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The relation of mansonones to resistance against Dutch elm disease and their accumulation, as induced by several agents

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Abstract

No evidence was found that mansonones E and F, which accumulated in elms after infection with *Ceratocystis ulmi*, were responsible for resistance against Dutch elm disease in *Ulmus hollandica* cl. 390. Furthermore, production of mansonones in *U. americana* was only about one-fifteenth of that in *U. hollandica* cl. 'Belgica'. Yet both are susceptible to Dutch elm disease.

Induction of these compounds was not specific, for accumulation occurred not only after introduction of crude toxin of *C. ulmi*, but also after inoculation with *Verticillium albo-atrum* and after introduction of H_2SO_4 or ethanol.

The dark discolouration of the wood after toxin treatment was restricted to the lower part of the stem. No mansonones could be detected in the non-discoloured higher parts of the stem or from the necrotic or wilted leaves.

Introduction

In wilt diseases, phenols and their oxidation products accumulate (Dimond, 1970).

Bell (1969) found a more rapid formation of gossypol-related compounds in resistant cotton plants than in susceptible ones during the first 3 days after infection with *Verticillium albo-atrum*. He suggested that resistance of cotton varieties might depend on the speed with which a 100% fungistatic dose of these compounds was synthesized at the site of infection in relation to the speed of fungal colonization. Overeem and Elgersma (1970) isolated fungitoxic compounds, identified as mansonones E and F, from elms infected by *Ceratocystis ulmi*.

In the present study, the accumulation of these products in susceptible and resistant elms was determined during the first days after inoculation. Accumulation of these compounds was also studied in susceptible elms after treatment with a crude toxin preparation from culture filtrates of *C. ulmi* (Rebel, 1969), after inoculation with *Verticillium albo-atrum*, and after treatment with heat-killed spores of *C. ulmi* and various chemicals.

The toxicity of extracts from culture filtrates was checked at various pH levels.

Materials and methods

Rooted callus cuttings of a susceptible clone (*Ulmus hollandica* cl. 'Belgica') and of a resistant clone (*U. hollandica* cl. 390) were grown in the greenhouse at 20–25 °C and

used during their second growing season (Elgersma, 1969). Nursery grown seedlings of *U. americana* about 3–4 m tall were inoculated with *C. ulmi* in the beginning of June.

The *C. ulmi* strain TX 21, isolated by Tchernoff (1965) was used. Inoculum was prepared and inoculations were carried out as described by Elgersma (1969). A spore suspension of 5.10^6 spores per ml was used.

Crude toxin was produced and isolated from culture filtrate according to Rebel (1969). The medium contained 20 g D-glucose, 2 g asparagine. H_2O, 1.5 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 20 mg $ZnSO_4.7H_2O$, 10 mg $FeCl_3$, 1 mg thiamine, 1 mg pyridoxine, 0.1 mg $CoCl_2$ and 1 mg $CuSO_4.5H_2O$ per liter of tap water. Aliquots of 250 ml were sterilized in 750 ml Erlenmeyer flasks at 120 °C for 20 minutes. The pH after sterilization was 6.2. After addition of 1 drop of a spore suspension, the flasks were incubated on a shaker for 3 weeks at 23 °C. Spores and mycelium fragments were centrifuged and the supernatant was concentrated to 1/15 of its original volume by means of vacuum distillation with slight heating (below 40 °C). To this rather viscous concentrate an equal volume of ethanol was added to precipitate the polysaccharides. After storage overnight at 4 °C, the polysaccharide fraction was removed by vacuum filtration and the filtrate was freed from small particles by filtration through a Sartorious Membrane filter (0.45 μm). The ethanol was evaporated by vacuum distillation and the water fraction dialyzed for 2 days against tap water and for 7 days against deionized water to which chloroform was added to prevent contamination. The water was renewed twice a day. After 4 or 5 days the tubes were replaced because they were affected by the dialysate. The nondialyzable fraction was freeze-dried. Dry weight of fungal material and polysaccharide was determined by drying at 80 °C until a constant weight was obtained.

Bud cells were obtained by growing the fungus for about 1 week in shake culture. The cells were centrifuged and washed 3 times with sterile tap water, heat-killed by sterilizing for 30 minutes at 110 °C and washed again 3 times. Leaching products from heat-killed spores were obtained by keeping the spore suspensions (10^9 spores per ml) at 4 °C for 2 days after washing and removing spores by centrifugation.

