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Dutch elm disease progression and quantitative determination of cerato-ulmin in leaves, stems and branches of elms inoculated with *Ophiostoma novo-ulmi* and *O. ulmi*

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The progression of disease and infection was monitored following inoculation of elm plants with the Dutch elm disease (DED) agents *Ophiostoma novo-ulmi* or *Ophiostoma ulmi*. The disease was more severe in plants inoculated with *O. novo-ulmi*, with a rapid appearance of foliar symptoms just 10 days after inoculation. The degree of progression of the two pathogens in elms was similar except for the plant sections below the inoculation point, where *O. novo-ulmi* grew more than *O. ulmi*. Neither pathogen could be reisolated from symptomatic leaves. The concentrations of cerato-ulmin (CU) in stems, branches and leaves was quantitatively determined by enzyme-linked immunosorbent assay (ELISA). Foliar symptoms were associated with high CU contents. The contents of CU were not significantly different in branches bearing symptomless or wilted leaves. These findings support the hypothesis that CU is produced by the pathogens in xylem vessels of stems and branches, and is then translocated into leaves where it accumulates, causing DED symptoms.

INTRODUCTION

Since the phytotoxic compound cerato-ulmin (CU) was detected in shake cultures of *Ophiostoma ulmi* (Syn. *Ceratocystis ulmi*) [*7*, *28*], much evidence has been produced indicating that CU plays a major role in Dutch elm disease (DED). CU is released in *in vitro* shake culture media only by the weakly pathogenic species *O. ulmi* (Buisman) Nannfeldt and the highly pathogenic *Ophiostoma novo-ulmi* Brasier [*14*]. Toxin production by the DED pathogens is correlated with their pathogenicity levels on elm [*6*, *8*]. CU displays some specificity to elm [*33*], producing internal and external symptoms similar to DED [*28*, *31*], causing a reduction in transpiration of elm cuttings, increasing the respiration rate and electrolyte loss from elm leaf tissue [*16*, *18*], and inhibiting division in tissue cultured cells of the susceptible host species *Ulmus carpinifolia* Gleditsch [*35*]. CU is present at detectable levels

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Abbreviations used in text: CU, cerato-ulmin; DED, Dutch elm disease.

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in naturally and artificially infected elms [*19*, *34*] and is readily absorbed by elm sucker cuttings, causing symptoms identical to those of DED [*16*, *21*].

Recently, a number of observations led to a reassessment of the role of CU as a major virulence factor of the DED pathogens. CU was found to be a structural component of the hyphal surface of both *O. novo-ulmi* and *O. ulmi* and of the non-pathogen of elm *Ophiostoma piceae* [*22*, *25*, *30*, *32*]. However, *O. piceae* did not secrete CU in culture under any culture conditions tested [*22*]. The toxin is believed to be involved in the developmental processes of DED fungi as a cell surface hydrophobin [*3*, *24*]. Wu *et al.* [*43*] reported that CU may protect DED fungi from elm phytoalexins. Interestingly, both natural and laboratory-induced non-CU producing mutants, as well as a mutant disrupted in the CU-encoding gene of *O. novo-ulmi*, were still pathogenic [*4*, *7*, *37*]. Production of the toxin in liquid shake cultures by *O. novo-ulmi* and *O. ulmi* critically depends on environmental factors such as temperature [*36*], and is reduced following infection with virus-like d-factors [*26*].

In order to evaluate further the role of CU in DED symptom expression, the content of the toxin in leaves and in stems and branches of plants artificially inoculated with *O. novo-ulmi* or *O. ulmi* was monitored in parallel with the progression of infection and appearance of DED symptoms.

MATERIALS AND METHODS

Fungal cultures

The origins of the isolate H328 of *O. novo ulmi* race EAN and isolate E2 of *O. ulmi* have been described previously [*36*, *37*]. Blastoconidial suspensions were prepared according to Scala *et al.* [*21*].

