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Five laboratory mutants of *Ophiostoma novo-ulmi* EAN isolate H328 were obtained from uv-irradiated and unirradiated blastoconidia. Although the mutants were initially selected on the basis of various characteristics, including failure to produce cerato-ulmin (CU) in the culture filtrate, growth-temperature responses and colony morphology, they all were eventually also found unable to produce CU when grown in liquid shake culture at 23 °C and 33°, or they produced CU only in negligible amounts. This association suggests possible linkages between CU production and other fungal metabolic pathways. Genetic analysis of crosses between the mutants and a wild type isolate of EAN *O. novo-ulmi* suggested that each mutant involved a change at a single-locus, at least as far as segregations for colony morphology were concerned. Pathogenicity trials using the five mutants were carried out on four-year old *Ulmus carpinifola* and *U. procera*. Two out of the five mutants showed a significant reduction in pathogenicity in comparison to the *O. novo-ulmi* wild type isolate H328.

Ophiostoma ulmi (Buisman) Nann. and *O. novo-ulmi* Brasier are the two fungal pathogens involved in the Dutch elm disease (D.E.D) (Brasier, 1991; Buisman, 1932; Melin & Nannefeldt, 1934). *O. ulmi*, responsible for the first pandemic during the 1920s–40s, is considerably less aggressive than *O. novo-ulmi*, the causal agent of the current, second pandemic of the disease (Brasier, 1979, 1990; Brasier & Gibbs, 1973; Gibbs, 1978; Guyot, 1921). They also differ in many important morphological, physiological and ecological characteristics, among which are growth-temperature responses, and the production of phytotoxic compounds. Different temperature regimes affect the linear growth rate of the two fungi on agar, though the differences are much less marked in liquid shake cultures. At higher temperatures, *O. ulmi* isolates are able to grow on Oxoid Malt Agar Extract, while *O. novo-ulmi* are not (Brasier, 1981; Brasier, Lea & Rawlings, 1981; Gibbs & Brasier, 1973; Tegli *et al.*, 1994).

The most important toxic metabolite discovered so far is cerato-ulmin (CU) (Richards & Takai, 1973; Takai, 1974; Takai & Richards, 1978). It is a protein with a molecular weight of 8 kDa and consists of 75 aminoacid residues, and has hydrophobic properties (Scala *et al.*, 1994*a*; Stevenson, Slater & Takai, 1979; Yaguchi *et al.*, 1993). Homogeneous preparations of CU are able to induce the internal and external symptoms of D.E.D., when absorbed by elm cuttings (Richards, 1993; Richards & Takai, 1984; Takai & Hiratsuka, 1984). Until now experiments for the detection of CU *in vivo* have been performed only in elms artificially or naturally infected with *O. novo-ulmi*, and not with *O. ulmi* (Richards, 1993; Takai, Richards & Stevenson, 1983). The toxin is also produced in the culture filtrate of the fungi grown in liquid shake culture. Considerable quantities are detectable in the case of *O. novo-ulmi* isolates incubated at 23°, while at the same temperature CU production by *O. ulmi* isolates is very low or close to zero (Barrett & Skidmore, 1975; Brasier *et al.*, 1990; Takai, 1974; Takai & Richards, 1978). Recently, Tegli *et al.* (1994) reported that *in vitro* CU production is modulated by temperature, with some *O. ulmi* isolates able to reach high CU levels, if grown at 33°.

CU appears to have other functions in addition to being a toxin. It has been detected in the cell wall and in the synnemata head fluid of *O. novo-ulmi* (Takai, 1980; Takai *et al.*, 1980; Svircev, Jeng & Hubbes, 1988), and recently Brasier, Kirk & Tegli (1994) reported the occurrence of wild isolates of *O. novo-ulmi* unable to produce CU and which exhibited altered colony phenotype. These results are in accordance with the proposal by Stringer & Timberlake (1993) and Bowden *et al.* (1994) that, on the basis of its published aminoacid sequence, CU is a fungal hydrophobin, a class of cell wall proteins characterized by a great number of hydrophobic residues and other structural properties.

With the object of better understanding the role of CU in the pathogenicity of the Dutch elm disease pathogens, we have followed the classical strategy of producing mutants of the fungus deficient in functions potentially related to pathogenicity/virulence (Beraha & Garber, 1971; Defago & Kern, 1983; Howell, 1976; McDonnell, 1962; Puhalla & Howell, 1975). Starting from a typical, highly pathogenic isolate of *O. novo-ulmi*, we obtained and characterised both uv-induced and spontaneous mutants, which had lost the capacity to produce CU.

MATERIALS AND METHODS

Fungal cultures

The isolate H328 of *O. novo-ulmi* Euroasian (EAN) race and the isolate E2 of *O. ulmi* were the same as used in our previous paper (Tegli *et al.*, 1994). The isolate H327 of *O. novo-ulmi* race EAN was from Dr Clive M. Brasier's collection.

The fungi were inoculated onto elm branches each spring to reduce loss of pathogenicity during *in vitro* culture, and in autumn the young twigs were stored at −18°. The fungus was re-isolated from the infected tissue on 90 mm diam. Petri dishes, each containing 20 ml Oxoid Malt Extract Agar (MEA) (Brasier, 1981) with the addition of 0·1 mg ml^{-1} Actidione™ (cycloheximide, Sigma) and 1 mg ml^{-1} streptomycin sulphate.

For short-term maintenance, the isolates were inoculated onto MEA, incubated at 21°, and subcultured once per month. Long term stocks were maintained at 4° and at −20° and subcultured every three months.

The fungi were grown in liquid shake culture using the medium of Takai & Richards (1978) with some modifications (TK) (Tegli *et al.*, 1994).

