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Susceptibility and possible mechanisms of resistance to Dutch elm disease

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A phytotoxic glycopeptide produced by *Ophiostoma ulmi* in several elm species and clones

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THE JAPANESE ELM SPECIES AND THEIR VALUE
FOR THE DUTCH ELM BREEDING PROGRAMME

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In its morphology and ecology Ulmus japonica is similar to the European U. carpinifolia, and U. laciniata to U. glabra Huds. The third species, U. parvifolia is quite different. In provenance experiments in the Netherlands, differences in height growth up to 90% occurred between provenances of U. japonica. Differences in growth rate between provenances explained 60% of differences in disease expression after inoculation. No resistant populations were found in U. japonica and laciniata; the search now is for relatively resistant individuals.

INTRODUCTION

The value of the three Japanese elm species *U. japonica* Sarg., *U. laciniata* (Tr.) Mayr and *U. parvifolia* Jacq. for breeding purposes has been mentioned earlier by Lester and Smalley (4) and Santamour (10). This paper adds some early findings of recent experiments, after giving a characteristic of the taxonomy and ecology of the three species. Comparisons with two European sister-species should help to clarify the picture. Experiences gained during a collecting trip through Japan in May 1977 are included.

U. JAPONICA AND U. LACINIATA

Taxonomy, morphology

These two species invite comparison with their European relatives: *U. japonica* with *U. carpinifolia* Gled. and *U. laciniata* with *U. glabra* Huds. Some botanists did not even grant them the status of separate species, reducing *U. japonica* to *U. carpinifolia*, and *U. laciniata* to *U. glabra*. I do not share that opinion: the two east-Asiatic taxa are clearly different from their European counterparts, and merit the status of species. The four species could be considered, however, to be pairs of vicariating species.

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In the latest subdivision of the genus, all four are placed in the section *Ulmus* (syn. *Madocarpus*); *U. carpinifolia* and *japonica* then come in the series *Nitentes*, while *U. glabra* and *laciniata* fit in the series *Ulmus* (syn. *Euglabrae*) (1).

It can still be doubted whether the species which is called *U. japonica* in this paper bears that name correctly. That depends whether it can be considered to differ at the species level from *U. davidiana* Planch., described in 1873 from the hills north of Peking. That material is in so far different as its samara is hairy over the seed, glabrous over the wing. *U. japonica* should have fruits which are glabrous all over. On this ground Kitagawa (3) considered them to be two different species. However, in some of our collections from Japan the samaras were hairy over the seed also. Maybe this character is variable and does not constitute a sufficient base to recognize a separate species; maybe it is in this case just part of a varietal or even individual variation within a single species of elm, as it is in *U. wallichiana* (7) or in *U. glabra* (e.g., 5 p. 17). Further study of continental material is needed to determine whether this view is correct. In that case, the combined "large" species should bear the older name *U. davidiana*. For the purpose of this paper, the name *U. japonica* is retained.

In the forest and when the trees are leafless, I found it more difficult to discern between *U. japonica* and *laciniata* than between *U. carpinifolia* and *glabra*. Differences are less striking. *U. laciniata* tends to have a flaky bark, the bark of *U. japonica* mostly shows a ridge pattern. However, the latter species may also have a flaky bark, even resembling the bark of *Zelkova*. Such trees were found in Kyushu and appeared to have a different vernacular name: "ishi - keyaki", or rock-*Zelkova*, because of the bark. To me they looked like normal *U. japonica* showing its inner layers of bark, as the outer layers deteriorated quickly in the humid climate and under thick layers of epiphytic mosses.

The biggest *U. japonica* seen was 30 m high and had a diameter of 1.40 m. *U. laciniata* is described in some publications as "a small tree". This is not true. The biggest I saw was 27 m high, diameter 104 cm.

Especially in *U. japonica*, a considerable variation in tree- and crown shapes was met. The entire range from wide fan-shaped, via globular, to narrow-crowned, single stemmed trees was found.

Ecology

When giving an ecological characteristic of *U. carpinifolia* and *U. glabra* Huds., it is of interest to know that in central

Europe, the first is called "field-elm", the latter "mountain elm". The field elm has several niches. First of all, it is the species of the forests on the river forelands in the plains, forests which are flooded in most springs when snow in the mountains melts, and occasionally in summer when there is much rain. These forests grow on slightly higher soil than the willow-poplar forests. They consist normally of oak, ash and field elm, the latter reaching the highest volume per ha. Owing to its regeneration by rootsuckers, the elm may occur in large clonal groups. Another typical site is at the foot of sandy hills, where minerals and some sand wash down; the bases of the trees may be buried slowly but the soil is rich and the trees survive. Third, the tree may be found in a more or less shrublike form on dry, grazed calcareous slopes; corky ridges on twigs, a thick bark, rootsuckers, a good regenerative capacity and drought resistance help the tree to survive here. It further feels at home at the borders of fields in the open, dry, lowland agricultural areas with a highly continental climate. It stands coppicing and pollarding. In the Alps, its altitudinal limit is at 600 m; its northern outposts are on the calcareous Baltic Islands of Öland and Gotland, where it even may have been brought by man.

