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Comparative determination of cerato-ulmin on cell surface and in mycelial extracts of pathogenic and non-pathogenic *Ophiostoma* species

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Cerato-ulmin (CU) presence was monitored on cell surface and quantitatively determined in mycelial extracts of the elm pathogens *Ophiostoma novo-ulmi* (races EAN and NAN) and *O. ulmi* and of the non-pathogenic *O. piceae*. CU was detected on the surfaces of *Ophiostoma novo-ulmi* (races EAN and NAN) and, for the first time, of the weak Dutch elm disease pathogen *O. ulmi* and the non-pathogen *O. piceae*. Quantitative determination of CU content in the mycelial extracts of the three species showed that high CU cellular content is associated with high CU content in culture filtrates. The content of CU in biomasses and in culture filtrates was influenced by temperature, growth phase and fungal species or race. CU synthesis occurred during the stationary phase and in the late logarithmic phase when fungi were grown at 23° and 32 °C, respectively. High temperatures of growth (32°) did not have a negative effect on the cellular CU content but severely hampered CU secretion in high CU-producers *O. novo-ulmi* isolates.

Cerato-ulmin (CU) is an 8 kDa protein only produced by *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, the causal agents of Dutch elm disease (DED). Richards (1993) reviewed the evidence which supports the involvement of CU in DED. An important point is that the production of CU by the two pathogens correlates with their virulence on elm: the highly virulent races EAN and NAN of *O. novo-ulmi* produce relatively large amounts of CU, whilst barely detectable amounts are produced by the weak pathogen *O. ulmi* (Brasier *et al.*, 1990; Brasier, 1991).

CU levels in liquid culture filtrates are temperature-dependent (Tegli *et al.*, 1994). At 23° *O. novo-ulmi* produces much more CU than *O. ulmi*, while at 31–33°, when the inoculum density is higher than 10^5 blastoconidia ml^{-1}, the amounts of CU detected in the cultures of the two species are similar but lower than those produced at 23° by *O. novo-ulmi*. Moreover, increase of temperature from 23 to 32° differentially influences growth of the two species on malt extract agar (Brasier, 1991) and virulence on elm (Smalley & Guries, 1993). CU is present in synnemata head fluid (Takai *et al.*, 1980) and on the hyphal surface of the race NAN of *O. novo-ulmi* (Takai & Hiratsuka, 1980; Svircev *et al.*, 1988).

The present paper reports the monitoring of CU in mycelial extracts and on cell walls of blastoconidia and germ-tubes of a worldwide collection of *Ophiostoma* isolates belonging to the pathogenic *O. novo-ulmi* and *O. ulmi* and to the non-pathogenic. *O. piceae*, a species closely related to *O. ulmi sensu* Hausner *et al.* (1993).

MATERIALS AND METHODS

Fungal cultures

The isolates of *Ophiostoma* used in the study, their classification, geographical origin and CU production index (c.p.i., Scala *et al.*, 1994) are reported in Table 1. Maintenance of isolates and culture conditions were as reported by Scala *et al.* (1994). Blastoconidia concentration was estimated by a haemocytometer or by determining the colony forming units (cfu) ml^{-1}. Given the differential effect of temperature on *Ophiostoma* species, determination of CU was carried out on fungi grown at both 23° and 32°.

Isolates of *Aspergillus niger* Tiegh., *Botrytis cinerea* Pers.:Fr., *Fusarium oxysporum* Schltdl.:Fr., *Penicillium chrysogenum* Thom and *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. to be used as putative negative controls were from the Culture Collection of the Institute of Plant Pathology of the University of Naples. *P. cactorum* was grown in V8 while all other fungi were grown in liquid Czapek–Dox medium.

Quantitative determination of CU in liquid cultures

CU production in liquid culture filtrate was determined according to the turbidimetric assay of Takai & Richards (1978) with slight modifications (Scala *et al.*, 1994).