Evaluation of in vitro *growth rate, colony characteristics and CU production*

Growth on agar has been assayed according the following method. Mycelial plugs of 2 mm square were removed from the active growth area in the outer portion of the colony and transferred to the centres of 90 mm diam. Petri dishes containing 20 ml MEA. Cultures were incubated in the dark at 15°, 21°, 23°, 28° and 33°. Growth of the fungus was measured at 2, 5, 7 and 12 days from inoculation along two diagonal lines inscribed at right angles on the bottom of the Petri dish. Measurements were converted to daily radial growth values (mm d^{-1}). Growth rate in liquid shake culture was measured in terms of blastoconidia concentration and expressed as colony-forming units (c.f.u.) ml^{-1}, according to the method of Scala *et al.* (1994*a*) and Tegli *et al.* (1994).

For colony morphology, cultures were examined after being incubated for a week at 21° in the dark, and for a further 10 days at the same temperature in diffuse lighting.

CU production in the culture filtrates of *Ophiostoma* isolates grown in liquid shake culture was evaluated following Scala *et al.*'s (1994*a*) modification of the turbidimetric method of Takai & Richards (1978) and expressed as an isolate's Cerato-ulmin Production Index (CPI). This index is based on the linear relation existing between the optical density at 400 nm of the shaken sample and the decimal logarithm of the dilution factor (Scala *et al.*, 1994*a*).

Mutagenesis procedure

A starter culture of the EAN isolate H328 of *O. novo-ulmi* was established in TK liquid medium and incubated at 23° on a rotatory shaker at 110 rpm for 12 h. A sample of blastoconidia from this culture was transferred to 100 ml Erlenmeyer flasks with 30 ml TK medium to yield a concentration of 3×10^4 blastoconidia ml^{-1}. The flasks were then maintained on the rotary shaker at 23° until exponential growth was reached.

Sterile serial decimal dilutions of the liquid cultures were prepared and blastoconidia concentrations determined by a haemocytometer. The dilution nearest the target concentration of 3×10^3 blastocondia ml^{-1} was brought to this value by adding sterile distilled water. Samples of 100 µl were plated evenly with a glass spreader onto 90 mm diam. Petri dishes containing 20 ml MEA plus 100 mg l^{-1} sodium deoxycholate (Bernier & Hubbes, 1990*a*, *b*; Tegli *et al.*, 1994). Dishes were exposed to uv radiation, wavelength 254 nm, at a distance of 30 cm from the lamp (4W, Spectroline CC-80). A range of exposure times was tested in preliminary experiments to obtain a survival curve. For the mutation trials, an exposure of 3·5 min was used in order to obtain a survival rate of approximately 10%. For each experiment, 40–50 Petri dishes of blastoconidia were treated with uv radiation.

Identification of non CU-producing mutants

To identify non CU-producing mutants, MEA dishes with irradiated blastoconidia of isolate H328 were incubated in the dark at 23° for 10 days and the number of surviving colonies on each Petri dish was counted. Each surviving colony was then transferred with a sterile toothpick to one of the 12 wells of a Cell Wells™ (Corning) containing 2 ml of sterile TK medium each, labelled, and incubated in the dark on a rotary shaker at 110 rpm for 10 days, after which the culture broth was examined with the naked eye for milkiness, the tell-tale sign of CU presence. From each well in which no CU was apparent, 0·1 ml of culture was transferred to an Erlenmeyer flask (100 ml) containing 30 ml sterile TK medium, shaken under standard conditions for 7 days, after which blastoconidia concentration was estimated and CU level assessed by the turbidimetric method. The isolates that still showed no CU production were designated putative non CU-producing mutants and were subcultured in liquid shake culture once every 10 days for a further three months to confirm the stability of the mutations.

Identification of 'heat-tolerant' mutants

After the uv treatment, MEA dishes with irradiated blastoconidia of isolate H328 from *O. novo-ulmi* were incubated in the dark at 33° for 10 days. Usually, *O. novo-ulmi* is not able to grow at 33°, whereas *O. ulmi* does. A number of control dishes including both irradiated and non-irradiated cultures, were incubated at 23° to provide a basis for assessing the selective pressure exerted by incubation at 33° on the irradiated colonies. The number of colonies surviving exposure to 33° was counted. Irradiated colonies that grew well were subcultured to the centre of 50 mm diam. Petri dishes containing 10 ml MEA, and incubated for a further 10 days at 33° in the dark. Mycelial plugs were then removed from all colonies with a diameter exceeding 10 mm, by means of a 6 mm diam. cork borer, and placed in the centre of 90 mm diam. Petri dishes containing 20 ml MEA. The threshold of 10 mm was chosen according to the growth rates

at 33° of numerous isolates of both *O. novo-ulmi* and *O. ulmi*. Heat-tolerant mutants were considered those isolates maintaining a growth rate $\geqslant 1$ mm day^{-1} after five months of subculturing on MEA at 33°.

Phenotypic characterization of mutants and pathogenicity tests

From the uv mutagenesis one non CU-producing mutant and one heat-tolerant mutant were obtained and numbered '4*cu*$^-$' and '5II', respectively. Three spontaneous colony morphology mutants of H328, which occurred in unirradiated cultures of the fungus on MEA, were also included in this study and numbered '110', '148' and '186'.

All the mutants were tested for *in vitro* growth rates at different temperatures both on agar and in liquid shake culture, and for CU production, according to the methods previously described.

Pathogenicity tests of the mutants and controls on 4 year-old clonal *Ulmus carpinifolia* Gleditsch and *U. procera* Salisbury were carried out as described in Brasier (1986). The *U. carpinifolia* were inoculated at the nursery of the Centro di Studio per la Patologia delle Specie Legnose Montane (CNR) Florence, Italy, and the *U. procera* at the Forestry Research Station at Alice Holt Lodge, Farnham, U.K. Inoculations were carried out in mid-May in Florence, and 4 weeks later at Farnham, following the method of Brasier (1981) using ten and four randomly selected trees per isolate, respectively. Twelve weeks after inoculation the percent defoliation was scored using three independent assessors (Brasier, 1986).

