



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

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

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Effect of temperature on cerato-ulmin production by *Ophiostoma ulmi* and *O. novo-ulmi* / S. Tegli; C. Comparini; C. Giannetti; A. Scala. - In: MYCOLOGICAL RESEARCH. - ISSN 0953-7562. - STAMPA. - 98:(1994), pp. 408-412.

Availability:

This version is available at: 2158/352624 since:

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)

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

Isolate	Origin	Date of isolation	Source*
H328	Soviet Union	1978	CMB
H322	Czechoslovakia	1979	CMB
SS21	Italy	1985	LM
165	Italy	1986	LM
166	Italy	1986	LM
169	Italy	1987	LM
171	Italy	1987	LM
175	Italy	1987	LM
1110	Italy	1987	LM
1117	Italy	1987	LM
NAN race			
RDT38	Germany	1975	CMB
H351	Belgium	1980	CMB
182	Italy	1980	LM
<i>Ophiostoma ulmi</i>			
E2	—	—	CvS
R21	Romania	1986	CMB
Yv99	Yugoslavia	1980	CMB
179	Italy	1987	LM
* LM, L. Mitterperger; CMB, C. M. Brasler; 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°C to prevent loss of fungus virulence. A small portion of the colonized wood was placed on MFA Agar Extract powder and 3.0 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, 1986a, b). Incubation was in the dark at 23°C.

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 $MgSO_4 \cdot 7H_2O$, 80 mg $ZnSO_4 \cdot 7H_2O$, 40 mg $FeCl_3 \cdot 6H_2O$, 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 MFA, and incubating at 23°C 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 , 10^2 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 MFA samples (100 µl for a 90 mm diam, Petri dish) from

serial dilutions of liquid cultures. Sodium-deoxycholate had been added to MFA (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 MFA plates after 3 d of incubation at 23°C 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 , 10^2 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 10^2 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).

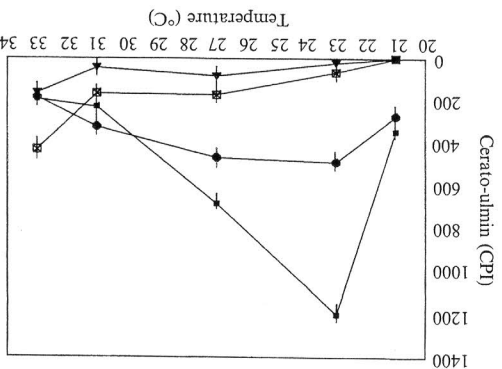


Fig. 2. CU production by *O. novo-ulmi* H328 isolate and *O. ulmi* E2 isolate grown in liquid shake culture at 21°, 23°, 27°, 31° and 33°. CU production was measured 7 and 10 d after inoculation and expressed as CPI (see Material and Methods). The symbols are as follows: 7 and 10 d CU production by H328 (■, ●) 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* (Brasler *et al.*, 1981; Brasler, 1986a; Brasler *et al.*, 1990; Kile & Brasler, 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 blastococonidia ml^{-1}). With the other isolates of *O. novo-ulmi* at a starting concentration of 10^5 blastococonidia 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