Elm inoculations and pathogenicity index

Elm hybrid clone FL025 (*Ulmus elliptica* × *U. pumila* S15) was used as the host plant [*27*]. The reactions of FL025 are close to that of clone 'Commelin'; it is rather susceptible to *O. novo-ulmi* but resistant to *O. ulmi*. Trials were conducted at the Antella Nursery, Florence, Italy. Three-year-old plants, whose height varied from 2·5 to 3·0 m, were inoculated at a height of 1·7–2·0 m above ground level. A cut was made in the sapwood of the main stem with a sterile scalpel blade, followed by application of 100 µl of a suspension of 10^6 blastoconidia ml^{-1}. The trees immediately absorbed the suspension and the wounds were then sealed with tape. Twelve plants were inoculated with isolate H328 (*O. novo-ulmi* race EAN), and twelve with the isolate E2 (*O. ulmi*). The two groups of twelve plants were divided into subgroups of three plants, and each subgroup was labelled with the numbers 5, 10, 34, and 60 to indicate on which day after inoculation plants were to be analysed. Plants mock inoculated with distilled water served as controls. Disease development was expressed as percentage of chlorotic, wilted leaves and/or defoliation of the crown, where 0 % = healthy tress and 100 % = trees completely wilted or defoliated.

Reisolation of pathogens

At 5, 10, 34 and 60 days after inoculation, the stem of each tree was cut into three parts of the same length (about 90 cm): the upper section (above the inoculation point), the

middle section (below the inoculation point) and the lower section. A 4 cm thick cylinder was cut from the middle of each section and about 30 pieces of tissue (2–5 mm^3) of xylem and adjacent tissues were aseptically removed from the tissues underneath the cortex and placed on malt extract agar (Oxoid). Reisolation of the pathogens was also performed from leaf sections of a total of 90 symptomatic or symptomless leaves per pathogen. *Ophiostoma novo-ulmi* and *O. ulmi* were identified by colony characteristics [*6*].

Extraction of CU from leaves, stems and branches

Random samples of ten symptomatic or symptomless leaves were lyophilized and stored in plastic bags under vacuum at −70 °C. Each bag contained a label describing the time after inoculation, the pathogen used for inoculation and the elm section from which leaves were collected. One gram of lyophilized leaves was homogenized at 4 °C in the presence of quartz sand, in 10 ml of the following buffer: Tris-HCl 30 mM, pH 6·8, $Mg(CH_3COO)_2$ 5 mM, KCl 50 mM, 2-mercaptoethanol 7·5 mM, Tween 20 0·5 % (v/v), polyvinylpyrrolidone K25 0·1 % (w/v), EDTA 2 mM. The homogenate was centrifuged at 11 000 ***g*** for 10 min at 4 °C and the supernatant was lyophilized and stored at −70 °C until required for enzyme-linked immunosorbent assay (ELISA).

Debarked stems and branches were chipped in a Bio80 chopper. Chips were lyophilized and then finely powdered in an electric cereal mill. Wood powder was stored in plastic bags under vacuum at −70 °C. CU was extracted from wood powder according to the procedure of Takai *et al.* [*34*] with slight modifications. Ten grams of wood powder were suspended in 200 ml of 0·05 % (v/v) Tween 20, and gently stirred overnight at 22 °C. The suspension was centrifuged at 11 000 ***g*** for 15 min at 4 °C. The supernatant (30 ml) was 60 % (w/v) saturated with $(NH_4)_2SO_4$, and again stirred overnight at 22 °C. The solution was centrifuged at 11 000 ***g*** for 15 min at 4 °C, and the pellet freeze-dried and stored at −70 °C until required for ELISA.