Determination of sexual mating types, sexual crosses and isolation of ascopore progenies

The sexual mating type of isolates to be tested was determined according to the method of Brasier (1981) and Brasier & Gibbs (1976). All five mutants were of sexual compatibility type B, like H328. Wild type EAN *O. novo-ulmi* isolate H327, of sexual compatibility type A, was crossed with the five mutants, and with wild type H328 as a control. Recipient colonies of isolates H327, H328 and the five mutants were established in 90 mm diam. Petri dishes containing 20 ml elm sapwood agar (ESA) following the method of Brasier (1981). The cultures were incubated in the dark at 21° until the colony had completely overgrown the dish, and then maintained in diffuse lighting at the same temperature for a further 2 weeks to allow the protoperithecia to mature.

To fertilize the protoperithecia, a 0·5 cm^2 piece of mycelium from a culture of a donor isolate which had been grown on MEA for 10 days was rubbed repeatedly on a similarly sized patch on the surface of the recipient. The fertilised recipient was then incubated further in diffuse light at 21° until the perithecia had matured. Perithecia were considered mature when the ascopore drop at the tip of the perithecial neck began to turn opaque. Single ascospore isolations were carried out as described in Brasier & Gibbs (1976) and Webber, Mitchell & Smith (1986). About 100 F1 ascospore progeny were collected from each cross.

For each cross 30 ascospore progeny were selected at random and tested for growth rate, colony morphology and sexual compatibility type. Among the mutant and wild type progenies, fifteen isolates were then randomly selected and tested for CU production.

RESULTS

Production and identification of the mutants

Three independent experiments to obtain non CU-producing mutants from *O. novo-ulmi* isolate H328 by exposure to uv radiation were performed. Almost all isolates that did not produce CU in the Cell Wells™ (Corning) became producers under normal growing conditions in Erlenmeyer flasks. At the end of three selection trials, of 11555 blastoconidia irradiated only one stable CU non-producing mutant was identified, giving a frequency of mutation of the order of *ca* 10^{-4}. This isolate was designated '4*cu*$^-$'. Its colony morphology on MEA differed from that of the 'parent' isolate H328, with areas of aerial mycelium alternating with more waxy areas (Fig. 1).

In another series of experiments, the cultures of H328 exposed to uv radiation were further incubated at 33° to select heat-tolerant mutants. An equal number of unirradiated plates were incubated at the same temperature, and used as control. None of the unirradiated cultures grew after 10 days. Of 40560 previously uv-irradiated blastoconidia, only 244 (0·6%) grew at 33°. Of these, only one strain was heat-tolerant in

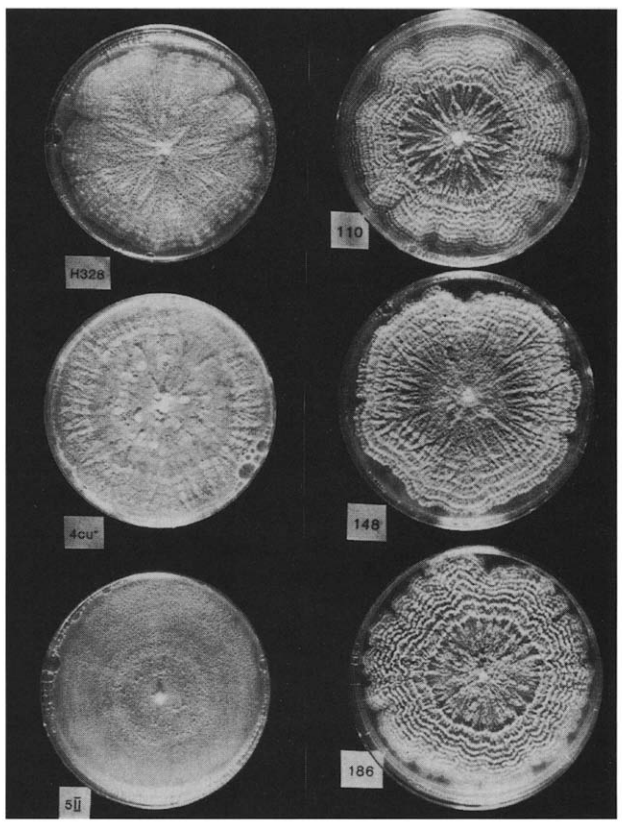

Fig. 1. Colony characteristics of *Ophiostoma novo-ulmi* wild type isolate H328, uv-induced mutants 4*cu*$^-$ and 5II, and spontaneous mutants 110, 148 and 186 grown onto MEA at 21°.

Table 1. Growth and CU production in liquid shake culture at 23° and 33°, and pathogenicity on *Ulmus* species, by laboratory-induced mutants from *Ophiostoma novo-ulmi* isolate H328 and the parental isolate.

	Blastoconidia ml^{-1} ($\times 10^7$)*†				Cerato-ulmin production index†				Pathogenicity (mean % defoliation)	
Days after inoculation...	3		6		7		10		84	84
	23°	33°	23°	33°	23°	33°	23°	33°	*Ulmus procera*§	*Ulmus carpinifolia*‖
H328	23±3	2±0·6	57±4	42±7	995±120	72±71	497±251	203±65	71·9±1·7^a	46·5±6·7^a
4*cu*$^-$	31±2	2±0·4	62±9	38±4	0	0	0	54±43	12·5±3·3^b	12·0±2·3^b
5II	41±5	1±0·7	59±4	35±3	0	65±21	0	114±49	7·1±2·0^b	5·0±1·0^b
110	29±5	2±0·3	55±9	46±5	0	0	0	0	56·0±3·0^a	17·0±2·8^{bc}
148	34±6	2±0·4	69±6	58±3	0	0	0	0	61·9±6·6^a	32·0±3·0^{ac}
186	30±4	2±0·5	67±6	42±5	0	0	0	0	75·2±8·7^a	44·5±7·5^a
E2‡	46±3	2±0·7	78±3	67±6	0	161±38	61±31	356±19	7·4±3·0^b	1·5±0·8^b

* Initial blastoconidia concentration was 5×10^4 ml^{-1}.

