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Naturally occurring non cerato-ulmin producing mutants of *Ophiostoma novo-ulmi* are pathogenic but lack aerial mycelium

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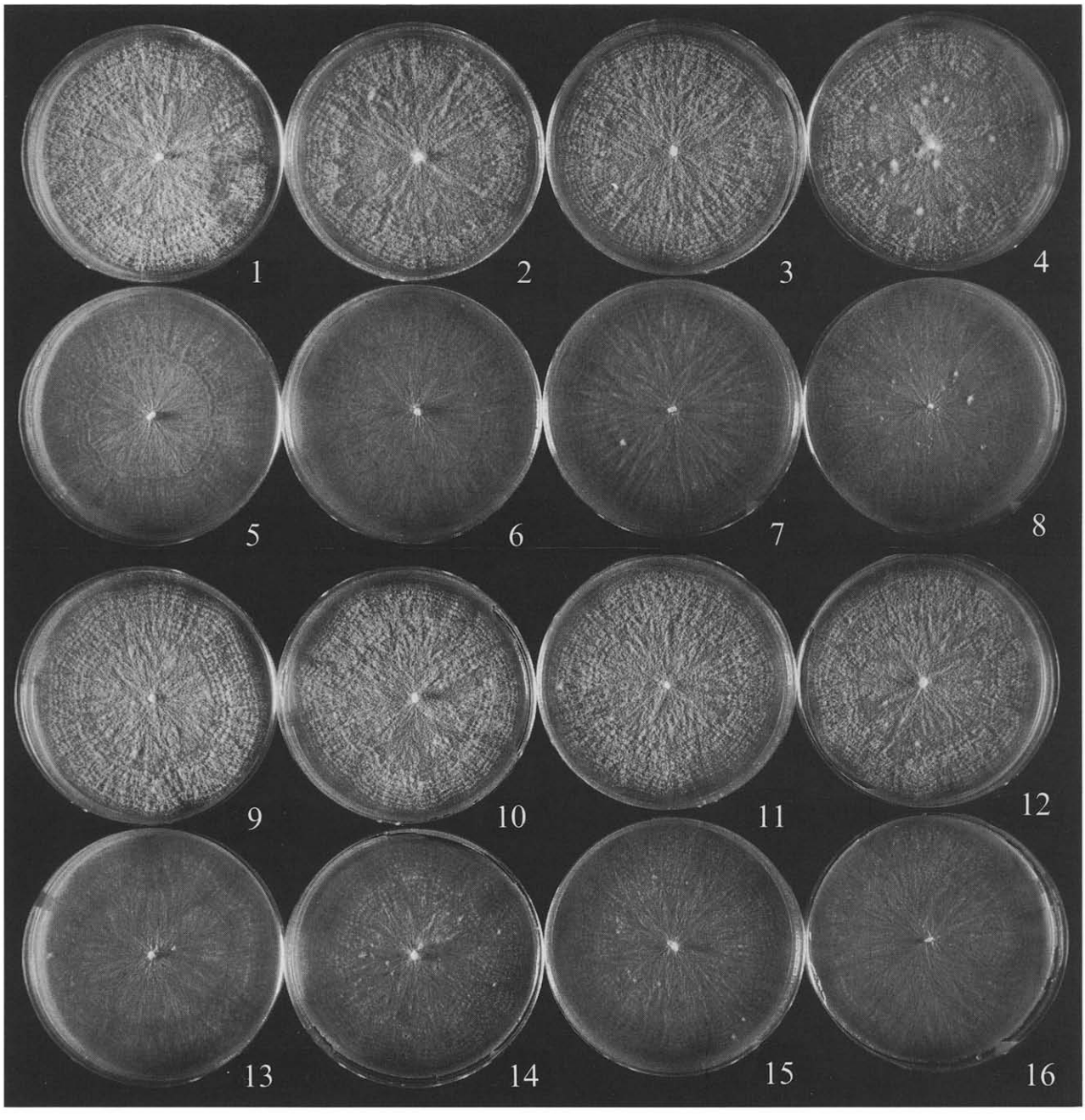
Cerato-ulmin is a protein implicated as a major toxin in the development of Dutch elm disease symptoms in elms infected with *Ophiostoma novo-ulmi*. *O. novo-ulmi* isolates typically produce fibrous-striate aerial mycelium on malt extract agar and secrete high levels of cerato-ulmin in liquid medium. However, two different genotypes of *O. novo-ulmi* (NAN race) from a population sample in Portugal, isolates MAFf8 and PG470, exhibited unusual flat-waxy 'non-aerial mycelial' colony types and were found to produce no detectable cerato-ulmin. The two isolates otherwise behaved as normal *O. novo-ulmi* isolates, including being highly pathogenic to *Ulmus procera*. When MAFf8 and PG470 were crossed with wild-type NAN *O. novo-ulmi* isolates, non-aerial mycelial colony phenotype and non cerato-ulmin production was shown in each case to be controlled by a single pleiotropic mutation, termed *cu*$^-$. A further cross showed the *cu*$^-$ locus was probably allelic in the two isolates. The influence of the *cu*$^-$ locus on colony phenotypes in *O. novo-ulmi* supports the view that cerato-ulmin is a fungal hydrophobin. The normal pathogenic ability of the two *cu*$^-$ isolates raises questions about the proposed role of cerato-ulmin as a wilt toxin.