Quantitative ELISA

An antiserum specific for CU from *O. novo-ulmi* EAN isolate H328 was used [*21*]. A freeze-dried fraction (15 mg) of each leaf or wood sample was extracted for 2 min with 500 µl of 60 % (v/v) ethanol and centrifuged at 13 000 ***g*** for 5 min at 4 °C. The supernatant was collected and dried under vacuum. The CU-containing residues were solubilized in 500 µl PBS and used to coat triplicate wells (50 µl per well) of Falcon 3911 Microtest flexible plates (Becton Dickinson Labware, Oxnard, CA, U.S.A.), which were incubated for 3 h at 37 °C. The wells were washed three times with PBS, and any remaining binding sites were blocked with 50 µl 0·5 % (w/v) gelatin in PBS for 2 h at 37 °C. Each assay included a standard calibration curve that was generated by preparing serial dilutions of homogeneous CU in PBS. Wells coated with 50 µl of extracts from plants inoculated with distilled water served as blanks. Following saturation, 50 µl anti-CU antiserum diluted 1 : 1000 in PBS containing 0·25 % (w/v) gelatin and 0·2 % (v/v) Tween 20 were added. Rabbit preimmune serum was used in control wells. Plates were incubated overnight at 4 °C and washed three times with PBS. Fifty microlitres of goat antirabbit IgG-peroxidase conjugate (Sigma) diluted 1 : 2000 in PBS containing 0·25 % (w/v) gelatin and 0·2 % (v/v) Tween 20 were added to each

well and incubated for 3 h at 37 °C. After three washes with PBS, 150 µl of substrate solution (0·4 mg ml^{-1} *o*-phenylenediamine 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 incubation with a Titertek Multiskan Plus MKII plate reader (Flow Laboratories Inc., McLean, VA, U.S.A.). OD_{492} for the blanks were subtracted from all test values. The concentration of CU in individual samples was determined by comparing the mean OD_{492} 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 give an OD_{492} of about 1·0 for the wells containing the highest concentration of CU. Slopes similar to that of the standard curve were obtained with serial dilution curves of samples. The sensitivity of the ELISA for CU was 10 ng ml^{-1}. All samples were tested together in a single experiment and each test was repeated three times in independent experiments. Negative samples always yielded an $OD_{492} \leqslant 0{\cdot}025$.

RESULTS

Pathogenicity

The symptoms of DED were more severe in elm plants inoculated with *O. novo-ulmi* than in plants inoculated with *O. ulmi* (Fig. 1). Ten days after inoculation, 8 % of leaves of plants inoculated with *O. novo-ulmi* were chlorotic or wilted. This value

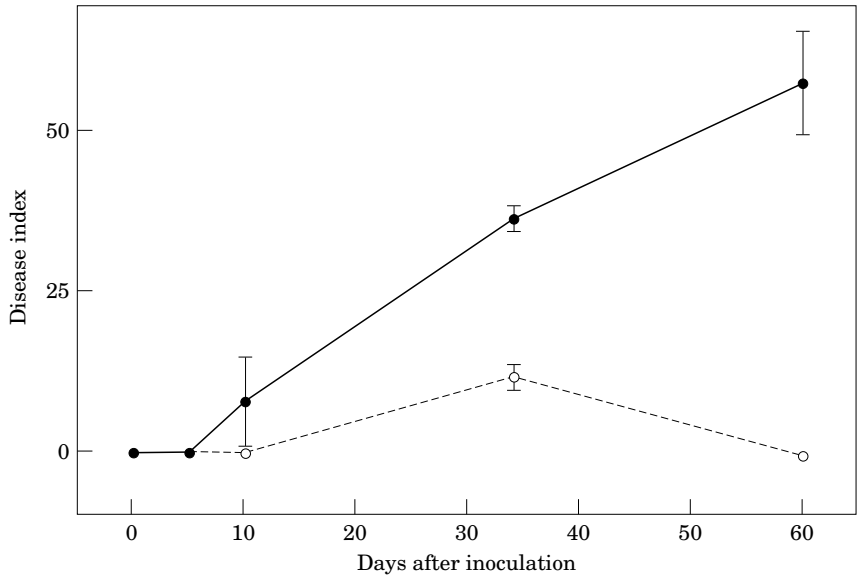

FIG. 1. Progress of Dutch elm disease (DED) in elm plants following artificial inoculation with *Ophiostoma novo-ulmi* isolate H328 (●) or *O. ulmi* isolate E2 (○). Date of inoculation: 13 May 1994. Disease index represented the mean percentage of the crown showing chlorotic, wilted leaves and/or defoliation. Vertical bars represent S.E.