† Results are the mean ± S.E.M. of two experiments with three replicates each.

‡ The isolate E2 of *O. ulmi* was tested as weakly pathogenic control.

§ Results are the mean ± S.E.M. of four replicates; means in the column followed by the same letter do not differ significantly at $P = 0{\cdot}05$, according to the Tukey test.

‖ Results are the mean ± S.E.M. of ten replicates; means in the column followed by the same letter do not differ significantly at $P = 0{\cdot}05$, according to the Tukey test.

subsequent tests, giving a mutation frequency of *ca* $2{\cdot}5 \times 10^{-5}$. This mutant was designated '5II'. Its colony morphology in MEA at 23° was much smoother than that of isolate H328 (Fig. 1).

Three spontaneous mutants of H328 occurred *in vitro* culture and were designated '110', '148' and '186'. All three mutants were of dark appearance when grown on MEA at 23°, and were named 'dark mutants' (Fig. 1). This was in part due to the large number of synnemata which developed in the colonies in concentric bands, an unusual character when the fungus is cultured on MEA.

Characterization of the mutants

The growth rates on MEA of the uv-induced mutants 4*cu*$^-$ and 5II, and the spontaneous mutants 110, 148 and 186 were determined at 15°, 21°, 23° 28° and 33°, in the dark. The *O. ulmi* isolate E2 and the *O. novo-ulmi* isolate H328 were tested as controls typical of their species, and the latter also because it was the parental isolate. The growth patterns of the mutants 4*cu*$^-$, 110, 148 and 186 were similar to that of the isolate H328, indicating that the mutation had not affected the growth temperature response of the fungus. Under the same conditions, the growth rate of the heat-tolerant mutant 5II was significantly different from that of H328 only at 33° as expected. At 33°, 5II grew as well as *O. ulmi* isolate E2 (data not shown).

Table 1 shows the development of 4*cu*$^-$, 5II and the three dark mutants in liquid shake culture at 23° and 33°. Starting from a concentration of 5×10^4 blastoconidia ml^{-1}, control isolates H328 and E2 behaved typically for their species. All five mutants grew well at both temperatures. As far as CU production is concerned, none of the three dark mutants produced CU in any of the experimental conditions examined, while both 4*cu*$^-$ and 5II were able to produce small amounts at 33° and behaved more similarly to the *O. ulmi* isolate E2 than to the *O. novo-ulmi* wild type isolate H328.

Table 1 also shows the results of pathogenicity tests, expressed as percentage of defoliation on clonal *Ulmus carpinifolia* (clone FL25) and *U. procera*, 12 weeks after inoculation with *O. novo-ulmi* isolate H328, the 4*cu*$^-$, 5II, 110, 148, 186 mutants, and *O. ulmi* isolate E2 as weakly pathogenic control. On *U. procera*, which is very susceptible to D.E.D., the dark mutants (110, 148, 186) caused defoliation similar to that produced by the parental isolate H328. For 4*cu*$^-$ and 5II the decrease of defoliation was statistically significant, giving a level comparable to that caused by *O. ulmi* isolate E2. On clone FL25 of *U. carpinifolia*, which is partially resistant to D.E.D., the percentages of defoliation caused by the parent H328 and the 148 and 186 mutants were statistically comparable to each other, and lower than those obtained on *U. procera*. In the case of mutant 110, defoliation was as low as that caused by the 4*cu*$^-$ and 5II mutants, and by *O. ulmi* isolate E2. One year after inoculation these last results have been confirmed, since 70% of the *U. carpinifolia* plants inoculated the previous year with the 110 mutant did not show symptoms of Dutch elm disease, neither did plants inoculated with both the 4*cu*$^-$ and 5II mutants and *O. ulmi* isolate E2. On the contrary, all plants inoculated with *O. novo-ulmi* isolate H328, and the 148 and 186 mutants were dead or severely affected.

Mutant × wild type crosses: cultural characteristics and CU production of F1 progenies

The isolate selected to be crossed with the five mutants was H327. This belongs to the EAN race of *O. novo-ulmi* and among all the possible candidate isolates it most nearly resembled H328 (data not shown). In this way, any novel characteristic in the F_1 progeny could with certainty be attributed to the mutant rather than the other parent. All the mutants were crossed reciprocally with A-type isolate H327. All mutants were female sterile, as they produced no perithecia, and sometimes not even protoperithecia, when used as

Table 2. Growth rate and colony phenotypes on MEA at 21° of F1 progenies from laboratory-induced mutants of *Ophiostoma novo-ulmi* isolate H328 and the parental isolate crossed with *O. novo-ulmi* isolate H327 as recipient.