Cerato-ulmin is a low molecular weight protein of *c.* 8000 daltons (Yaguchi *et al.*, 1992; Scala *et al.*, 1994) produced by the Dutch elm disease pathogens *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier. The molecule has unusual physical properties. Its individual 'units' assemble into rods and fibrils at air-water interfaces (Takai, 1974), it has a large proportion of hydrophobic residues (Stevenson, Slater & Takai, 1979), and from its apparent combination of hydrophobic and hydrophilic properties, it is considered a probable surfactant (Russo *et al.*, 1981). The protein is produced in considerable quantity in liquid cultures by the highly aggressive *O. novo-ulmi*, and at generally much lower levels by the less aggressive *O. ulmi* (Takai, 1974, 1978; Barrett & Skidmore, 1975; Brasier *et al.*, 1990; Tegli *et al.*, 1994). It also induces characteristic wilting and internal disease symptoms in elm cuttings, can be detected immunologically *in vivo* in infected elm shoots, and shows some host specificity to elm (Takai, 1974; Takai & Richards, 1978; Russo *et al.*, 1981, 1982; Richards & Takai, 1984; Okamoto *et al.*, 1986; Richards, 1993). It has therefore been proposed to be the major extracellular toxin involved in the development of vascular wilt symptoms in Dutch elm disease (Takai, 1974; Takai & Richards, 1978; Richards, 1993).

Cerato-ulmin also occurs on the hyphal surfaces, spore surfaces and in the synnemetal head fluid of *O. novo-ulmi* (Takai, 1980; Takai *et al.*, 1980; Svircev, Jeng & Hubbes, 1988). Moreover, Stringer & Timberlake (1993) have noted that the hydrophobicity plot of cerato-ulmin produced by Bolyard & Sticklen (1992), based on the amino acid sequence derived by Stevenson *et al.* (1979) and Yaguchi *et al.* (1992), possesses the structural features of a hydrophobin. These are a recently discovered class of molecules considered to confer hydrophobicity to hyphal and spore surfaces, and implicated in spore dispersal and in pathogen adhesion processes (Beever & Dempsey, 1978; Doyle & Rosenberg, 1990; Stringer *et al.*, 1991). By using mutants Wessels *et al.* (1991*a*, *b*; see also Wosten, DeVries & Wessels, 1993; Wosten *et al.*, 1994) have demonstrated that in *Schizophyllum commune* hydrophobins are also involved in cell wall polymerization, and in the development of more differentiated non-assimilative hyphae from more juvenile assimilative hyphae. A general implication is that hydrophobins are a universal structural feature of fungal mycelia with important developmental functions (Wessels, 1992). This raises the issue of the comparative role of cerato-ulmin as a hydrophobin versus its role as a putative elm toxin.