Toxin solutions (1 %), suspensions of heat-killed spores (10^9 spores per ml), leaching products of spores, or chemicals were introduced into the vascular system of rooted elm callus cuttings by means of a cone of modelling clay tightly fixed around the stems. Into these cones 5 ml of solution was poured. Incisions below the level of the solution were made with a Stanley trimknife and the solution was absorbed.

The toxicity of this crude toxin preparation was checked on elm sprouts and on rooted elm callus cuttings of *U. hollandica* cl. 'Belgica' by the method of Rebel (1969). For each test, 10 sprouts were placed in 12 ml of a solution containing 300 mg of crude toxin per liter. During the test period the sprouts were kept lighted by Philips TL 4F33W/40 lamps at 22 °C.

Stem samples from inoculated or otherwise treated trees were cut into small pieces by means of pruning-shears and boiled for at least 2 h in 50 ml of 80 % ethanol. The solution was extracted with 100 ml of chloroform and the organic layer dried over Na_2SO_4. The solvent was evaporated in vacuo, the precipitate redissolved in 0.5 ml of chloroform and eventually stored at –20 °C in the dark. The mansonones E and F were separated by means of thin layer chromatography on silica gel plates eluted with chloroform-ethyl acetate (9:1). The quantity was determined with a CF4 Optica spectrophotometer by measuring absorption in ethanol at 443 nm (mansonone E) and at 551 nm (mansonone F).

Accumulation of mansonones in diseased seedlings of *U. americana* was determined as described by Overeem and Elgersma (1970).

Experiments and results

Thirty-five rooted callus cuttings of each of the resistant clone 390 and of the susceptible clone 'Belgica' were inoculated with *C. ulmi*. After 1, 2, 3, 4 and 7 days, stem segments 8 cm long were cut 1 cm above the site of inoculation. These segments were extracted and mansonones E and F determined (Table 1). The first day after inoculation the mansonones appeared only as very faint spots and could not be determined quantitatively with the technique used. As the table shows, no more mansonones were present in resistant elms than in susceptible ones during the period tested. In fact, after 4 and 7 days the content of mansonone E was even less in the resistant than in the susceptible plants.

Substances related to the mansonones have been found in some *Ulmus* species, but not in others (Lindgren and Svahn, 1968). It was not known if species of the genus *Ulmus* other than *U. hollandica* could produce mansonones. Therefore the susceptible elm, *U. americana*, was inoculated with *C. ulmi* and checked for mansonone accumulation. About 4 weeks after inoculation infected twigs were extracted and the amount of mansonones E and F determined. *U. americana* contained only 15 mg of mansonone E and 4 mg of mansonone F per 10 kg (fresh weight) of wood.

In order to find out how specific the production of mansonones is, an effort was made to induce their synthesis by introducing toxins of *C. ulmi*, *V. albo-atrum*, dead spores, or chemicals into the xylem system.

The pH of 3-week-old shake cultures was higher than 6, in contrast to Rebel's report (1969) of a pH between 3.7 and 4.4. Feldman *et al.* (1950) found that toxic activity of culture filtrate was inactivated above pH 6; therefore we compared the toxicity of compounds isolated from filtrates of 1, 2, and 3-week-old shake cultures. The pH of these cultures were respectively 4.0, 6.2 and 7.0.

Table 1. Average content of mansonones E and F of 7 stem pieces of susceptible (S) and resistant (R) elms (*U. hollandica*) cut from 1 to 9 cm above the site of inoculation.

Days after inoculation	Mansonone E (μg)		Mansonone F (μg)	
	R	S	R	S
1	trace	trace	trace	trace
2	3	3	1	1
3	7	9	2	2
4	11*	21	4	5
7	39**	63	11	14

* Significant, Wilcoxon's two sample test; $\alpha = 0.01$

** Significant; $\alpha = 0.1$

Tabel 1. Gemiddelde gehalte aan mansononen E en F uit 7 stamgedeelten van vatbare en resistente iepen genomen van 1 tot 9 cm boven plaats van inoculatie.