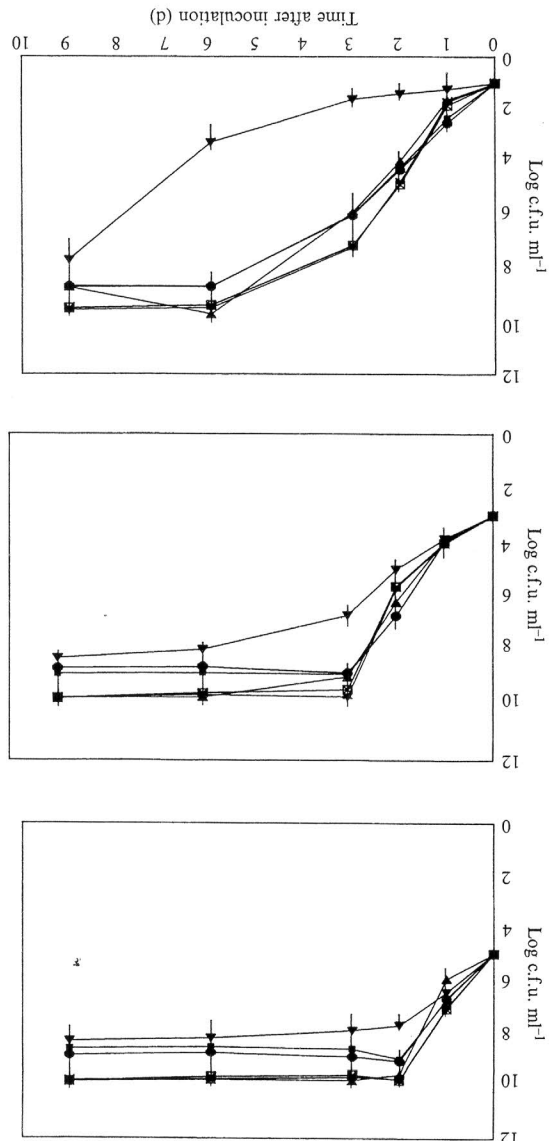


Fig. 1. Growth of *O. novo-ulmi* isolate H328 and *O. ulmi* isolate E2 in liquid shake culture at 21°, 23° and 33°, estimated 1, 2, 3, 6 and 9 d after inoculation and expressed as decimal logarithm of colony-forming units (c.f.u.) ml^{-1} . Different initial inocula were used for each species and at 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), 10^6 (b) and 10^7 (c) blastococonidia ml^{-1} . Values are the mean \pm 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} blastococonidia, except for some isolates (H322, I75, RD38 and I82) that had grown on

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

Days after inoculation ...	Colony-forming units ml ⁻¹ (× 10 ⁹)*				Cerauto-ulmin production index			
	23°	33°	23°	33°	23°	33°	23°	33°
H328	476 ± 624	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
165	156 ± 22	227 ± 43	319 ± 4	443 ± 54	414 ± 114	145 ± 28	406 ± 37	166 ± 35
169	359 ± 23	263 ± 13	596 ± 31	467 ± 19	559 ± 103	177 ± 95	697 ± 153	189 ± 24
171	154 ± 17	146 ± 21	299 ± 24	342 ± 33	423 ± 102	174 ± 13	599 ± 120	170 ± 31
175	46 ± 10	0.9 ± 0.03	383 ± 25	4 ± 0.1	707 ± 190	147 ± 51	501 ± 36	407 ± 5
110	256 ± 13	271 ± 34	532 ± 18	641 ± 29	516 ± 65	215 ± 212	441 ± 49	164 ± 51
117	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
182	215 ± 13	8 ± 1	534 ± 27	75 ± 3	1249 ± 213	150 ± 47	969 ± 178	241 ± 72
E2	4713 ± 69	6551 ± 93	5809 ± 79	6126 ± 54	164 ± 24	0	61 ± 31	356 ± 19
R21	1641 ± 25	985 ± 15	3224 ± 87	2149 ± 36	144 ± 24	200 ± 38	31 ± 7	21 ± 39
Y99	679 ± 11	497 ± 7	1870 ± 43	963 ± 15	0	0	0	0
179	772 ± 25	843 ± 14	1403 ± 95	871 ± 26	58 ± 28	0	99 ± 48	0

* Initial blastoconidia concentration was 10⁸ ml⁻¹.
 † Results are the mean ± s.e.m. of two experiments with three replicates each.

REFERENCES

Bates, M. R., Buck, K. W. & Braster, C. M. (1993). Molecular relationships between *Ophostoma ulmi* and the NAN and EAN races of *O. novo-ulmi* determined by restriction fragment length polymorphisms of nuclear DNA. *Mycological Research* **97**, 449-455.

Berner, L. & Hubbs, M. (1989). Mutations in *Ophostoma ulmi* induced by N-methyl-N'-nitro-N-nitrosoguanidine. *Canadian Journal of Botany* **68**, 225-231.

Braster, C. M. (1979). Dual origin of recent Dutch elm disease outbreaks in Europe. *Nature* **281**, 78-79.

Braster, C. M. (1982). Occurrence of three subgroups within *Ceratocystis ulmi*. In *Proceedings of the Dutch elm disease Symposium and Workshop* (ed. E. S. Kondo, Y. Hiratsuka & W. B. G. Dwyer), pp. 298-331. Manitoba Department of Natural Resources: Winnipeg, Manitoba.

Braster, C. M. (1983). The future of Dutch elm disease in Europe. In *Research on Dutch Elm Disease in Europe* (ed. D. A. Burdekin). *Forestry Commission Bulletin* **60**, 96-104. HMSO: London.

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

Braster, C. M. (1986b). The population biology of Dutch elm disease: its principal features and some implications for other host-pathogen systems. In *Advances in Plant Pathology*, vol. 5 (ed. D. S. Ingram & P. H. Williams), pp. 55-118. Academic Press: London.

Braster, C. M. (1987a). Recent genetic changes in the *Ophostoma ulmi* populations: the threat to the future of the elm. In *Populations of Plant Pathogens* (ed. M. S. Wolfe & C. E. Caten), pp. 213-226. Blackwell Scientific Publications: Oxford, UK.