Antiserum preparation

CU was purified from the culture filtrate of the *O. novo-ulmi* isolate H328 (Scala *et al.*, 1994). An antiserum against purified CU was obtained as previously described (Scala *et al.*, 1994).

Table 1. *Ophiostoma* isolates used in this study

	Source* and origin	Date of isolation	†Cerato-ulmin production μg ml^{-1}
Ophiostoma novo-ulmi			
EAN race			
H327	CMB, ex Czechoslovakia	1979	28 ± 5
H328	CMB, Soviet Union	1978	42 ± 7
AST27	CMB, Iran	Unknown	22 ± 4
H590	CMB, Germany	1980	31 ± 7
NAN race			
182	LM, Italy	1985	47 ± 8
H161	CMB, U.S.A.	Unknown	36 ± 5
H351	CMB, Belgium	1980	15 ± 2
RDT38	CMB, Germany	1975	15 ± 8
Ophiostoma ulmi			
E2	CvS	Unknown	0
R21	CMB, Romania	1986	6 ± 1
Yu99	CMB, ex Yugoslavia	1980	0
Ophiostoma piceae			
H988	CMB, Tadjikistan	1986	0
H1042	CMB, U.K.	1987	0
H2053	CMB, U.K.	1989	0

* LM, L. Mittempergher; CMB, C. M. Brasier; CvS, Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).
† Data are the means ± S.E. of numerous independent determinations performed in our laboratory on liquid shake cultures grown for 7 d at 23°.

Immunofluorescence (IF) assay

All isolates were grown in liquid-shake culture for 7 d as described by Scala *et al.* (1994). Samples (10 ml) were collected daily and immediately prepared for IF assay. Fungal biomasses, mainly constituted of blastoconidia and mycelial germlings, were collected by centrifugation at 5000 ***g*** for 10 min, washed three times with 0·9% (w/v) NaCl and once with distilled water and then resuspended to a concentration of 5×10^6 cfu ml^{-1}. For IF assay, 20 μl of this suspension were dispensed onto 12-well Multitest slides (Flow Laboratories, McLean, VA, U.S.A.) and dried at 60°. Wells were washed with PBS (Phosphate Buffered Saline 0·15 M, pH 7·2), filled with 20 μl of anti-CU antiserum diluted 1:100 in PBS and incubated for 2 h at room temperature. Conidia and vegetative hyphae of isolates of *Aspergillus*, *Botrytis*, *Fusarium*, *Penicillium* and *Phytophthora* were used as negative controls. Control wells were made by using pre-immune serum diluted 1:100. After three washings with PBS, wells were filled with 20 μl of a 1:40 dilution of goat anti-rabbit IgG-fluorescein isothiocyanate conjugate (Sigma) in PBS and incubated for 2 h at room temperature. Slides were washed with distilled water and examined with a Zeiss Axioskop microscope equipped for epifluorescence with an HBO 50 lamp (exciter blue filter, BP 450–490 and barrier filter LP 520).

The intensity of fluorescence was recorded on an arbitrary 0–3 scale, where 0 = no visible fluorescence; 1 = faint fluorescence; 2 = moderate fluorescence; 3 = intense fluorescence.