It has an even wider ecological amplitude, though. In the extreme oceanic and stormy climate of Brittany and Cornwall, a special subspecies of the field-elm, *U. carp. spp. cornubiensis*, syn. *U. stricta*, is the most successful tree species.

The mountain elm, on the other hand, is a forest species, preferring the humid valleys and rich moist soils. Another typical site is on steep slopes with more or less loose boulders under which moving water is found. Its preference or tolerance for a cool humid climate allows it to occur higher in the mountains (up to 1400 m in the Alps) and further to the north (up to 67° in Norway, 65°60' in Sweden, 65°20' in Finland). Its bark is thin; it regenerates by seedlings, not rootsuckers.

(The Mediterranean species, *U. procera*, with its outlying occurrence in Great Britain, where it rarely sets seed, is not considered here.)

When we compare the ecological characteristics of the Japanese elms, *U. japonica* and *U. laciniata* with those European relatives, it appears that similar tendencies can be recognized, but that the differences between the species are smaller.

In the phytosociological literature, the forests of *U. japonica* in Japan are described as "swamp forests on young alluvial soils" (6), "on wet soils" (9).

Several authors agree that large tracts of the now cultivated plains once had vast elm forests. The present stands

are no more than a few remote relics. The intensive Japanese agriculture, bordering on horticulture, apparently left no niches for elm between its rice fields. On Kyushu, the tree was found to occur on disturbed soil along a road on a steep hillside. That the elm has similar capacities as its European relative to mix into the cultivated landscape can be seen on the island of Hokkaido with its recent agriculture of a more Western type. In Sapporo, it is planted as a street tree.

No reference or example has been found of the growth of Japanese elm on the borders of calcareous grasslands. This may be related to the fact that Japan lacks the traditional European grazing systems. It must be admitted, however, that no clear evidence has been found of propagation by rootsuckers and of the concurrent shrublike behaviour of the tree.

Ulmus laciniata, on the other hand, is more typically a tree of the hills and the mountains, Sasaki (11) mentions it as a character species of the humid "ravine forests" ("Schluchtwälder") together with *Cercidiphyllum japonicum*, *Aesculus turbinata* and *Pterocarya rhoifolia*. We saw it on steep humid boulder slopes just like *U. glabra* in Europe, as well as on a poorly drained plateau with heavy soil.

U. laciniata is less represented at lower latitudes and altitudes than *U. japonica*. This is shown by Horikawa (2), (pages 48 and 535). In the southern parts of Japan, it retreats to the hills. When comparing these data to the natural vegetation zones as given by Schwind (12, 13), it can be seen that *U. laciniata* hardly penetrates the upper evergreen broadleaves' zone "Immergrüne Laubwaldzone", while *U. japonica* fills it. With regard to their northern and altitudinal limits the two species seem to differ little.

The two species thus reflect to some extent the ecological characteristics of their European counterparts, though the contrast is smaller. Stands can be found in which the two species occur together. It is conceivable that on the Asiatic continent, with its wider range of ecological conditions, the contrast will be found to be more pronounced than on the Japanese islands.

This ecological characteristic may help to explain why *U. japonica* seems to be the more promising species for our selection program. We try to produce elms for use not in the forest but in the towns and in the open, hot agricultural landscape. At these sites, air humidity can be low, and trees are fully exposed to winds with their desiccating effect. Under these conditions, *U. japonica*, like *U. carpinifolia* seems to succeed, while *U. laciniata*, at home in humid places only, incurs damage to its leaves and stops growing. It might, however, be useful for hybridization.

U. PARVIFOLIA

This is the third Japanese species. It occurs in southern Japan only, where it is confined to the zone of the "evergreen broadleaved forests" (2, 13). It occurs also in southern Korea, in the coastal zone of the Chinese mainland, on Taiwan and Hainan. On the latter island, it overlaps with the closely related tropical species *U. lanceaeifolia* which extends westward into Nepal and southward across the equator in Indonesia. The two species constitute the separate section *Microptelea* in the genus *Ulmus*. *U. parvifolia* thus has no counterpart in Europe. In several parts of its area it is widely planted, thus perhaps raising some doubt about the exact limits of its "natural" area.

The species does not seem to be mentioned in the Japanese phytosociological literature. I saw it occurring in pioneer situations on disturbed soils along roads, on the borders of fields in the hills, on a dike along a river. It is planted in parks and gardens.