Braster, C. M. (1987b). Some genetic aspects of necrotrophy with special reference to *Ophostoma ulmi*. In *Genetics and Plant Pathogenesis* (ed. P. R. Day & G. J. Jellis), pp. 297-310. Blackwell Scientific Publications: Oxford, UK.

Braster, C. M. (1988). Rapid changes in genetic structure of epidemic populations of *Ophostoma ulmi*. *Nature* **332**, 538-541.

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

Thanks are due to Professor Lorenzo Mittemperger for kindly providing fungal isolates, to Mr F. Ferrini and Mr A. Fagnani for technical assistance in maintaining the fungal cultures and Mr L. Bonuomo for technical assistance in drawing the figures.

Research was supported by the National Research Council

- Brasier, C. M. (1991). *Ophostoma novo-ulmi* sp. nov., causative agent of current Dutch elm disease pandemics. *Mycopathologia* **115**, 151-161.
- 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 subgroups of *Ophostoma ulmi*. *Plant Pathology* **39**, 231-236.
- Compartini, C., Tegli, S., Zanoli, C. & Scala, A. (1993). Search for new models in the study of Dutch elm disease and use of peroxidase activity as resistance marker. *Giornale Botanico Italiano* **127**, 179-180.
- Gibbs, J. N. & Brasier, C. M. (1973). Correlation between cultural characters and pathogenicity in *Ceratocystis ulmi* from Europe and North America. *Nature* **241**, 381-383.
- Kile, G. A. & Brasier, C. M. (1990). Inheritance and inter-relationship of fitness characters in progeny of an aggressive x non-aggressive cross of *Ophostoma ulmi*. *Mycological Research* **94**, 541-522.
- Niiske, J. & Fritsche, W. (1989). Phasolotoxin production by *Pseudomonas syringae* pv. *phaseolicola*: the influence of temperature. *Journal of Basic Microbiology* **29**, 441-447.
- Richards, W. C. & Takai, S. (1984). Characterization of the toxicity of cerato-ulmin, the toxin of Dutch elm diseases. *Canadian Journal of Plant Pathology* **6**, 291-298.
- Richards, W. C. & Takai, S. (1988). Production of cerato-ulmin in white elm following artificial inoculation with *Ceratocystis ulmi*. *Physiological and Molecular Plant Pathology* **33**, 279-285.
- Scala, A., Tegli, S., Compartini, C., Pancoski, A. & Mittemperger, L. (1992). [Biological activities of cerato-ulmin.] *Pertica* **2**, 222-223. [In Italian.]
- Schaffer, R. J., Heybroeck, H. M. & Elgersma, P. M. (1980). Symptom expression in elms after inoculation with combination of an aggressive and a non-aggressive strain of *Ophostoma ulmi*. *Netherlands Journal of Plant Pathology* **86**, 315-317.
- Accepted 16 September 1993
- Stieklen, M. B., Bolyard, M. G. & Cheng, J. (1990). Methods for *in vitro* selection of Dutch elm disease resistant American elms and expression of synthetic DNA encoding a Dutch elm disease toxin in *E. coli*. In *Proceedings of the International Energy Agency Symposium on the Exchange of Genetic Material* (ed. V. Steenackers & P. Smets), pp. 57-65. Poplar Research Center: Gerardsbergen, Belgium.
- Svircev, A. M., Jeng, R. S. & Hubbes, M. (1988). Detection of cerato-ulmin on aggressive isolate of *Ophostoma ulmi* by immunochromatography 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. (1978). Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: cultural factors affecting cerato-ulmin production by the fungus. *Phytopathologische Zeitschrift* **91**, 147-158.
- Takai, S. (1980). Relationship of the production of the toxin cerato-ulmin with synemata formation, pathogenicity, mycelial habit and growth of *Ceratocystis ulmi*. *Canadian Journal of Botany* **58**, 658-662.
- 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., Compartini, C., Cianetti, C. & Scala, A. (1993). Effect of cerato-ulmin on survival of *in vitro* elm callus and cells. *Giornale Botanico Italiano* **127**, 190-191.
- Yaguchi, M., Putzai, M., Roy, C., Surewicz, W. K., Carey, P. R., Stevenson, K. J., Richards, W. C. & Takai, S. (1992). Amino acid sequence infrared and raman spectroscopy of Dutch elm disease toxin cerato-ulmin. In *Abstracts of 1992 International Dutch Elm Disease Workshop*, Recent approaches to the Dutch Elm Disease problem' (ed. M. B. Stieklen & J. Sherald), p. 15. Michigan State University: East Lansing, U.S.A.