Cross ♀ × ♂	No. of progeny	Growth rate (mm day^{-1}) Progeny mean*	Progeny range	Parent mid point	No. of wild type	No. of mutant	χ^2†
H327 × H328	30	4·27 ± 0·09^{a}	3·45–5·45	4·02	30	0	n.m.‡
H327 × 4*cu*$^-$	30	3·89 ± 0·16^{ab}	2·45–5·03	4·39	11	18	1·67
H327 × 5II	30	2·64 ± 0·09^{c}	1·85–3·50	4·14	14	16	0·13
H327 × 110	30	4·18 ± 0·11^{a}	3·37–6·10	3·96	16	14	0·13
H327 × 148	30	3·63 ± 0·18^{b}	1·68–5·70	3·71	10	12	2·27
H327 × 186	30	4·80 ± 0·08^{d}	3·95–5·85	4·06	17	13	0·53

* Results are the mean ± S.E.M.; values followed by the same letter do not differ significantly at $P = 0·05$, according to the Tukey test.

† χ^2 values for goodness of fit to a 1 : 1 wild type mutant phenotype ratio in crosses between H327 and the mutants. In cross between H327 and the parental isolate H328, all progenies have a wild type phenotype, as was expected. Progeny with non-parental colony types produced in crosses H327 × 4cu$^-$ and H327 × 148 omitted from the segregations.

‡ n.m. = not measurable.

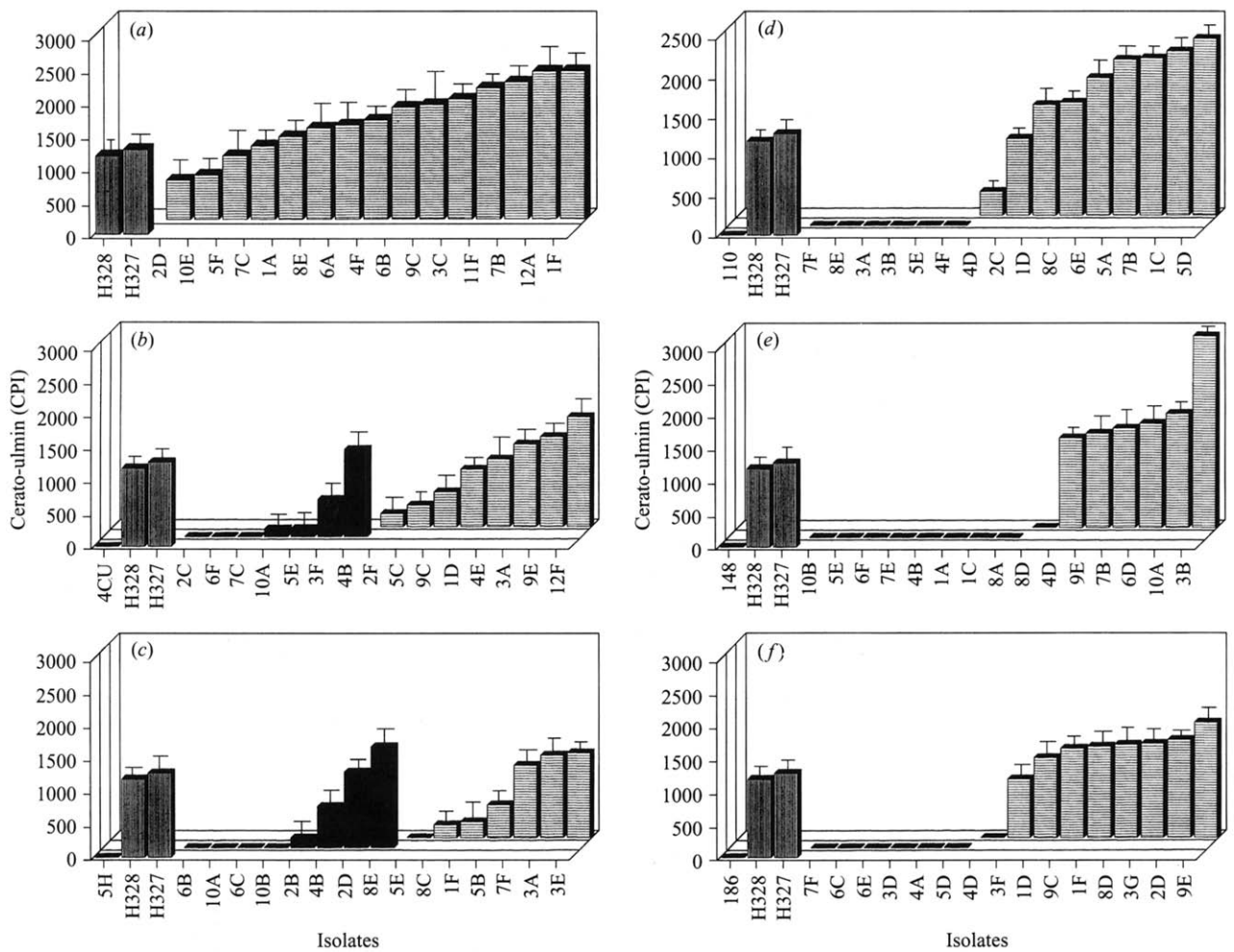

Fig. 2. Production of cerato-ulmin of parents and F1 progenies in crosses between the *Ophiostoma novo-ulmi* isolate H327 and *O. novo-ulmi* isolate H328 (a), and its uv-induced mutants 4*cu*$^-$ (b) and 5II (c), and spontaneous mutants 110 (d), 148 (e) and 186 (f). The isolates were grown in liquid shake culture on TK at 23°C. The distribution of colony types within production classes is shown as follows: (▥) parents, (▤) F1 wild type and (■) F1 mutant type. Cerato-ulmin levels are expressed as C.P.I. (Cerato-ulmin Production Index). Values are for 15 randomly selected F1 isolates and are the means of three replicates for each isolate.

recipient. The F_1 progenies examined were therefore limited to the crosses in which the mutants were donors.