Quantitative ELISA

A freeze-dried aliquot (10 mg) of each sample was extracted for 2 min with 500 μl of 60% (v/v) ethanol and centrifuged for 5 min at 13000 ***g***. The supernatant was dried under vacuum. The residue was solubilized in 500 μl PBS and used for coating the wells of Falcon 3911 Microtest flexible plates (Becton Dickinson Labware, Oxnard, CA, U.S.A.). Test samples (50 μl) were added to triplicate wells for 3 h at 37°. The wells were washed three times with PBS and any remaining binding sites were blocked with 50 μl per well of 0·5% (w/v) gelatin in PBS for 2 h at 37°. Each assay included a standard calibration curve that was generated by preparing serial dilutions of CU, purified and quantified as previously described (Scala *et al.*, 1994), in PBS. Wells coated with 50 μl of 0·5% (w/v) gelatin in PBS served as blanks. Optical density values (OD) for the blanks were subtracted from all test values. Mycelial extracts from isolates of *Aspergillus*, *Botrytis*, *Fusarium*, *Penicillium* and *Phytophthora* were assayed as negative controls. Following saturation, 50 μl of anti-CU antiserum diluted 1:1000 in PBS containing 0·25% (w/v) gelatin and 0·2% (v/v) Tween 20 were added. Rabbit pre-immune serum was used in control wells. Plates were incubated overnight at 4° and washed three times with PBS. Fifty μl per well of goat anti-rabbit IgG–peroxidase conjugate (Sigma) diluted 1:2000 in PBS containing 0·25% (w/v) gelatin and 0·2% (v/v) Tween 20 were added and allowed to incubate for 3 h at 37°. After three washes with PBS, 150 μl per well of substrate solution (0·4 mg ml^{-1} *o*-phenylenediammine dihydrochloride, 0·012% (v/v) H_2O_2 in 0·1 M citrate–phosphate buffer pH 5·0) were added. Optical density at 492 nm (OD_{492}) was measured after 30 min of incubation with a Titertek Multiskan Plus MKII plate reader (Flow Laboratories Inc., McLean, VA, U.S.A.). The concentration of CU in individual samples was determined by comparing the mean OD obtained for triplicate wells to a standard curve using linear regression analysis. Standard calibration curves had a linear correlation coefficient $\geqslant 0{\cdot}98$ using purified CU over a concentration range of 0·01–10 μg ml^{-1}. The conditions of the assay were optimized to have an OD_{492} of about 1·0 for the wells containing the highest concentration of CU. Slopes similar to that of standard

curves were obtained with serial dilution curves of samples. The sensitivity of the ELISA for CU was 10 ng ml^{-1}. Negative samples always yielded an OD < 0·025.

RESULTS

All isolates of *Ophiostoma* grew in the yeast-like form at 23°. However, some blastoconidia produced germ-tubes. Therefore, the presence of CU was analysed in both blastoconidia and germlings. Table 2 summarizes the results of IF assays obtained for the isolates of *O. novo-ulmi* (races EAN and NAN), *O. ulmi* and *O. piceae* after 7 d of growth at 23°. All *Ophiostoma* isolates tested gave a positive reaction. Fluorescence intensity varied from moderate to intense, except in isolate Yu99 of *O. ulmi* where it was faint. Vegetative hyphae of *Aspergillus*, *Botrytis*, *Fusarium* and *Penicillium* did not show fluorescence, nor did the *Ophiostoma* control samples treated with preimmune serum.

Table 2 also reports the cellular content of CU as determined by quantitative ELISA. Five isolates of *O. novo-ulmi* showed amounts of CU significantly higher than those of *O. ulmi* and *O. piceae*. No CU was detected in extracts of the isolates of other genera. Figs 1 and 2 show the time-courses of CU cellular content and the fluorescence index of blastoconidia and germ-tubes for the isolates H328 of *O. novo-ulmi* (race EAN), 182 of *O. novo-ulmi* (race NAN), E2 of *O. ulmi* and H988 of *O. piceae*, grown at 23° and 32°. The analysis was conducted on these isolates because they have been used as representatives of the respective *Ophiostoma* species and races in our previous studies (Scala *et al.*, 1994; Tegli *et al.*, 1994; Tegli & Scala, 1996). The growth rate of the four isolates, determined as cfu ml^{-1}, and CU concentration in liquid culture, determined as c.p.i. and transformed in μg ml^{-1}, are also reported. The four isolates reached a plateau density after 2 d of growth at 23°. At 32° the isolate of *O. ulmi* was close to the plateau density after 2 d, when the other three isolates were only at the beginning of the logarithmic phase of growth. After 3 d the concentration of all four isolates was similar. In the cultures of EAN and NAN races grown at 23°, CU began to be detected when the plateau was reached, and became very abundant after 7 d. At 32°, no isolate produced CU before the fourth day. However, after 7 d, low amounts of CU (< 10 μg ml^{-1}) were produced by *O. novo-ulmi* and *O. ulmi*, but not by *O. piceae*.

