Effect of temperature on cerato-ulmin production by Ophiostoma ulmi and O. novo-ulmi.

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Effect of temperature on growth and cerato-ulmin production of *Ophiostoma novo-ulmi* and *O. ulmi*

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Ophiostoma novo-ulmi and *O. ulmi*, previously named 'aggressive' and 'non-aggressive' subgroups of the old species *O. ulmi*, are distinguished by a wide range of morphological, molecular, genetical and physiological characters, observed both *in vivo* and *in vitro*. They show different virulence on elms of moderate resistance, that seems to be correlated with the ability to synthesize phytotoxic compounds, of which the most important is cerato-ulmin (CU). To date, *O. novo-ulmi* isolates have been considered the greatest producers of CU in contrast to *O. ulmi* isolates, that synthesize very little or no toxin. We demonstrate that the synthesis of CU is temperature-dependent and that the two species seem to have different temperature optima for its production. However, while the difference in CU production is linked to temperature, it is not a consequence of differences in fungus growth in liquid shake culture caused by these temperature differences.

In the northern hemisphere two Dutch elm disease pandemics are known to have occurred in this century. The first was in the 1920s to 1940s and the second began in the 1970s and is still in progress. Two distinct subgroups of the ascomycete *Ophiostoma ulmi* (Buisman) Nannf., termed 'non-aggressive' and 'aggressive', have been considered the causative agents of the first and the second epidemic, respectively (Gibbs & Brasier, 1973; Brasier, 1979, 1983, 1986*a*, *b*, 1988). The aggressive subgroup is divided into two races, the Eurasian (EAN) and the North American (NAN), according to their initial geographical distribution. Recently Brasier (1991) elevated each of these subgroups to species. The aggressive subgroup, still including the EAN and NAN races, was named *O. novo-ulmi Brasier*, distinguishing it from the non-aggressive subgroup, now *O. ulmi*. As suggested by the old terminology, aggressiveness is greater in *O. novo-ulmi* isolates than in *O. ulmi* on elms of moderate resistance (Brasier, 1982, 1986*a*, *b*, 1987*a*, *b*).

The two species show strong reproductive isolation and differ in a wide range of morphological, molecular, genetical and physiological characters (Bates, Buck & Brasier, 1993; Brasier, 1991; Brasier, Lea & Rawlings, 1981; Brasier *et al.*, 1990; Kile & Brasier, 1990). Included among these are marked differences in optimum temperature for linear growth rate and differences in CU production in liquid shake cultures at 23 °C. *O. novo-ulmi* isolates have growth temperature optima at 20–22°; *O. ulmi* at 27·5–30°. *O. ulmi* isolates are also able to grow at 33°, while *O. novo-ulmi* isolates are not.

Many papers have been written on CU, its chemical structure and its significance in the physiology of Dutch elm disease (Takai, 1974, 1978; Takai & Richards, 1978; Takai, 1980; Takai, Richards & Stevenson, 1983; Richards & Takai, 1984; Richards & Takai, 1988; Svircev, Jeng & Hubbes, 1988; Brasier *et al.*, 1990). It is a highly hydrophobic protein of about 8 kDa and 75 amino acid residues, among which eight cysteines are important in maintaining the tertiary structure by the formation of four disulphide bounds (Takai & Richards, 1978; Yaguchi *et al.*, 1992). Pure preparations of CU induce wilting if injected into elms susceptible to the pathogen, and reproduce both external and internal symptoms with the same host specificity when absorbed by elm cuttings (Takai, 1978; Takai & Richards, 1978; Scheffer, Heybroeck and Elgersma, 1980; Richards & Takai, 1984; Comparini *et al.*, 1993; Scala *et al.*, 1992). In contrast, the effects of purified CU on elm callus and cell cultures are not as clear, and sometimes appear to contradict the findings using elm cuttings (Sticklen, Bolyard & Cheng, 1990; Tegli *et al.*, 1993).