Table 2. Amounts of crude toxin, polysaccharide and fungal cells collected from culture filtrates of various pH levels. Experiments were performed in duplicate. The culture filtrate of 4 flasks was used for each replication.

Week of incubation	Toxin (mg)	Polysaccharides (mg)	Fungal cells (g)	pH
1	211	714	4.8	4.0
	187	584	4.4	4.1
2	230	1032	4.6	6.2
	184	1016	4.3	6.3
3	345	868	4.4	7.0
	217	930	4.6	7.1
6	339	611	3.9	7.2
	361	686	4.5	7.1

Tabel 2. Opbrengst aan ruw toxine, polysacchariden en schimmelmateriaal verzameld uit cultuurfiltraat van verschillende pH. De experimenten werden in duplo uitgevoerd waar bij telkens 4 kolven met cultuurfiltraat werden gebruikt.

After 1, 2, 3, and 6 weeks of incubation the crude toxin, the polysaccharides, and the fungal cells were collected. The experiments were done in duplicate, using 4 flasks each time (Table 2). After 1 week of incubation, toxin production was already rather high and maximum growth had been attained. The crude toxin content continued to increase gradually for several weeks. The amount of polysaccharides however, decreased after the second week. No differences were observed in toxicity of the crude toxin isolated after 1, 2, and 3 weeks.

In bioassay tests of toxin activity the first leaf symptoms appeared within 24 h. Leaves of sprouts showed up-curled-margins, interveinal necrosis, and wilting. After introduction of the toxin into the xylem system of 1-year-old callus cuttings, the interveinal tissue died, the whole leaves curled upward and became desiccated.

These rooted callus cuttings were extracted and checked for presence of mansonones one week after the crude toxin solution had been absorbed. Wood discolouration occurred only in the lower part of the stem and was never macroscopically observed higher than 35 cm above the site of toxin introduction although the total length of each stem was about 1.5 m. Mansonones E and F could easily be detected in extracts of the discoloured wood, but non-discoloured parts, cut more than 70 cm above the site of inoculation, did not contain these substances. No mansonones were found in necrotic and wilted leaves of these trees. Similar results were obtained with a crude toxin solution heated for 30 minutes at 110 °C or with heat-killed spores of *C. ulmi* (10^9 spores per ml), but leaching products of these heat-killed spores induced only a slight discolouration of the wood and no mansonones could be detected. Introduction of 0.05 n H_2SO_4 or 50% ethanol also resulted in discolouration of the wood and in accumulation of mansonones. No mansonones were detected and only slight wood discolouration was observed after plants were treated with sterile culture medium, carbowax (10% solution, mol. wt 20,000), $CuCl_2$ (10^{-3}M) or McIlvaine's buffer pH 4.5.

Elms inoculated with one-tenth ml of a spore suspension containing 10^6 spores per ml of *Verticillium albo-atrum* (an isolate from tomato, probably non-pathogenic for elms) showed low levels of mansonone production. Only faint spots of mansonones

appeared on thin layer chromatography plates from extracts of plants made 1 week after inoculation. When the concentration of spores was increased to 10^8 spores per ml and approximately 3 ml of suspension were introduced by means of a cone, the mansonones could easily be detected after 1 week.

The leaves of plants treated with carbowax curled-up and showed interveinal necrosis. By means of a sprout test the toxicity of the crude toxin (300 mg per liter) were compared with a solution of carbowax (400 mg per liter). Sprouts treated with toxin or carbowax showed similar symptoms.

Discussion

There is no evidence that fungitoxic compounds, such as mansonones E and F, which are formed after infection, play a separate role in the resistance of *U. hollandica* cl. 390 against Dutch elm disease, as no higher amounts of mansonones could be detected in this resistant clone than in the susceptible *U. hollandica* cl. 'Belgica'. Furthermore, Overeem and Elgersma (1970) found that *U. hollandica* cl. 'Belgica' accumulated about 15 times as much mansonones as did *U. americana* following inoculation with *C. ulmi*, although both are susceptible to Dutch elm disease. Mansonones might, however, be at least partly responsible for the decrease in fungal propagules after the first week of infection in both resistant and susceptible elms (Elgersma, 1969). They might have some effect against infection by *C. ulmi*, as a supplement to anatomical factors (Elgersma, 1969, 1970) which, in resistant clones, retard the transport of spores in the vascular system. However, since *C. ulmi* can tolerate a relatively high concentration of these substances (Overeem and Elgersma, 1970) their effect must not be over-estimated.