Blastoconidia and germ-tubes of the four isolates always gave a weak positive reaction in the IF assay during the logarithmic phase of growth (Figs 1, 2). In races EAN and NAN, at 23°, the fluorescence index markedly increased after 3 or 4 d of growth when the cultures had already reached the plateau and began to produce CU in the liquid culture. At 32° an increase in the fluorescence index was observed after 2 d, during the exponential phase of growth. The fluorescence curves of *O. ulmi* and *O. piceae* appeared substantially flat with moderate signals in the first 4 d of culture at 23° and in the first 2 d at 32°. However, after 7 d the fluorescent signal of these two fungi increased to a value comparable to those of *O. novo-ulmi*.

The amount of cellular CU varied among the four isolates. The highest cellular CU amounts were detected after 3 or 4 d at both growth temperatures, with the exception of *O. ulmi* in which cellular CU increased slightly until day 7.

Temperature did not influence the CU content of isolates 182 and H988 which showed similar amounts of CU at 23 and 32°. For isolates H328 and E2, the amounts of CU detected at 32° were respectively about 8 and 4 times higher than those at 23°. After 3 or 4 d of growth at 23° detectable amounts of CU were already present in culture filtrates of isolates of EAN and NAN races whereas, at 32°, CU was detected in filtrates of both EAN and NAN races of *O. novo-ulmi* and in *O. ulmi* only at day 7.

Table 2. Cellular content and surface presence of cerato-ulmin (CU) in the EAN and NAN races of *Ophiostoma novo-ulmi*, *O. ulmi* and *O. piceae**

		Surface presence (IF)‡	
	Cellular content†	Blastoconidia	Germ-tubes
Ophiostoma novo-ulmi			
EAN race			
H327	85·83 ± 3·65[a]	3	3
H328	30·70 ± 1·49[b]	2	2
AST27	29·91 ± 10·94[b]	2	2
H590	6·10 ± 0·71[cd]	3	3
NAN race			
182	13·73 ± 0·85[c]	2	2
H161	39·18 ± 4·16[b]	2	3
H351	8·62 ± 0·61[cd]	3	3
EDT38	3·99 ± 0·27[cd]	2	2
Ophiostoma ulmi			
E2	1·41 ± 0·10[d]	3	3
R21	1·32 ± 0·06[d]	2	2
Yu99	0·75 ± 0·05[d]	1	3
Ophiostoma piceae			
H988	0·67 ± 0·01[d]	3	3
H1042	0·63 ± 0·02[d]	3	3
H2053	0·85 ± 0·05[d]	3	3
Aspergillus niger§	0	0‖	0¶
Botrytis cinerea§	0	0‖	0¶
Fusarium oxysporum§	0	0‖	0¶
Penicillium chrysogenum§	0	0‖	0¶
Phytophthora cactorum§	0	n.d.	n.d.

* Mixtures of the shake liquid cultures collected at 7 d after inoculation.

† Expressed as ng of CU mg^{-1} lyophilized fungal biomass ± S.E., assayed by ELISA; means followed by the same letter do not significantly differ at $P = 0·05$ according to the Tukey's test.

‡ Expressed as fluorescence index, determined on a scale, where 0 = no visible fluorescence; 1 = faint fluorescence; 2 = moderate fluorescence; 3 = intense fluorescence. Data are based on observations of 30 microscopy fields in three independent experiments.

§ Used as controls.

‖ Conidia.

¶ Vegetative hyphae.

n.d. not determined.

DISCUSSION

In this paper we demonstrate that CU is present in mycelial extracts and on cell surfaces of highly pathogenic, weakly pathogenic and non-pathogenic species of *Ophiostoma*.