The role of CU in the pathogenic process is still unclear. One hypothesis concerning this role is based on the fact that *O. novo-ulmi* isolates growing *in vitro* in liquid shake culture are the greatest producers of CU, while *O. ulmi* isolates generally produce little or no toxin (Takai, 1980; Takai *et al.*, 1983; Svircev *et al.*, 1988; Brasier *et al.*, 1990).

In this paper we compare the influence of temperature on growth in liquid culture and CU production for these two species *in vitro*. CU synthesis was found to be a temperature-modulated process for all isolates of *O. novo-ulmi* and *O. ulmi* examined.

MATERIALS AND METHODS

Fungal cultures

Isolates were kindly provided by Professor Lorenzo Mittem-

Stefania Tegli a

Table 1. *Ophiostor*

Isolate
Ophiostoma novo-u
EAN race
H328
H322
SS21
I65
I66
I69
I71
I75
I110
I117
NAN race
RDT38
H351
182
Ophiostoma ulmi
E2
R21
Yu99
I79

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Table 1. *Ophiostoma novo-ulmi* and *O. ulmi* isolates used in this study

Isolate	Origin	Date of isolation	Source*
Ophiostoma novo-ulmi			
EAN race			
H328	Soviet Union	1978	CMB
H322	Czechoslovakia	1979	CMB
SS21	Italy	1985	LM
I65	Italy	1986	LM
I66	Italy	1986	LM
I69	Italy	1987	LM
I71	Italy	1987	LM
I75	Italy	1987	LM
I110	Italy	1987	LM
I117	Italy	1987	LM
NAN race			
RDT38	Germany	1975	CMB
H351	Belgium	1980	CMB
182	Italy	1980	LM
Ophiostoma ulmi			
E2	—	—	CvS
R21	Romania	1986	CMB
Yu99	Yugoslavia	1980	CMB
I79	Italy	1987	LM

* LM, L. Mittempergher; CMB, C. M. Brasier; CvS, Centraalbureau voor Schimmelcultures (Baarn, NL).

pergher (Centro per lo Studio della Patologia delle Specie Legnose e Montane, CNR, Firenze, Italy). The isolates, their classification and their geographical origin are listed in Table 1.

Isolates were artificially inoculated on elms in early spring of 1991. In autumn the young twigs were stored at −18° to prevent loss of fungus virulence. A small portion of the colonized wood was placed on MEA prepared by suspending 33·0 g of Oxoid Malt Agar Extract powder and 10 g Agar (Difco) in 1 l distilled water, and autoclaving at 0·6 atm for 20 min (Brasier, 1986*a*, *b*). Incubation was in the dark at 23°.

The modified Takai medium (TK) (Takai, 1978; Takai & Richards, 1978) used to grow the liquid cultures consisted of the following ingredients: 20 g sucrose, 2 g L-asparagine, 2 g yeast extract, 6 g KH_2PO_4, 200 mg $MgSO_4 . 7H_2O$, 80 mg $ZnSO_4 . 7H_2O$, 40 mg $FeCl_3 . 6H_2O$, distilled water to 1 litre, pH 4·80.

To produce the liquid cultures, Erlenmeyer flasks (100 ml), each with 30 ml of TK, were sterilized at 0·6 atm for 20 min. Starter cultures were obtained by inoculating each flask with a plug (3 mm diam.) from the edge of actively growing mycelium on MEA, and incubating at 23° in the dark, on a rotary shaker at 110 rpm for a week. From these cultures, other flasks (100 ml) with 30 ml of TK were inoculated, providing the following concentrations of blastoconidia: 10^1, 10^3 and 10^5 blastoconidia ml^{-1}. The cultures were then incubated at various temperatures (21°, 23°, 27°, 31° and 33°) in the dark, on rotary shakers at 110 rpm for 10 d. Since under these conditions both species of *Ophiostoma* developed mainly as continuously budding conidia, their growth was measured in terms of blastoconidia concentration.