increased to 59% 60 days after inoculation. Twenty-one days after inoculation, wilt symptoms began to be observed on branches below the inoculation point (data not shown). Starting from day 25, numerous leaves dried, and some of them abscised. In plants inoculated with *O. ulmi*, 34 days after inoculation, 12% of crown showed chlorotic and/or wilted leaves. Subsequently, symptomatic leaves fell and a complete recovery from disease took place. At 60 days no wilt symptoms were present nor crooking of the tips and death of small shoot tips occurred; the recorded disease level was 0%. Control plants did not show any DED symptoms.

Reisolation of pathogens

On day 5 after inoculation, *O. novo-ulmi* and *O. ulmi* were reisolated only from the upper stem sections (Fig. 2). On day 10 both fungi had spread into the middle and lower sections. No differences in reisolation frequencies were observed between the isolates. After 34 days, *O. novo-ulmi* was reisolated from all plant sections (upper, middle and lower), with percentages ranging from 65% in the lower to 100% in the middle section. In contrast, *O. ulmi* was reisolated from middle and lower sections with frequencies of 12% and 5%, respectively.

The two fungi were never reisolated from either symptomatic or symptomless leaves, nor from control plants.

Extraction and assay of CU

The variation of CU content in leaves, stems and branches of plants inoculated with *O. novo-ulmi* and *O. ulmi* is shown in Fig. 3. CU was always found in relatively low concentration ($\leqslant 0{\cdot}13 \pm 0{\cdot}08$ ng mg^{-1} d. wt tissue) in stems and branches of plants inoculated with *O. novo-ulmi* or *O. ulmi* whether or not the plants bore symptomatic or symptomless leaves. No CU was detected in stems and branches of plants mock inoculated with distilled water.

In symptomless leaves the highest level of CU was found after 60 days ($0{\cdot}52 \pm 0{\cdot}20$ ng mg^{-1} d. wt tissue from plants inoculated with *O. ulmi* and $0{\cdot}24 \pm 0{\cdot}10$ ng mg^{-1} d. wt tissue from plants inoculated with *O. novo-ulmi*). In plants inoculated with *O. ulmi*, the CU content of symptomatic leaves, which was present only in the sample collected on day 34, was $3{\cdot}45 \pm 1{\cdot}04$ ng mg^{-1} d. wt tissue. In plants inoculated with *O. novo-ulmi*, the CU content in symptomatic leaves was $1{\cdot}21 \pm 0{\cdot}47$ and $7{\cdot}48 \pm 3{\cdot}87$ ng mg^{-1} d. wt tissue after 10 and 34 days, respectively. Sixty days after inoculation leaves were completely dried and CU concentration was $0{\cdot}23 \pm 0{\cdot}05$ ng mg^{-1} d. wt tissue. No CU was detected in leaves of plants mock inoculated with distilled water.

The cumulative CU contents of leaves, stems and branches is shown in Table 1. Statistical analysis shows that values from leaves with symptoms are significantly different from the others. The average concentrations of CU in extracts of symptomless leaves removed from elm plants 5, 10, 34 and 60 days after inoculation with *O. ulmi* or *O. novo-ulmi* were $0{\cdot}21 \pm 0{\cdot}06$ and $0{\cdot}12 \pm 0{\cdot}03$ mg mg^{-1} d. wt leaf tissue, respectively. In stems and branches of plants inoculated with *O. ulmi* or *O. novo-ulmi* bearing leaves with or without symptoms, the average concentrations of CU were not significantly different from those found in symptomless leaves. The concentration of CU in symptomatic leaves was $3{\cdot}45 \pm 1{\cdot}04$ ng mg^{-1} d. wt leaf tissue in plants inoculated