Two fresh wild isolates of *O. novo-ulmi* from a population sample of several hundred NAN race isolates collected in Portugal during 1985 and 1986 (Brasier, 1988) were found to have an atypical colony phenotype for the species in Oxoid malt extract agar (MEA; see Brasier, 1981). Isolate PG470 was obtained from a fresh xylem infection in an elm at Mafra, and isolate MAFf8 from diseased elm bark of another tree near the same location. Both isolates exhibited a flat, waxy colony type lacking any significant development of aerial mycelium (Figs 5, 13) in contrast to the usual fibrous striate petaloid appearance of *O. novo-ulmi* NAN colonies (Figs 1, 9). MAFf8 was of sexual compatibility type A, and PG470 of type B, and the two isolates were each of a different vegetative incompatibility type (see Brasier, 1988). Hence they were of



Figs 1–16. Colony characteristics of parents and progenies in crosses between wild-type and 'non-aerial mycelial' NAN *O. novo-ulmi* isolates. Crosses: H351 × PG470 (Figs 1–8) and PG402 × MAFf8 (Figs 9–16). **Fig. 1,** wild-type parent H351. **Figs 2–4,** wild-type F_1s. **Fig. 5,** non-aerial mycelial parent PG470. **Figs 6–8,** non-aerial mycelial F_1s. **Fig. 9,** wild-type parent PG402. **Figs 10–12,** wild-type F_1s. **Fig. 13,** non-aerial mycelial parent MAFf8. **Figs 14–16,** non-aerial mycelial F_1s. The isolates were grown on Oxoid malt extract agar at 20 °C as described in Brasier (1981).

different background genotype, but of possibly related genetic origin via a sexual recombination event at the Mafra site.

It was initially suspected from their unusual colony phenotypes that MAFf8 and PG470 might be products of rare introgression between *O. ulmi* and *O. novo-ulmi* as a result of the intermingling of the two species at current epidemic fronts (Brasier & Kirk, 1994). To examine this possibility, they were biologically characterized in terms of their temperature-growth relationships, sexual fertility barrier against *O. ulmi* both as donor (♂) and as a recipient (♀), vascular wilt ability in elm xylem, and pathogenicity to elm bark using the methods described in Kile & Brasier (1990); and for their extracellular cerato-ulmin production index (CPI) in *in vitro* liquid cultures using the method of Takai & Richards (1978). Their pathogenicity to the susceptible *Ulmus procera* and to the more resistant Commelin elm (Table 1) fell within the normal range for *O. novo-ulmi* (see Brasier, 1986), and they conformed to *O. novo-ulmi* in all the other properties tested with the exception of their CPI. This was found to be close to zero in contrast to the normal high CPI readings of NAN *O. novo-ulmi* isolates. The two isolates were also shown to have typical NAN *O. novo-ulmi* nuclear DNA patterns following

Table 1. Pathogenicity of non-aerial mycelial and wild-type NAN *O. novo-ulmi* isolates

Isolates	Pathogenicity (mean % defoliation)* at 12 wk		Mean lesion area (cm^2)
	2m *Ulmus procera*	3m *U.* × Commelin	in *U. procera* bark at 10 wk
Non-aerial mycelial NAN isolates			
MAFf8	72·5	36·0	22·1 ± 11·6
PG470	80·0	41·1	14·1 ± 12·3
NAN wild-type controls**			
H351	nt	50·4	nt
PG402	66·8	71·9	15·6 ± 10·3‡
PG426	89·1	nt	13·9 ± 11·6
PG465	72·2	26·1	nt
O. ulmi control			
PG401	19·5	0	7·6 ± 6·1

*, Results of inoculation experiments conducted from mid-June to mid-September 1987.

**, Control isolates PG402, PG426, PG465 and PG401 are from Portugal (see Brasier, 1988; Kile & Brasier, 1990) and H351 from Belgium (see Brasier, 1986).

nt, Not tested.

‡, Lesion size of PG402 was assessed at 13 wk in a separate experiment.

random amplification of their DNA via the polymerase chain reaction using eleven random oligonucleotide probes which distinguish *O. ulmi* from *O. novo-ulmi* (N. Pipe, K. W. Buck and C. M. Brasier, unpublished observations).