The blastoconidia concentration was estimated by plating on MEA samples (100 µl for a 90 mm diam. Petri dish) from serial dilutions of liquid cultures. Sodium-deoxycholate had been added to MEA (100 mg l^{-1}), to limit radial growth (Bernier & Hubbes, 1989). The concentration of blastoconidia was calculated on the basis of the number of colonies formed on MEA plates after 3 d of incubation at 23° in the dark, and expressed as colony-forming units (c.f.u.) ml^{-1}, since each colony corresponded to one blastoconidium. For the starter cultures blastoconidia were counted with a haemocytometer.

Evaluation of CU production

Samples from liquid shake cultures were taken 7 and 10 d after inoculation. They were centrifuged for 30 min at 4° and 8000 *g* (r_{av} 8 cm), and the supernatant filtered through a 0·45 µm Millipore membrane. The filtrate was assayed for CU concentration, using the turbidometric method (Takai & Richards, 1978) with a Shimadzu spectrophotometer mod UV-160. The unit of measurement for CU concentration is the so-called Cerato-Ulmin Production Index (C.P.I.), turbidity at 400 nm × dilution factor × 100.

RESULTS

Growth in liquid shake cultures of O. novo-ulmi *and* O. ulmi *at various temperatures*

Fig. 1 shows the growth in liquid shake culture of *O. novo-ulmi*, isolate H328, and of *O. ulmi*, isolate E2, at various temperatures. Three initial concentrations of blastoconidia were used (10^1, 10^3 and 10^5 blastoconidia ml^{-1}). The cultures were grown at 21°, 23° and 33°, and c.f.u. ml^{-1} was estimated.

Blastoconidia production by the E2 isolate was not affected by temperature at any concentration: all the growth curves followed the same trend.

Isolate H328 was able to grow quickly at all temperatures, if starting from an inoculum concentration of 10^5 blastoconidia ml^{-1}. Cultures from lower initial cell densities, such as 10^3 blastoconidia ml^{-1}, and even more significantly 10^1 blastoconidia ml^{-1}, were slower in growth at 33° but not at 21° or 23°.

CU production at different temperatures

CU production by H328 and E2 at various temperatures after 7 and 10 d starting from a concentration of 10^5 blastoconidia ml^{-1} is shown in Fig. 2. *O. novo-ulmi* isolate H328 produced the greatest amount of CU on the 7th day of culture at 23°, and much less at the other temperatures, and particularly at 31° and 33°. *O. ulmi* isolate E2 also produced appreciable amounts of CU when incubated for 10 d at the higher temperatures; after 10 d at 33° it produced quantities of CU comparable to those of isolate H328.

Moreover, these data showed that the production of CU by *O. novo-ulmi* and *O. ulmi* was not a consequence of the temperature of 33° inhibiting fungal growth, since at the initial concentration of 10^5 blastoconidia ml^{-1} the growth of *O. novo-ulmi* and *O. ulmi* was not influenced by the temperatures considered (data not presented).