IF assays indicate that comparable amounts of CU are present on surface of all isolates tested with the only exception of blastoconidia of isolate Yu 99 of *O. ulmi*, which displays a faint but still clear signal. The presence of CU in

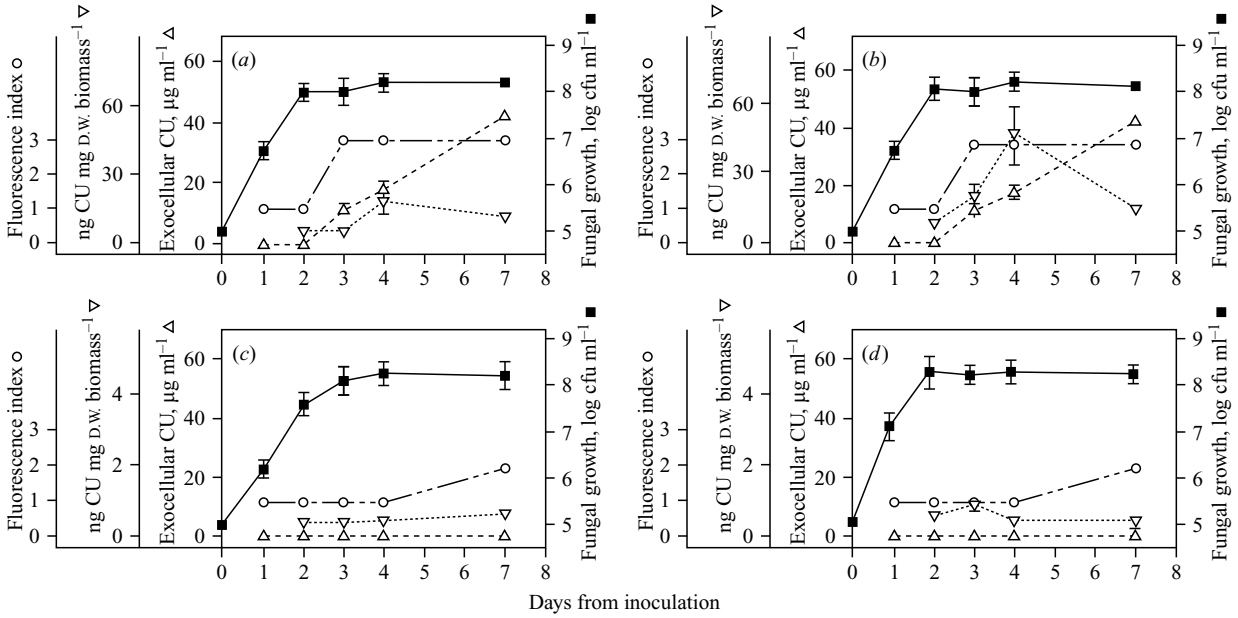

Fig. 1. Time-course determination of CU on cell surface, in mycelial extracts and in culture filtrates of isolates (*a*) H328 (*O. novo-ulmi* EAN race), (*b*) 182 (*O. novo-ulmi* NAN race), (*c*) E2 (*O. ulmi*) and (*d*) H988 (*O. piceae*) grown at 23°. Bars represent standard error.