Table 2 shows the growth rate data on MEA at 21° of F_1 progenies from the crosses between H327 and the mutants, and the cross H327 ♀ × H328 ♂ as a control. It also shows for each cross the segregation of colony phenotypes: 'wild type' (characteristic of the EAN race of *O. novo-ulmi*), and 'mutant' (characteristic of the mutant parent isolate). One and eight

unstable non-parental colony types, with a slow growing, ameboid and waxy mycelium, appeared in F_1 progenies from the $4cu^-$ and 148 crosses, respectively. Up to now, their origin is unknown. As far as the colony morphology is concerned, in all the crosses between H327 and the mutants, the progenies segregated in a 1:1 ratio wild type:mutant type, indicating the involvement of a single locus, though a certain amount of morphological heterogeneity was caused by the different genetic backgrounds.

The growth rate distribution was normal and fairly continuous for all crosses (data not shown). The progeny growth rate mean for H327 ♀ × H328 ♂ cross was 4·27 mm day^{-1} similar to those of the crosses H327 ♀ × 110 ♂ and H327 ♀ × $4cu^-$ ♂, that were 4·18 and 3·89 mm day^{-1}, respectively. The slowest progenies were those deriving from crosses involving the mutants 5II and 148, with values of 2·64 and 3·63 mm day^{-1}, respectively. The highest growth rate (4·80 mm day^{-1}) was that of F1 progeny from H327 ♀ × 186 ♂. Despite the differences in progeny means the parental mid point values were quite similar for all crosses. Moreover, in all crosses, growth rate generally seemed to be independent of the colony type. Although there was a tendency for the wild type progeny to grow faster, this was not the case for the crosses in which $4cu^-$ and 148 were the donors. When these progeny were of the mutant type, they more often fell in a high growth rate class than when they were of the wild type (data not shown).

CU production in F1 progenies

Fig. 2 shows for each cross H327 ♀ × mutant ♂ the distribution of CU production in liquid shake culture at 23°, and the distribution of colony types within production classes.

The H327 ♀ × H328 ♂ progeny shows a mean CPI of 1515, that is higher than both parental mean values: in fact, H327 and H328 exhibit CPI values of approximately 987 and 995, respectively. Generally the mean CPI in the F_1 progenies, where the mutants were donors, were lower than in F_1 progeny from the control H327 ♀ × H328 ♂ cross, with mean CPI values of approximately 533 for 5II, 622 for $4cu^-$, 921 for 110, 700 for 148 and 726 for 186 progenies. A statistically significant correlation between colony morphology and CU production exists in the crosses where $4cu^-$, 110, 148 and 186 mutants were the donors. The results plotted in Fig. 2 were evaluated using the χ^2 test, according to the expected independent segregation ratio of 1:1:1:1 for the phenotypical classes wild type/cu^+, wild type/cu^-, mutant type/cu^+ and mutant type/cu^-, respectively. The calculated χ^2 values (8·73 for $4cu^-$, 3·4 for 5II, 16·2 for 110, 11·93 for 148 and 186) indicated that the observed ratios agreed with the expected (at $P = 0{\cdot}05$, with three degree of freedom) only in the case of 5II. Thus colony morphology and CU production are associated in four of the five mutant crosses.

DISCUSSION

The aim of our work was to understand the role of CU in the pathogenicity of Dutch elm disease pathogens by producing mutants of the EAN *O. novo-ulmi* isolate H328, altered either just in CU production or in other functions potentially related to pathogenicity. We obtained five mutants that have been characterized for their growth on MEA and in liquid shake culture, and CU production in the culture filtrates, at various temperatures. The results show that all the mutants had maintained a normal ability to survive and to grow.

Of these mutants, $4cu^-$ was the only one selected because of its failure to produce CU after uv-mutagenesis. The others had been selected for different reasons: 5II because of its ability to grow on MEA at 33°, and the dark mutants because of their unusual colony phenotypes on MEA at 23°. Colony aspect has been always related to the aggressiveness of the isolate (Brasier, 1991). Surprisingly, both 5II and the three dark mutants were eventually also found to be unable to produce CU in the culture broth at 23°. 5II did produce CU when grown at 33°, as was demonstrated for *O. ulmi* in our previous work (Tegli *et al.*, 1994). Any contamination by *O. ulmi* can be excluded in the isolation of the mutants, since in our crosses the mutants always behaved as donors, while interspecific crosses between *O. novo-ulmi* × *O. ulmi* are possible only when *O. ulmi* is used as recipient. Moreover, they all belong to the same sexual mating type B of the parental isolate H328. Thus, it can be assumed that the production of CU was in some way related to the other functions. This seems to have been confirmed by the inheritance patterns of F1 progenies of the crosses between the *O. novo-ulmi* EAN isolate H327 and our mutants, when colony phenotype and *in vitro* CU production were examined. The ratio between the wild and the mutant colony phenotype in the progenies were always 1:1, suggesting that a single-locus mutation was involved. Moreover, a statistically significant relation exists between colony morphology and CU production in the F1 progenies, at least as far as $4cu^-$, 110, 148 and 186 mutants are concerned. Thus, the mutations appear to have involved a locus controlling both morphology and CU production. Actually, it is uncertain whether these mutants are affected in CU production or in its secretion, since also alterations on biochemical mechanisms of secretion of CU from fungal cells could determine a lower accumulation of the toxin in the culture broth. When 5II is considered, the correlation was not found and it is reasonable to suppose that thermotolerance (the character we selected for) involved further unknown metabolic changes one of which affected CU production.