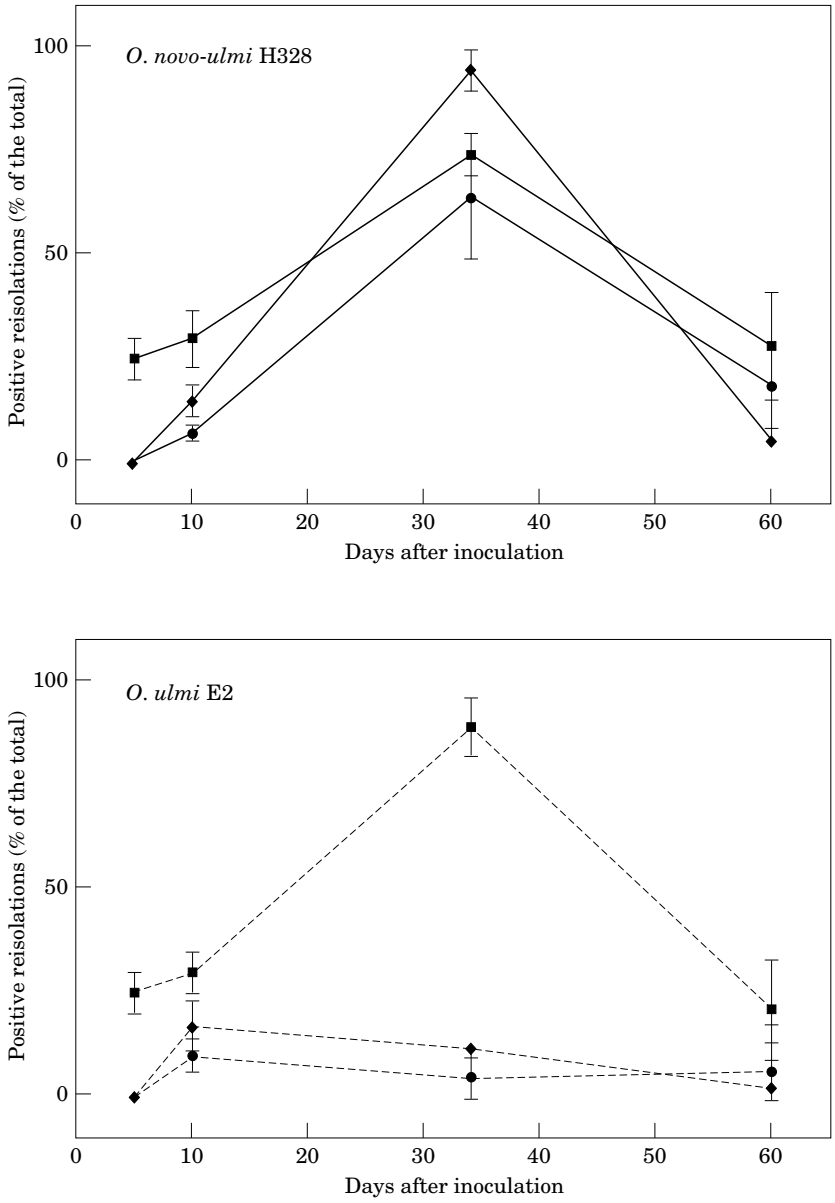

FIG. 2. The number of fungal reisolations from different elm plant sections at various times after inoculation with *Ophiostoma novo-ulmi* isolate H328 or *O. ulmi* isolate E2. Date of inoculation: as in Fig. 1 Vertical bars represent S.E. (■) elm upper section; (◆) elm medium section; (●) elm lower section.

with *O. ulmi* and $2{\cdot}69 \pm 1{\cdot}27$ ng mg^{-1} d. wt leaf tissue in plants inoculated with *O. novo-ulmi*. If we exclude the values of CU concentration in leaves assayed 60 days after inoculation with *O. novo-ulmi*, the value of the average concentration of CU in leaves with symptoms was $4{\cdot}79 \pm 2{\cdot}88$ ng mg^{-1} d. wt leaf tissue (data not shown).