Because of the mild climate of its area, one might expect the species to be tender in North America. Surprisingly, this is not the case. I saw it growing happily in Ames, Iowa, which is in hardiness zone 5a. It seems a contradiction that seedlings of the species (and even seedlings derived from the Ames specimens) suffer badly from the mild Dutch winters. They mostly die back considerably. Maybe our cool summers do not allow them to harden off sufficiently. Older specimens suffer much less. The species' subtropical connections as well as its adaptability are illustrated by the fact that it tends to be evergreen under Californian conditions, whereas it sheds its leaves after an ample display of fall colours in cooler areas.

In Japan it seems to remain a smaller tree than the other two elm species. Its form is variable: next to fan-shaped, umbrella-like trees, often globular, single-stemmed trees were seen.

PROVENANCE RESEARCH

In May 1977, a one-month collecting tour was made through Japan, selecting elm trees of the three species. Seed was to be collected from these trees by the local forest authorities later, and sent over by air mail. No vegetative reproductive material was sent over, partly for fear of introducing new parasites. In total, batches of seed were obtained from some 110 mother trees, originating from 32°41' to 44°03' latitude and from sea level up to 1580 m above sea level. With the resulting seedlings, provenance experiments were laid out in the nursery in 1978, 1979 and 1980. Some seedling populations from non-Japanese elm species were included. Height growth was measured annually, winter frost damage

was assessed. In the second and third year after planting, part of the seedlings in each experiment were inoculated with *Ceratocystis ulmi* in order to compare levels of resistance. A longer term provenance test, at the same time a gene bank, with 52 populations was laid out in 1981.

Results of the nursery experiments are still coming in. Some early results will be given below.

Height Growth

Growth rate differs considerably between provenances. Figure 1 shows a typical situation, average heights per population ranging from 105 to 195 cm.

There appears to be a clear trend in the geographical variation of height growth of *U. japonica*. In this particular provenance test, there was no correlation to be found between height growth of the families and the height above sea level of their mothertree, while there was a weak correlation ($r = -0.66$) between population height and the geographical latitude of the mothertree. The correlation was raised to $r = -0.75$, however, when height above sea level and geographical latitude were combined in a single index, in which each 100 m altitude is taken as equivalent to one degree latitude (Fig. 1) (14).

The other provenance tests showed the same trend.

Frost Resistance

Plants in the experiment laid out in 1978 kept growing until late fall in 1978. By the end of November many were still in green leaves. Spring 1979 found many frozen back. The lowest temperatures recorded in Wageningen at 1.50 m above ground during short cold spells in that winter were as follows:

November	-	7.8°C
December	-	16.8°C
January	-	18.8°C
February	-	8.5°C
March	-	2.7°C

Not surprisingly, only the southern provenances, from the island Kyushu, showed this sort of damage. Though most plants recovered during 1979, and the damage was not repeated in the following winters, it shows that the southern provenances are not dependable for our conditions. From the preceding section it follows that in this way the fastest growing populations are discarded for our use. Similarly, we might be little interested in the more northern/higher altitude provenances because of their slower growth. It may be surmised, however, that the two southern

provenances could be a perfect fit for the conditions of Italy, while the two northern ones might prove excellent for Denmark. This is why an abridged version of this same provenance experiment is repeated in all countries of the European Common Market, including the two mentioned ones. This will test the above surmisions, and perhaps allow a broader use of the whole range of provenances collected.

Resistance to DED

At least some disease resistance is needed if the material is to be useful for our breeding programme. Testing is under way. Inoculations of 1981 produced severe disease symptoms in general, allowing a good differentiation. Some early data are given in Figure 2.

Looking at *U. japonica* first, the correlation between plant height and disease severity is striking. It illustrates the general experience that fast-growing elms get more severely diseased than slow-growing ones. It shows that "resistance" can easily be obtained by scarificing growth. It also shows that, if we want health and growth combined, crossing the resistant (slow growing) with the fast growing (susceptible) genotype will not easily yield the resistant and fast growing tree. For our breeding purposes, resistance should rather be characterized as the deviation from the trend, which is the distance of the population mean from the regression line.

When we make geographical groupings of these families and compare their resistance defined in this way, there appears no geographical pattern in resistance. Some clear pattern would have induced us to concentrate a second seed collection on certain areas where a high level of resistance could be expected; as the matter stands, no such area can be indicated.

A wider variation among populations would have been welcome. It can only be hoped that the individual variation within the populations will be sufficiently large so that individuals figuring in the lower right-hand corner will be found. Time will tell.

In the experiment, one population of typical Dutch *U. carpinifolia* is included. Its mean value is clearly on the wrong side of the regression line of *U. japonica*, confirming the general opinion that *U. japonica* has more resistance.

Several provenances of *U. pumila* were included as well. They reacted to the inoculation in this particular year with more severe symptoms than expected, but as a group they are clearly more resistant than *U. japonica*. As yet little can be said about *U. parvifolia* because of its poor initial growth and because the

slight symptoms seen may have had other causes. The results so far do not conflict with the general experience that the species has a high level of resistance.