The outstanding properties of PG470 and MAFf8 therefore were an absence of detectable extracellular cerato-ulmin production and a 'non-aerial mycelial' colony type. Their cerato-ulmin production was therefore retested using Scala *et al.*'s (1994) refinement of the turbidometric test of Takai & Richards (1978), in which the cerato-ulmin production index (CPI) of an isolates culture filtrate is calculated from a series of aqueous dilutions (1:1, 1:2, 1:3 to 1:*n*). Each dilution is first shaken to induce turbidity of the cerato-ulmin. Its optical density at 400 nm is then compared to that of a still sample at the same dilution, and plotted against the decimal logarithm of the dilution factor. It was confirmed that the mean CPI's of MAFf8 and PG470 lay close to zero (at 1·9 and 2·0 in this test) compared with the high CPI's of control NAN *O. novo-ulmi* isolates PG402 and ES376 (at 1080·2 and 1012·7 respectively). In the same test control *O. ulmi* isolate H200 gave a typically low mean CPI reading for *O. ulmi* of 8·6.

To investigate the genetic control of their unusual colony phenotype and non cerato-ulmin production, each isolate was crossed with an *O. novo-ulmi* isolate exhibiting a normal wild-type colony pattern and high level of cerato-ulmin production; PG470 with A-type isolate H351 (from Belgium; see Brasier, 1985) and MAFf8 with B-type isolate PG402 (from Portugal, see Kile & Brasier, 1990). Crosses and isolation of random ascospores were carried out as described by Kile & Brasier (1990). From each cross 102 and 100 progeny respectively were examined. They segregated in a near 1:1 ratio for wild type: non-aerial mycelial colony types (52:50 and 53:47 respectively), indicating the involvement of a single locus in each case. The two phenotypes also segregated independently of sexual compatibility type. Otherwise the progeny showed typical growth rate variation and in the case of the wild-type progeny typical colony patterns for F_1 progeny samples in crosses between NAN isolates (Figs 1–16), i.e. both isolates 'bred true' to *O. novo-ulmi*. Ten wild-type and 10 non-aerial mycelial progeny of each cross were then selected at random and tested for cerato-ulmin production using the method of Scala *et al.* (1994). All 20 non-aerial mycelial progeny isolates produced no detectable cerato-ulmin, whereas the 20 wild types produced high levels of cerato-ulmin comparable to that of PG402 and H351 (Table 2). This indicated that in each case a single locus was probably responsible both for the non-aerial mycelial phenotype and for the absence of cerato-ulmin production. To test for possible allelism of the loci in MAFf8 and PG470, these two isolates were crossed and 300 ascospore progeny characterized. All 300 F_1s exhibited the non-aerial mycelial phenotype. The loci involved are therefore most probably allelic. The locus has been designated the cu^- (non cerato-ulmin producing) locus, and its wild-type equivalent the cu^+ locus.

This evidence for pleiotropic control of the non-production of a hydrophobin-like molecule and an absence of aerial mycelium parallels the more detailed observations made by Wessels *et al.* (1991*a*, *b*) with *Sc* mutants in *Schizophyllum commune*, and supports the view of Stringer & Timberlake (1993) that cerato-ulmin is a hydrophobin. It suggests that in *O. novo-ulmi*, cerato-ulmin may have a similar function in hyphal development and in spore protection to that proposed for hydrophobins in other fungi (Beever & Dempsey, 1978; Stringer *et al.*, 1991; Wessels, 1992). With the exception of the cu^- locus and its effects, MAFf8 and PG470 otherwise behave as normal *O. novo-ulmi* isolates. The identification of the cu^- locus therefore offers the opportunity to examine the effects of a hydrophobin on *O. novo-ulmi* development, as has already been done in *S. commune* and *Aspergillus* (e.g. Stringer *et al.*, 1991; Wessels, 1992; Wosten *et al.*, 1993, 1994). Of obvious interest would be to compare viability of conidia and ascospores and comparative development of fruiting structures in cu^+ and cu^- isolates. Another approach could be to study the function of the cu^- allele by inserting it directly into *O. ulmi* by genetic transformation. It should be noted, however, that while MAFf8 cu^- and PG470 cu^- are most probably