When the mutants were inoculated onto clonal *U. carpinifolia*, partially resistant to Dutch elm disease, and *U. procera*, highly susceptible, 148 and 186 maintained the same pathogenicity level as the parent H328, whereas $4cu^-$ and 5II caused a much lower defoliation, on both *Ulmus* species. On the more susceptible elm species, the behaviour of the 110 mutant was closer to the parent H328. Moreover, naturally occurring non CU-producing mutants of *O. novo-ulmi* that are highly pathogenic are known (Brasier, Kirk & Tegli, 1994). The unexpected pathogenicity of these mutants might have been due to a particular interaction between the mutant and the host plant, leading to the *in planta* production of CU at levels sufficient for the expression of symptoms. Besides, *in vitro* CU production has already been shown to be a modulated process (Tegli *et al.*, 1994). It would be of primary importance to check how much CU is produced and the time

course of its production in the xylem vessels during the infection by both the mutants and the wild type isolates of *O. novo-ulmi* and *O. ulmi*. Whether, our mutants are deficient in producing or in secreting CU, and whether CU secretory pathway(s) can be influenced by unknown host factors are still under investigation. To this end attempts at targeted gene disruption using the gene encoding CU are in progress.

Our data do not exclude the possibility that CU has other roles in addition to being a wilting toxin, as previously shown and already suggested by Stringer & Timberlake (1993) and Brasier, Kirk & Tegli (1994). This protein has been detected by immunofluorescence tests not only in the cell wall of *O. novo-ulmi* (Svircev *et al.*, 1988) but also in that of *O. ulmi* and *O. piceae* (Scala *et al.*, 1994*b*). This last species is completely unable to produce CU in the culture filtrate in any condition of growth. It is a saprotroph on elm and it is considered the direct ancestor of the weak pathogen *O. ulmi*, from which *O. novo-ulmi* probably originated (Brasier, 1990). Thus, it would be reasonable to suppose that during evolution CU, a protein component of the cell wall, had been secreted as a casual event, and has subsequently assumed more than a structural function, as has been shown for other hydrophobins (Bell-Pedersen, Dunlap & Loros, 1992; Lauter, Russo & Yanofsky, 1992; Stringer *et al.*, Wessel, 1992).

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REFERENCES

Barrett, D. K. & Skidmore, A. M. (1975). Metabolite of *Ceratocystis ulmi* and its association with pathogenicity. *Transactions of the British Mycological Society* **65**, 469–475.

Bell-Pedersen, D., Dunlap, J. C. & Loros, J. J. (1992). The *Neurospora* circadian clock controlled gene *ccg-2*, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes Development* **6**, 2382–2394.

Beraha, L. E. & Garber, E. D. (1971). Avirulence and extracellular enzymes of *Erwinia carotovora*. *Phytopathologische Zeitschrift* **70**, 335–341.

Bernier, L. & Hubbes, M. (1990*a*). Mutations in *Ophiostoma ulmi* induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Canadian Journal of Botany* **68**, 225–231.

Bernier, L. & Hubbes, M. (1990*b*). Meiotic analysis of induced mutations in *Ophiostoma ulmi*. *Canadian Journal of Botany* **68**, 232–235.

Bowden, C. G., Hintz, W. F., Jeng, R., Hubbes, M. & Horgen, P. A. (1994). Isolation and characterization of the cerato-ulmin toxin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. *Current Genetics* **25**, 323–329.

Brasier, C. M. (1979). Dual origin of the recent Dutch elm disease outbreaks in Europe. *Nature* **281**, 78–80.

Brasier, C. M. (1981). Laboratory investigations of *Ceratocystis ulmi*. In *Compendium of Elm Diseases* (ed. R. J. Stipes & R. J. Campana), pp. 76–79. American Phytopathological Society: St Paul, Minnesota, U.S.A.

Brasier, C. M. (1986). Comparison of pathogenicity and cultural characteristics in the EAN and NAN aggressive subgroups of *O. ulmi*. *Transactions of the British Mycological Society* **87**, 1–13.

Brasier, C. M. (1990). China and the origin of Dutch elm disease: an appraisal. *Plant Pathology* **39**, 5–16.

Brasier, C. M. (1991). *Ophiostoma novo-ulmi* sp. nov., causative agent of the current Dutch elm disease pandemics. *Mycopathologia* **115**, 151–161.

Brasier, C. M. & Gibbs, J. N. (1973). Origin of the Dutch Elm Disease epidemic in Britain. *Nature* **242**, 607–609.

Brasier, C. M. & Gibbs, J. N. (1976). Inheritance of pathogenicity and cultural characters in *Ceratocystis ulmi*: hybridization of aggressive and non-aggressive strains. *Annals of Applied Biology* **83**, 31–37.

Brasier, C. M., Kirk, S. & Tegli, S. (1994). Naturally occurring non cerato-ulmin producing mutants of *Ophiostoma novo-ulmi* are pathogenic but lack aerial mycelium. *Mycological Research* **99**, 436–440.

Brasier, C. M., Lea, J. & Rawlings, M. K. (1981). The aggressive and non-aggressive strains of *Ceratocystis ulmi* have different temperature optima for growth. *Transactions of the British Mycological Society* **76**, 213–218.

Brasier, C. M., Takai, S., Nordin, J. H. & Richards, W. C. (1990). Differences in cerato-ulmin production between the EAN, NAN and non-aggressive sub-groups of *Ophiostoma ulmi*. *Plant Pathology* **39**, 231–236.

Buisman, C. (1932). *Ceratostomella ulmi*, de geschlachtelijeke vorm van *Graphium ulmi* Schwartz. *Review of Applied Mycology* **38**, 1–5.

Defago, G. & Kern, H. (1983). Induction of *Fusarium solani* mutants insensitive to tomatine, their pathogenicity and aggressiveness to tomato fruits and pea plants. *Physiological Plant Pathology* **22**, 29–37.

Gibbs, J. N. (1978). Intercontinental epidemiology of Dutch elm disease. *Annual Review of Phytopathology* **16**, 312–315.

Gibbs, J. N. & Brasier, C. M. (1973). Correlation between cultural characters and pathogenicity in *Ceratocystis ulmi* from Britain, Europe and America. *Nature* **241**, 381–383.