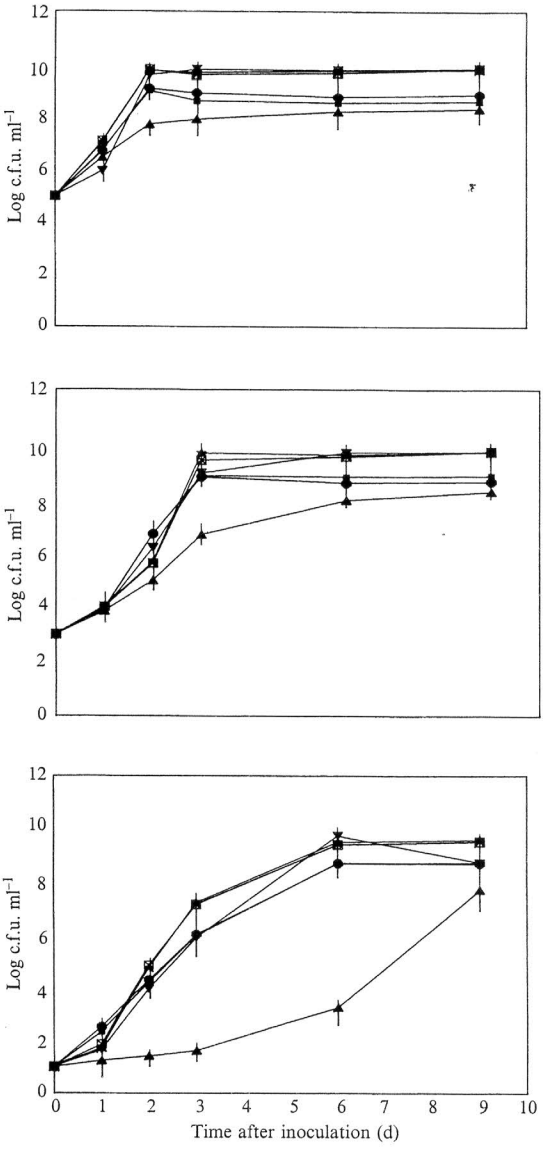

Fig. 1. Growth of *O. novo-ulmi* isolate H328 and *O. ulmi* isolate E2 in liquid shake culture at 21°, 23° and 33°, estimated 1, 2, 3, 6 and 9 d after inoculation and expressed as decimal logarithm of colony-forming units (c.f.u.) ml^{-1}. Different initial inocula were used for each species and at each temperature, and they are represented in the figure as follows: H328 at 21° (■), 23° (●) and 33° (▲) and E2 at 21° (⊠), 23° (★) and 33° (▼), at the initial concentrations of 10^5 (*a*), 10^3 (*b*) and 10^1 (*c*) blastoconidia ml^{-1}. Values are the mean ± S.E.M. of two experiments with three replicates each.

Examination of 13 isolates from *O. novo-ulmi* and 4 isolates from *O. ulmi* generally confirmed the findings for H328 and E2 (Table 2). The production rate of budding cells for all *O. novo-ulmi* isolates was similar at both temperatures, with a starting concentration of 10^5 ml^{-1} blastoconidia, except for some isolates (H322, I75, RDT38 and 182) that had grown on

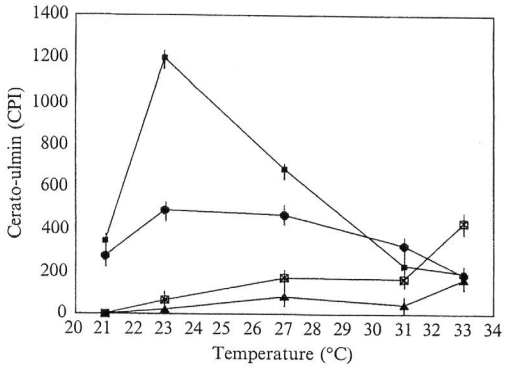

Fig. 2. CU production by *O. novo-ulmi* H328 isolate and *O. ulmi* E2 isolate grown in liquid shake culture at 21°, 23°, 27°, 31° and 33°. CU production was measured 7 and 10 d after inoculation and expressed as CPI (see Material and Methods). The symbols are as follows: 7 and 10 d CU production by H328 (■, ●) and by E2 (▲, ⊠), respectively. Each curve is the mean count of two experiments with three replicates each.

the 3rd day more slowly at 33° than at 23°. However, lesser differences of growth occurred on the 6th day. As far as CU production was concerned, all *O. novo-ulmi* isolates generally showed greater values of cerato-ulmin production index at 23° than at 33°, on both the 7th and the 10th days of culture. *O. ulmi* isolate generally grew well at both 23° and 33°, but they showed a more heterogeneous behaviour as far as CU production was concerned. Among the four isolates examined, only one (Yu 99) was unable to produce CU in any cultural condition. I79 produced very little CU and only at 23°. E2 and R21 produced appreciable quantities of CU, in particular when grown in liquid shake culture at 33°.