Table 2. Cerato-ulmin production *in vitro* by non-aerial mycelial and wild-type NAN *O. novo-ulmi* isolates and their F_1 progenies

Parent and F_1 isolates	Turbidity of shaken culture filtrate*	Cerato-ulmin production index (CPI)†		
		No. of isolates tested	Mean CPI	Range
Cross H351 × PG470				
Wild-type parent H351	High	1	230	
Non-aerial mycelial parent PG470	Zero	1	0	
10 wild-type F_1s	All high	5	206	117–318
10 non-aerial mycelial F_1s	All zero	10	0	
Cross PG402 × MAFf8				
Wild-type parent PG402	High	1	324	
Non-aerial mycelial parent MAFf8	Zero	1	0	
10 wild-type F_1s	All high	5	240	105–387
10 non-aerial mycelial F_1s	All zero	10	0	
O. ulmi controls				
R21	Near zero	1	5	
H200	Zero	5	0	

*, High turbidity, spectrophotometer reading (at 400 nm) > 2·5 at the 1:1 aqueous dilution. Zero turbidity, spectrophotometer reading (at 400 nm) < 0·004 at the 1:1 aqueous dilution.

†, Mean of two replicate dilution series.

naturally occurring mutants of *O. novo-ulmi*, there remains a possibility that their cu^- locus was derived from *O. ulmi* via rare introgression.

The present observations also raise interesting questions about the role of cerato-ulmin as a putative toxin during Dutch elm disease pathogenesis. MAFf8 cu^- and PG470 cu^- are as pathogenic to elm xylem or elm bark as most NAN *O. novo-ulmi* isolates (Table 1; Brasier, 1986). Barrett & Skidmore (1975) showed that white 'feltoid' cultural variants of NAN *O. novo-ulmi* (a spontaneous degenerate variant; cf. Brasier, 1982) also produced little or no cerato-ulmin *in vitro* yet remained highly pathogenic to elms. To account for cerato-ulmin's role as a toxin (Richards, 1993), it is possible that in the case of the cu^- and 'feltoid' isolates much higher levels of cerato-ulmin production are induced *in planta* than are produced *in vitro*, though this seems unlikely in view of the high constitutive CPI levels normally achieved by wild-type isolates in liquid cultures (cf. Table 2). It is also possible that the negligible *in vitro* CPI levels of the cu^- and feltoid isolates are sufficient for normal expression of pathogenicity *in vivo*. A further possibility is that, even in the absence of significant cerato-ulmin production, the remaining armoury of toxins and enzymes available to *O. novo-ulmi* is sufficient for pathogenic function. The above issue may be clarified if the levels of cerato-ulmin production by cu^- isolates *in planta* and *in vitro* can be compared by immunological methods. Critical to the success of such an approach, however, is the development of assays that are sufficiently sensitive and quantitative.

It is a characteristic of the less aggressive *Ophiostoma ulmi* that CPI levels in liquid cultures tend to be low or near zero (e.g. Brasier *et al.*, 1990). In this species, therefore, the molecule might function mainly as a cell surface hydrophobin. Unfortunately, interspecific crosses between *O. ulmi* and *O. novo-ulmi* show a rather complex pattern of inheritance for cerato-ulmin production (Brasier, 1987; Kile & Brasier, 1990) which could well reflect the fact that the protein is structurally different and differently regulated in the two species (Brasier *et al.*, 1990). Thus the typically higher *in vitro* CPI levels of *O. novo-ulmi* might be achieved through multiple copies of the relevant structural genes, through an alternative regulation system, or through a mechanism of enhanced cerato-ulmin excretion (cf. Sutherland & Brasier, 1994). Indeed the cu^- locus could conceivably be a locus affecting cerato-ulmin excretion ability rather than one directly affecting intracellular production levels of the protein.

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