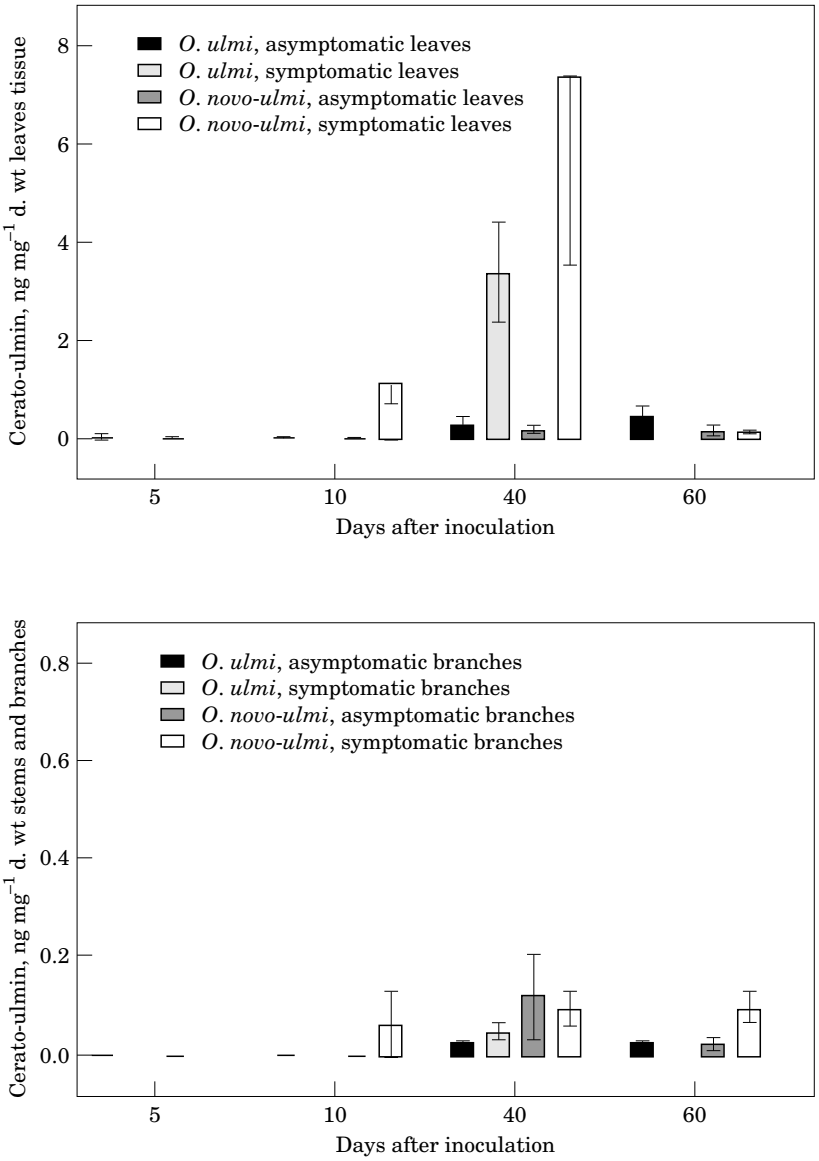

FIG. 3. Cerato-ulmin (CU) content (expressed as ng mg^{-1} d. wt tissue) in elm leaves and stems and branches at various times after artificial inoculation with *O. novo-ulmi* isolate H328 or *O. ulmi* isolate E2. Vertical bars represent CU concentration with S.E. Absence of bars indicates absence of CU from the sample.

DISCUSSION

As commonly found by other workers [*5, 10*], plants inoculated with *O. novo-ulmi* developed more progressive foliar symptoms than those which occurred after inoculation with *O. ulmi*. Differences in disease severity were observed within 10 days after inoculation. In contrast, at this time, infection progression, as determined by

Table 1
Cerato-ulmin content ± S.E. in plant tissues from elms artificially inoculated with Ophiostoma novo-ulmi *or* O. ulmi.