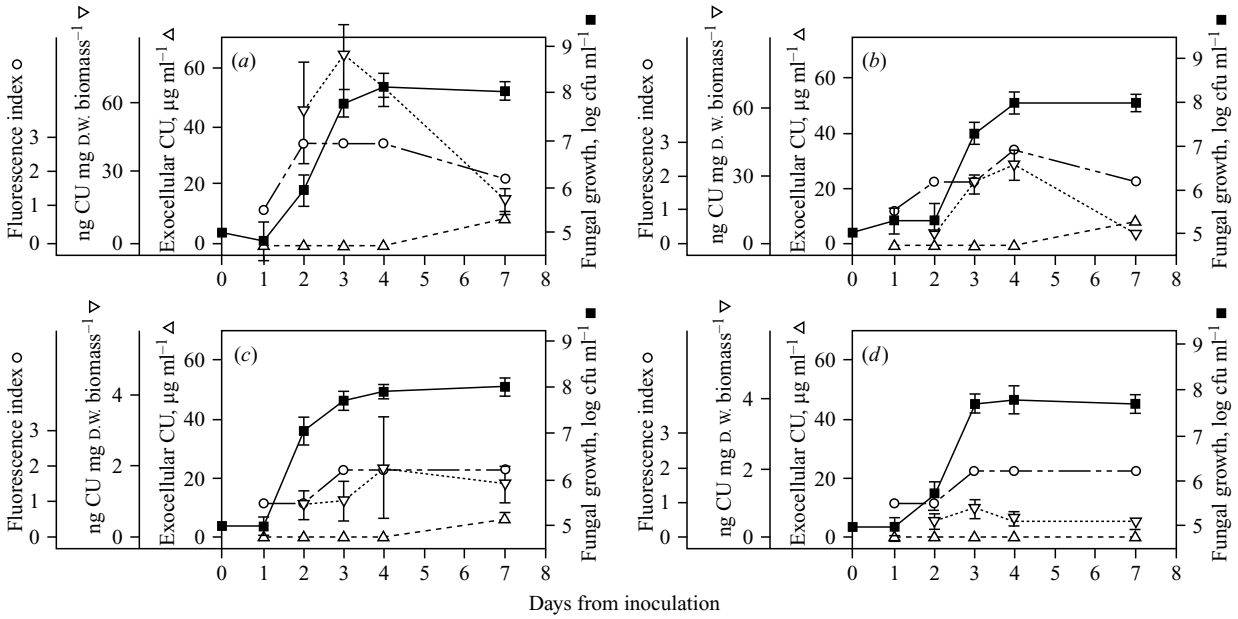

Fig. 2. Time-course determination of CU on cell surface, in mycelial extracts and in culture filtrates of isolates (*a*) H328 (*O. novo-ulmi* EAN race), (*b*) 182 (*O. novo-ulmi* NAN race), (*c*) E2 (*O. ulmi*) and (*d*) H988 (*O. piceae*) grown at 32°. Bars represent standard error.

mycelial extracts and on the surface of the non-pathogenic *O. piceae* may be meaningful in defining the functions of this protein. Generally, CU is associated with DED symptoms and considered a virulence factor of the fungus (Brasier *et al.*, 1990; Brasier, 1991; Richards, 1993).

The amounts of CU in blastoconidia and germ-tubes of races EAN and NAN were higher than those found in the other two species when grown at 23° for 7 d. By contrast, similar levels were observed on the surface of all *Ophiostoma* isolates tested by IF. This difference could depend on the fact that IF reveals only the CU present on the cell wall surface while ELISA detects the CU present in the whole cell.

Takai & Hiratsuka (1980) showed by SEM analysis that the cell surface of two *Ophiostoma* CU-produder isolates had some characteristics typical of the CU in aqueous solution, whereas two isolates which produced negligible amounts of CU did not have these properties. Furthermore, Svircev *et al.* (1988) observed, by associating immunocytochemistry and SEM, a strong gold label on the surface of two American aggressive isolates of *O. ulmi*, now classified as *O. novo-ulmi*, race NAN, whereas a non-aggressive isolate of *O. ulmi* presented only a very low concentration of gold label.

In this work we found that eight isolates belonging to the races EAN and NAN of *O. novo-ulmi* which were high CU-

producers showed fluorescence signals comparable to those of the low or nil CU-producer isolates of *O. ulmi* and *O. piceae*. At 23° only the isolate Yu 99 appeared to have a low level of fluorescence (value of 1) while all the other isolates had values of 2 or 3.