The induction of synthesis of mansonones appears not to be specific, since accumulation of these substances occurs not only with injection of *C. ulmi* spores or crude toxin extracted from culture filtrates of *C. ulmi*, but also after inoculation with *V. albo-atrum* or after injury caused by unrelated chemicals.

It is not clear why after introduction of the crude toxin preparation only a restricted extent of the stem was discoloured. Discouloration of the stem up to the leaves would be expected after introduction of these amounts of toxin. Possibly the toxin is absorbed by the xylem vessel walls and only in the lower parts reaches a level that could induce dark discolouration of the stem. The discolouration might be evoked by any injury that causes membrane damage, as suggested by Rai and Strobel (1969) for the toxin isolated from *Corynebacterium michiganense*. In the leaves it might only interfere with the water economy of the plant by blocking the small veinlets (Hodgson et al. (1949) or by accumulating in pit membranes and reducing lateral and longitudinal flow of water (Dimond, 1967). The concentration of toxin, however, might be too low in this part of the plant to induce mansonone production. Elm sprouts placed in a solution of carbowax showed symptoms similar to those of sprouts on crude toxin solution. This supports the idea that at least the crude toxin causes mechanical plugging.

Another explanation might be that more than one toxin is involved, each having a different effect on the host. One might be absorbed on the vessel walls and causing discolouration of the stem, another might interfere with water economy in the leaves. The chemical composition of the crude toxin is not fully understood. According to Rebel (1969), 90–95 % of the crude toxin is a glycoprotein with a molecular weight of about

30,000 and rather heterogeneous with regard to its chemical composition. The rest is a second, not yet identified, compound with a molecular weight of about 1,000,000 and probably not toxic.

Holmes (1954) showed pectinase activity in a spore suspension of *C. ulmi* and suggested that these enzymes may cause softening of the pit membranes leading to formation of tyloses in the xylem vessels. Beckman (1956) showed cellulase activity of the culture filtrate of *C. ulmi* and these enzymes affected probably the dialyzing tubes in our experiments. Rebel (1969), however, observed no cellulase, polygalacturonase or pectin methylesterase activity of the crude toxin. Moreover, the toxic principle appeared to be heat-stable, so enzyme activity seems to be unrelated to the role of toxins in the symptom expression under consideration, whether or not enzymes play an indirect role in evoking symptoms.

The introduction of high concentrations of heat-killed spores into the xylem system causes dark discolouration of the wood and accumulation of mansonones. Products leaching from the dead cells may be toxic and induce this syndrome. Leachates obtained in vitro, however, did not induce detectable amounts of mansonones. This discrepancy might be explained by differences in concentration.

The significance of all of these toxic compounds to internal symptom development in comparison to the effects of cellulases or pectindegrading enzymes produced by *C. ulmi* (Holmes, 1954; Gagnon, 1967; Dimond, 1970) remains uncertain.

The mansonones do not appear to be the determining factor in resistance against Dutch elm disease, but they may well play an important role in resistance of elms to other micro-organisms that may invade or be introduced into the vascular tissue of elms.

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Samenvatting

De betekenis van mansononen E en F voor de resistentie tegen de iepziekte en hun accumulatie veroorzaakt door verschillende middelen

Er werd geen aanwijzing gevonden dat mansononen E en F, die na infectie met *C. ulmi* gevormd worden, verantwoordelijk zijn voor de resistentie tegen de iepziekte in *U. hollandica* cl. 390 (Tabel 1).

U. americana bevat ongeveer 15 maal zo weinig van deze stoffen als *U. hollandica* cl. 'Belgica', terwijl beide vatbaar zijn.

De inductie van de synthese van deze stoffen bleek niet specifiek te zijn, omdat bij chemische beschadiging met H_2SO_4 of ethanol ook mansononen gevormd werden.

De donkere verkleuring van het hout bleek na toxine toediening beperkt tot het

onderste gedeelte van de stam. Geen mansononen werden gevonden in hoger gelegen, niet verkleurde stamgedeelten of in de necrotische en verwelkte bladeren.

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