Plant tissues	Inoculum	Cerato-ulmin concentration ng mg^{-1} d. wt tissue	
Stems and branches (S.B.)	Water (control)	§0	($n = 30$)
S.B. bearing asymptomatic leaves	*O. ulmi*	0·03 ± 0·00a	($n = 88$)
S.B. bearing symptomatic leaves	*O. ulmi*	0·05 ± 0·02a	($n = 8$)
S.B. bearing asymptomatic leaves	*O. novo-ulmi*	0·08 ± 0·05a	($n = 73$)
S.B. bearing symptomatic leaves	*O. novo-ulmi*	0·10 ± 0·02a	($n = 44$)
Leaves	Water (control)	0	($n = 30$)
Asymptomatic leaves	*O. ulmi*	0·21 ± 0·06a	($n = 78$)
Symptomatic leaves	*O. ulmi*	3·45 ± 1·04b	($n = 9$)
Asymptomatic leaves	*O. novo-ulmi*	0·12 ± 0·03a	($n = 71$)
Symptomatic leaves	*O. novo-ulmi*	2·69 ± 1·27b	($n = 39$)

§Results of ELISA analyses are the mean ± S.E. of values obtained from samples collected at 5, 10, 34 and 60 days from inoculation; each value is the average of three wells. Means followed by the same letter do not differ significantly at $P = 0·05$, according to Tukey test. n = total number of samples analysed.

percentage of pathogen reisolation, was similar in the upper sections of stems. In the following days, *O. ulmi* progression was slower in middle and lower sections. These differences in reisolation frequencies of the two fungi may depend on both structural and chemical defence mechanisms of elm plants, which are known to be more timely and effective against *O. ulmi* [*15*, *23*]. Because the two pathogens were never reisolated from leaves, it seems likely that the DED fungi colonized only the xylem vessels of stems and branches. These results suggest that CU was produced by the two pathogens in the xylem vessels. The toxin was then readily translocated into leaves, where it accumulated. The CU concentrations in the leaves were up to 150 times higher than in the stems and branches. This conclusion is in agreement with the finding that CU is taken up by stem sucker cuttings and is then translocated to leaves [*21*, *28*].

O. novo-ulmi and *O. ulmi* produced different quantities of CU in elm plants during the pathogenic phase of DED. The relative amount of CU accumulated in leaves could be calculated. For instance, by multiplying the values of the CU concentration in symptomatic leaves 34 days after inoculation (7·48 and 3·45 ng mg^{-1} d. wt leaves from plants inoculated with *O. novo-ulmi* and *O. ulmi*, respectively; see Fig. 3) with the corresponding disease index values (37% and 12% for *O. novo-ulmi* and *O. ulmi*, respectively; see Fig. 1), we estimate that *O. novo-ulmi* produced about seven times more CU than *O. ulmi*. This value is very similar to the values (10 and 8) obtained by dividing the values of CU production previously reported for the same isolates grown for 10 days at 23 °C in liquid shake culture [*36*, *37*]. Differences in the amounts of CU produced by the two pathogens occur 10 days after inoculation, when both fungi were reisolated with the same frequency from plant sections, and increase after 34 days, when the level of infection caused by *O. novo-ulmi* was higher than that of *O. ulmi*. Our results extend the previous conclusions of Brasier [*6*] and confirm that *O. novo-ulmi* and *O. ulmi* are separated also on the basis of relative CU production *in*

planta during pathogenesis. Recently, another criterion of use in their differentiation has been found which outlines a different pathological strategy of the two DED pathogens [*2*]. *Ophisotoma novo-ulmi* produced higher extracellular laccase activity than *O. ulmi*, thus suggesting that *O. novo-ulmi* might be facilitated in both survival in the bark and detoxification of host endogenous or post-infectional phenols.