Differences between our data and those reported in the literature could simply reflect differences in the isolates tested. Moreover, in our work, fungi were grown in liquid shake cultures and not on solid media. Liquid shake cultures favour both the production of CU *in vitro* and massive formation of blastoconidia as it occurs in xylem vessels during the pathogenetic phase of DED (Brasier, 1988). Moreover, we found that other factors, such as growth phase and temperature can also have a relevant influence on surface content of CU (Figs 1, 2).

The higher cellular CU content in *O. novo-ulmi* compared to *O. ulmi* and *O. piceae* may reflect a difference in CU synthesis regulation in the three species grown under standard culture conditions (liquid shaken culture at 23°). Relatively high CU contents in mycelial extracts are associated with secretion in culture filtrate. Low cellular contents in *O. ulmi* and *O. piceae* isolates only partly explain the absence of CU in culture filtrate which may depend on differences in CU sorting and secretion mechanisms occurring in the three species.

The temperature influenced the cell content in the biomasses and in the culture filtrates and the relationship between the curves of the cellular CU content and of the growth phase. In the three *Ophiostoma* species at 23° the peak of cellular CU was observed one or more days after the isolates reached the stationary phase of growth, while at 32° the peak occurred in the late logarithmic phase. The 1-d lag in growth of the four isolates at 32° could depend on the necessity of adaptation to a non-optimal growth temperature. In EAN race a relevant amount of CU accumulated in the cell during this phase whereas in NAN race CU content seems to be unaffected by temperature. Moreover, the cellular CU content seemed to be higher at 32° than at 23° and correspondently a delay in accumulation of CU in the filtrates was observed. These results may indicate that temperature affects both CU synthesis regulation and the mechanisms of CU secretion. Thus, CU synthesis is anticipated under stress conditions supporting the hypothesis that it could be involved in protection of fungi from environmental stresses (Wu *et al*., 1989). Moreover, since the increase of temperature negatively affects CU production in culture filtrates of *O. novo-ulmi* but not cellular CU content, we can conclude that mechanisms of CU secretion are severely hampered at temperatures near the upper limit for growth.

In this paper we show for the first time that CU occurs on the cell surface of different species of *Ophiostoma*, regardless of their pathogenticity towards elm. It has been found that CU is a hydrophobin (Stringer & Timberlake, 1993; Bowden *et al*., 1994; Templeton *et al*., 1994) that may be involved in recognition phenomena or in functions other than the toxic activity specific towards elm. The detection of CU on the cell wall surface of *O. piceae*, a saprotroph on elm, confirms this hypothesis.

It has been demonstrated (Hausner *et al*., 1993), by analysis of partial nucleotides of SSrDNA and LSrDNA, that *O. piceae* is a species closely related to *O. ulmi* (*sensu lato* including both *O. ulmi* and *O. novo-ulmi*). Our results indicate the presence of pronounced differences in CU production and secretion between *O. novo-ulmi* and *O. ulmi* and marked similarities between *O. ulmi* and *O. piceae*, thus suggesting a close phylogenetic relationship between the latter two species. On the basis of RFLPs analysis of nuclear DNA, Bates *et al*. (1993) suggested the evolutionary direction: *O. ulmi* → *O. novo-ulmi* EAN → *O. novo-ulmi* NAN. We can hypothesize that *O. ulmi* derives from *O. piceae* or from a common non-pathogenic ancestor. *O. ulmi* was the first species inhabiting elm that, by developing the ability to secrete CU, had a potential evolutionary advantage because CU was selectively toxic to elm.

From our results it appears that the understanding of the mechanism of CU sorting and secretion is of much interest in studies on DED and evolutionary relationships among *Ophiostoma* species. Recently, mutants which do not produce CU in culture but are still able to cause DED have been identified (Brasier *et al*., 1994; Tegli & Scala, 1996). These mutants represent ideal candidates for a better elucidation of the mechanisms of CU synthesis and secretion and of the role of CU as a pathogenicity factor in DED fungi.

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