DISCUSSION

Up to now, linear growth rates at given temperatures and CU production have been considered two of the most important *in vitro* characteristics to distinguish isolates of *O. novo-ulmi* from those of *O. ulmi* (Brasier *et al.*, 1981; Brasier, 1986*a*; Brasier *et al.*, 1990; Kile & Brasier, 1990). However, both parameters have always been estimated only under particular standard conditions: solid culture (MEA) to measure the effect of temperature, and liquid shake culture at 23° to measure CU production. Under these conditions, *O. novo-ulmi* failed to grow at 33° and produce a large amount of CU, and *O. ulmi* grew well at 33° but produced little or no CU.

In our tests temperature did not reliably discriminate the two species when they grew in liquid shake culture (rather than on solid medium), since the *O. novo-ulmi* isolate H328 was inhibited by 33° only when starting inoculum was very low (10^1 and in part 10^3 blastoconidia ml^{-1}). With the other isolates of *O. novo-ulmi* at a starting concentration of 10^5 blastoconidia ml^{-1}, a minor inhibitory effect occurred only for some and mainly in the first few days of culture.

As far as regulation of *in vitro* CU production is concerned, Takai (1978) demonstrated the importance that nutritional

Table 2. Growth and CU production in liquid shake culture at 23° and 33° by some *O. novo-ulmi* (*a*) and *O. ulmi* (*b*) isolates

	Colony-forming units ml^{-1} ($\times 10^6$)*				Cerato-ulmin production index			
Days after inoculation...	3		6		7		10	
	23°	33°	23°	33°	23°	33°	23°	33°
(*a*)								
H328	476±62†	96±11	621±54	179±12	1225±120	172±71	597±151	203±65
H322	77±5	0·3±0·09	360±21	15±3	512±99	19±3	402±12	431±93
SS21	12±3	34±9	440±19	70±6	643±106	211±12	386±41	251±13
I65	156±22	227±43	319±4	443±54	414±114	145±28	406±37	166±35
I69	359±23	263±13	596±31	467±19	559±103	177±95	697±153	189±24
I71	154±17	146±21	299±24	342±33	423±102	174±13	599±120	170±31
I75	46±10	0·9±0·03	383±25	4±0·1	707±190	147±51	501±36	407±5
I110	256±13	271±34	532±18	641±29	516±65	215±212	441±49	164±51
I117	363±25	276±14	546±21	483±25	764±222	229±83	850±142	293±65
RDT38	30±7	0·2±0·02	521±9	32±7	405±204	0	311±54	0
H351	31±3	29±6	384±11	66±9	394±40	108±41	290±18	85±41
I82	215±13	8±1	534±27	75±3	1249±213	150±47	969±178	241±72
(*b*)								
E2	4713±69	6551±93	5809±79	6126±54	0	161±38	61±31	356±19
R21	1641±25	985±15	3224±87	2149±36	164±24	200±38	31±7	21±39
Yu99	679±11	497±7	1870±43	963±15	0	0	0	0
I79	772±25	843±14	1403±95	871±26	58±28	0	99±48	0

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

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

factors have in this process. The effect of temperature on the production of phaseolotoxin by *Pseudomonas syringae* var. *phaseolicola* is known: high temperatures disable the bacteria, promoting the transcription but not the translation of the specific mRNA for phaseolotoxin, as is demonstrated using chloramphenicol together with temperature shifts (Nüske & Fritsche, 1989).

We demonstrated that temperature affects CU production, independently of fungal cell multiplication. Temperature not only strongly influenced CU production, but most *O. novo-ulmi* and some *O. ulmi* isolates were able to synthesize large quantities of CU at 23° and 33°, respectively. The greater homogeneity in CU production shown by the *O. novo-ulmi* isolates suggests that this character may have been selected during evolution as a consequence for a greater virulence.

Since most isolates of both species synthesize a similar amount of CU under the right conditions, both *O. novo-ulmi* and *O. ulmi* should have the structural gene for CU synthesis, with some 'inducing factors', such as temperature, regulating the expression of this gene at a previously unknown level. It is, therefore, of great importance to study what are the other triggers of the toxin synthesis both in *in vitro* fungal culture and in the *in vivo* host–pathogen system, in order to contribute towards finding an effective method to control Dutch elm disease.

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