There have been a number of studies of the role of CU in DED symptoms expression, but they have been rather inconclusive. Takai *et al.* [*34*] and Richards and Takai [*19*] demonstrated that CU was detectable in stems of DED-affected elms using double immuno-diffusion assays. However, these assays were not quantitative, and sampling was only performed 2–3 weeks after inoculation. Van Alfen and Mac Hardy [*40*] hypothesized that CU, because of its high molecular weight, could interfere with water conduction in the tree. Ipsen and Abul-Hajj [*11*] concluded that elm foliar wilting was caused by the presence of CU on bordered pit membranes, which would impede the flow of water to the leaves, possibly causing cavitation. However, in the same work CU was reported as not being detectable in the stem, although leaf wilting symptoms were present. Russo *et al.* [*20*] hypothesized that elm leaf wilting is caused only by mechanical blockage of the vascular system because of CU's ability to trap fine air bubbles in the xylem sap. In such a case, the CU concentrations would have been significantly higher in branches bearing wilted leaves than in branches with healthy leaves. In the present paper no significant differences in CU content occurred between stems and branches bearing either asymptomatic or symptomatic leaves, while foliar symptoms were associated with the presence of relatively high CU concentrations.

Identification of the site of action of fungal toxins in wilted hosts is more difficult than in localized diseases [*39*]. Furthermore, the relationship between pathogen produced toxins and plant wilting symptoms has been widely debated, with the aim to define where and how wilt toxins act [*1*, *13*, *38*, *39*]. The inconclusiveness of this debate, and related controversies, appeared in the conclusion of Van Alfen [*39*] that the best example of a toxin acting as suggested in the traditional wilt-toxin concept was that of fusicoccin, a toxin produced by *Fusicoccum amygdali*, a canker pathogen of almond and peach trees. In the present paper we demonstrate the existence of a strong positive correlation between the level of leaf wilting symptom and the amounts of CU in the leaves. This demonstration is consistent with the observations that CU causes direct damage to leaf tissues, increases respiration and electrolyte loss [*18*], and inhibits division *in vitro* of tissue cultured cells of the susceptible species *U. carpinifolia*, but not of the resistant *U. pumila* [*35*]. Furthermore, we have observed that, *in vitro*, elm cells died 3 days after CU was added to the culture (unpublished work). It is thus concluded that CU plays a major role in the expression of DED symptoms by inducing a direct phytotoxic action on elm leaves, and that the wilting of elm leaves is strictly correlated with the presence of CU *in situ*. This obviously does not exclude CU from having other functions that derive from some of its chemical and physical properties.

However, the relationship that we found between leaf symptom levels and CU concentrations contrasts with the evidence that non-CU-producing *O. novo-ulmi* mutants [*7*, *37*] and the recombinant CU-knock out mutant [*4*] cause DED symptoms. *In vitro* CU production by the DED fungi is a process modulated by numerous factors, such as temperature [*36*], the carbon and nitrogen source, and the salt concentration of culture medium [*29*]. Moreover, the gene encoding for CU is under some type of

nutritional regulation in wild type isolates of *O. novo-ulmi* [P. A. Horgen, personal communication]. Therefore, it cannot be concluded that the above-mentioned mutants of *O. novo-ulmi* do not produce CU *in planta* during pathogenesis. Like all fungal vascular diseases, DED is a result of a complex interaction between pathogen aggressivity factors and plant resistance mechanisms. When CU is not produced (i.e., in the case of the mutants of *O. novo-ulmi*), other aggressivity factors could play a major role. This could also be the case of the recombinant CU-knock out mutant of Bowden *et al.* [*4*]. Recently, Keen [*12*] stated that ''gene disruption mutants which cannot be demonstrated to show a significant difference in virulence should be viewed with great caution''. He discussed the case of mutants in which genes encoding for enzymes, for which substantial biochemical and physiological data already suggested an important role in pathogenicity or virulence, did not exhibit any reduction in virulence. These results may not be fully informative if virulence assays are not performed under stringent conditions, unidentified redundant gene functions are present, or environmental factors are not optimal. For instance, for virulent mutants of *Fusarium solani* f.sp. *pisi* disrupted in the gene encoding pisatin demethylase [*41*], the possibility existed that the disruption did not affect all pisatin demethylase genes present in the fungus. Recently VanEtten and colleagues demonstrated that virulence of natural pisatin demethylase non-producing strains can be substantially increased after transformation with a pisatin demethylase-encoding gene, even if high virulence of the fungus on pea needed additional genes [*9*, *42*].

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