standard et les variants naturels des Phytophthoras hybrides de l'aune. La viabilité des oospores de l'hybride standard, estimée par la méthode au bromide de tétrazolium, était faible (environ 31–36%). Aucune germination n'a été observée parmi plus de 4000 oospores, alors que la germination avait lieu chez *Phytophthora cactorum*, *Phytophthora citricola* et *Phytophthora cambivora* pris comme témoins. Ceci est cohérent avec les irrégularités méiotiques connues chez cet hybride. La viabilité moyenne des oospores des variants naturels était significativement différente ($p < 0001$), allant de 24% chez le variant de la Grande-Bretagne à 75% chez celui des Pays-Bas; aucune germination d'oospore n'a non plus été observée. Les zoospores et l'extrémité des hyphes de l'hybride standard et des variants de Suède et des Pays-Bas ressemblaient phénotypiquement à l'isolat 'parent'. Les variants d'Allemagne et de la Grande-Bretagne, cependant, différaient souvent du type parental. Le variant de la Grande-Bretagne était extrêmement variable et de façon continue, pour l'aspect des colonies, la vitesse de croissance, les relations croissance-température, et les niveaux de fertilité. Ces résultats ne confirment pas l'idée que les variants naturels se forment par altération génétique de l'hybride standard, mais cette possibilité ne peut pas encore être exclue. La non viabililité apparente des oospores suggère que le mycélium et les zoospores sont le principaux responsables de la survie et de la dispersion des Phytophthoras de l'aune dans la nature.

Zusammenfassung

Oosporenvitalität und Variation von Hyphenspitzenkulturen bei Phythopthora-Hybriden an Erlen

Die Vitalität und Keimfähigkeit der Oosporen sowie die phaenotypische Variation bei Isolaten aus Zoosporen und Hyphenspitzen wurden beim Standardtyp und bei natürlich vorkommenden Varianten der *Phythophthora*-Hybriden, die an Alnus vorkommen, untersucht. Die Vitalität der Oosporen des Standardhybriden, ermittelt mit der Tetrazoliumbromid-Färbung, war niedrig (≈ 31–36%). Bei über 4000 Oosporen konnte keine Keimung beobachtet werden, obwohl in den Kontrollen *Phythophthora cactorum*, *Phythophthora citricola* und *Phythophthora cambivora* keimten. Dies bestätigt die bekannten Meiosestörungen bei diesen Hybriden. Die durchschnittliche Vitalität der Oosporen war bei den natürlich vorkommenden Varianten signifikant unterschiedlich ($p < 0.001$) und reichten von ≈ 24% in der englischen Variante bis zu ≈ 75% in der holländischen Variante. Auch hier wurde keine Oosporenkeimung beobachtet. Subkulturen aus Zoosporen und Hyphenspitzen des Standardhybriden sowie der schwedischen und holländischen Varianten ähnelten phänotypisch der Ausgangskultur. Subkulturen der deutschen und der englischen Varianten unterschieden sich jedoch häufig von der Ausgangskultur. Die Subkulturen der englischen Variante variierten sehr stark und kontinuierlich in

ihrer Kulturmorphologie, der Wachstumsrate, der Temperaturabhängigkeit des Wachstums und in ihrer Fertilität. Diese Ergebnisse widersprechen der Hypothese, dass die natürlich vorkommenden *Phytophthora*-Varianten Produkte eines genetischen Zusammenbruchs des Standardhybriden sind. Trotzdem kann diese Möglichkeit aber nicht vollständig ausgeschlossen werden. Die geringe Vitalität der Oosporen deutet darauf hin, dass die Erlen-Phythophthoras unter natürlichen Bedingungen vorwiegend mit Hilfe ihres Myzels und der Zoosporen überleben.

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