Guyot, M. (1921). Notes de pathologie vegetale. *Bulletin de la Societè de Pathologie Vegetale de France* **7**, 132–136.

Howell, C. R. (1976). Use of enzyme deficient mutants of *Verticillium dahliae* to assess the importance of pectolytic enzymes in symptom expression of *Verticillium* wilt of cotton. *Physiological Plant Pathology* **9**, 279–283.

Lauter, F. R., Russo, V. E. A. & Yanofsky, C. (1992). Developmental and light regulation of *eas*, the structural gene for rodlet protein. *Genes Development* **6**, 2373–2381.

McDonnell, K. (1962). Relationship of pectic enzymes and pathogenicity in the *Fusarium* wilt of the tomatoes. *Transactions of the British Mycological Society* **45**, 55–62.

Melin, E. & Nannfeldt, J. A. (1934). Researches into the blueing of ground woodpulp. *Sven Skogsvardsfoeren Tidkr* **32**, 397–616.

Puhalla, J. E. & Howell, C. R. (1975). Significance of endo-polygalacturonase activity to symptom expression of *Verticillium* wilt in cotton, assessed by the use of mutants of *Verticillium dahliae* Kleb. *Physiological Plant Pathology* **7**, 147–152.

Richards, W. C. (1993). Cerato-ulmin: a unique wilt toxin of instrumental significance in the development of Dutch elm disease. In *Dutch Elm Disease Research. Cellular and Molecular Approaches* (ed. M. B. Sticklen & J. L. Sherald), pp. 89–151. Springer-Verlag.

Richards, W. C. & Takai, S. (1973). Novel technique for isolating microstructures present in shake cultures of the fungus *Ceratocystis ulmi*. *Applied Microbiology* **26**, 443–444.

Richards, W. C. & Takai, S. (1984). Characterization of the toxicity of cerato-ulmin, the toxin of Dutch elm disease. *Canadian Journal of Plant Pathology* **6**, 291–298.

Scala, A., Tegli, S., Comparini, C., Mittempergher, L., Scala, F. & Del Sorbo, G. (1994*a*). Influence of fungal inoculum on cerato-ulmin production; purification of cerato-ulmin and detection in elm sucker cuttings. *Petria* **4**, 53–63.

Scala, A., Tegli, S., Del Sorbo, G. & Scala, F. (1994*b*). [Role of cerato-ulmin in *Ophiostoma piceae* – *O. ulmi sensu lato* system]. *Petria* **4**, 320–321 [in Italian].

Stevenson, K. J., Slater, J. A. & Takai, S. (1979). Cerato-ulmin, a wilting toxin of Dutch elm disease fungus. *Phytochemistry* **18**, 235–238.

Stringer, M. A., Dean, R. A., Sewall, T. C. & Timberlake, W. T. (1991). Rodletless, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Development* **5**, 1161–1171.

Stringer, M. A. & Timberlake, W. T. (1993). Cerato-ulmin, a toxin involved in Dutch Elm Disease, is a fungal hydrophobin? *The Plant Cell* **5**, 145–146.

Svircev, A. M., Jeng, R. S. & Hubbes, M. (1988). Detection of cerato-ulmin on aggressive isolates of *Ophiostoma ulmi* by immunochemistry and scanning electron microscopy. *Phytopathology* **78**, 322–327.

Takai, S. (1974). Pathogenicity and cerato-ulmin production in *Ceratocystis ulmi*. *Nature* **252**, 124–126.

Takai, S. (1980). Relationship of the production of the toxin cerato-ulmin to synnemata formation, pathogenicity, mycelial habitat and growth of *Ceratocystis ulmi* isolates. *Canadian Journal of Botany* **58**, 658–662.

Takai, S. & Hiratsuka, Y. (1984). Scanning electron microscope observations of internal symptoms of white elm following *Ceratocystis ulmi* infection and cerato-ulmin treatment. *Canadian Journal of Botany* **62**, 1365–1371.

Takai, S., Hiratsuka, Y., Krywienczyk, J., Richards, W. C. & Davies, Y. P. (1980). Evidence for the presence of the toxin cerato-ulmin in the synnemata head fluid of *Ceratocystis ulmi*. *Canadian Journal of Botany* **58**, 669–675.

Takai, S. & Richards, W. C. (1978). Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: isolation and some properties of cerato-ulmin from the culture of *C. ulmi*. *Phytopathologische Zeitschrift* **91**, 129–146.

Takai, S., Richards, W. C. & Stevenson, K. J. (1983). Evidence for the involvement of cerato-ulmin, the *Ceratocystis ulmi* toxin, in the development of Dutch elm disease. *Physiological Plant Pathology* **23**, 275–280.

Tegli, S., Comparini, C., Giannetti, C. & Scala, A. (1994). Effect of temperature on growth and cerato-ulmin production of *Ophiostoma novo-ulmi* and *O. ulmi*. *Mycological Research* **98**, 408–412.

Webber, J. F., Mitchell, A. G. & Smith, F. (1986). Linkage of the genes determining mating type and fungicide tolerance in *Ophiostoma ulmi*. *Plant Pathology* **35**, 512–516.

Wessels, J. G. H. (1992). Gene expression during fruiting in *Schizophyllum commune*. *Mycological Research* **96**, 609–620.

Yaguchi, M., Puszatai, M., Roy, C., Surewicz, W. K., Carey, P. R., Stevenson, K. J. Richards, W. C. & Takai, S. (1993). Aminoacid sequence, infrared and raman spectroscopy of Dutch elm disease toxin cerato-ulmin. In *Dutch Elm Disease Research. Cellular and Molecular Approaches* (ed. M. B. Sticklen & J. L. Sherald), pp. 152–170. Springer-Verlag.

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