The data of the single population of *U. chenmoui* Cheng look promising. Seeds of this new species were kindly provided by the Arboretum of the Academy of Forestry in Peking. In spite of their provenance from the hills near Chu-hsien in the province of Anhwei with its rather mild climate, the plants survived our winter of 80/81 unscathed. The species seems to fit in the series *Nitentes* of the section *Ulmus*. Thus it might become a more useful crossing partner for breeding than *U. pumila*, whose progeny does not do well in our climate.

In this provenance experiment, there was no population of *U. laciniata*. In another experiment, it proved more susceptible than *U. japonica*. This agrees with earlier experiences with the two species.

DISCUSSION

Natural Japanese populations of *U. japonica* and *laciniata* do not exhibit much resistance to Dutch elm disease. There is, however, the real possibility that individuals with an interesting level of resistance can be found. Such an individual is the chance seedling of *U. laciniata* from near Lake Chuzenji in the Nikko area which was obtained by the Arnold Arboretum in 1905 and which was described as *U. laciniata* var. *nikkoensis*. This clone has given relatively resistant progeny in our breeding program. Several resistant clones of *U. japonica* have been found in Canada, Wisconsin and the Netherlands.

It should be added that in Japan no traces have been found of Dutch elm disease up to now, even though the elm bark beetle, *Scolytus japonicus* Chapuis is widespread, and two other *Scolytus* species occur on elm (8).

Ulmus parvifolia could become an attractive tree for northwestern Europe if populations or individuals adapted to the local climate can be found.

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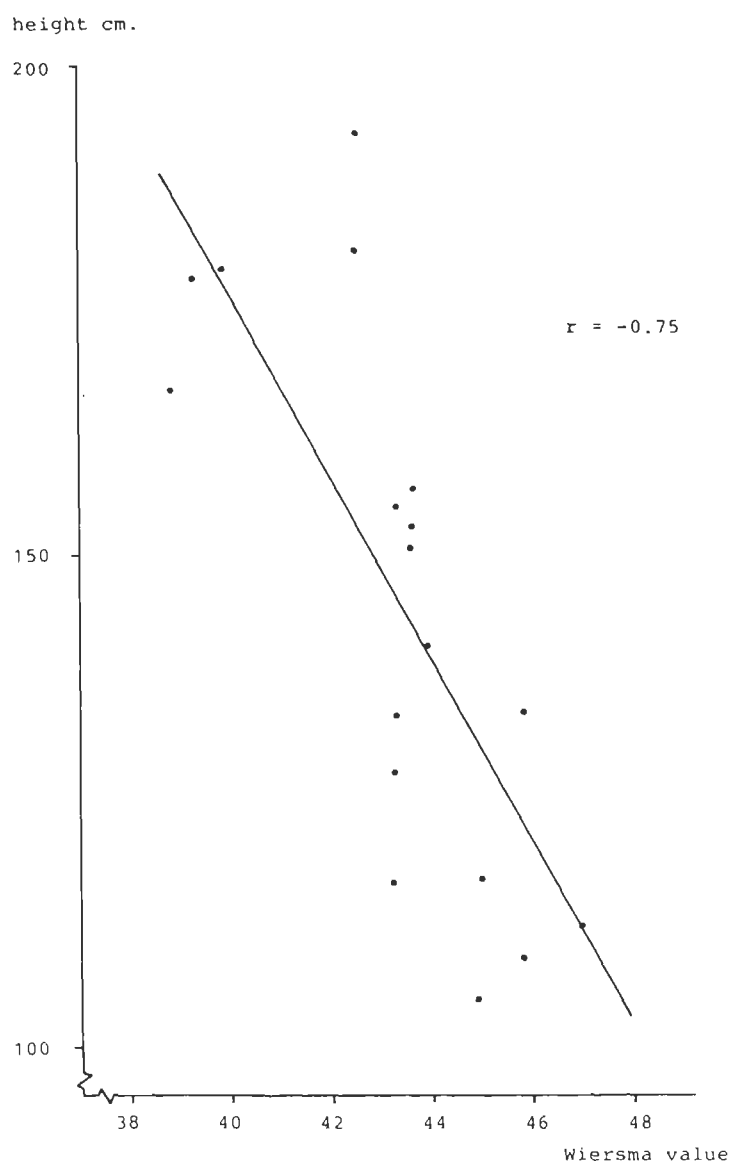
and evaluation of the provenance tests were under supervision of W. Tolkamp, who was assisted by A.M. van den Maagdenberg, R. Sonnelitter and G. Miller. Guidance in statistical matters was given by S. Heisterkamp and co-workers.

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Figure 1. Height growth of 2-year-old half-sib seedling populations of *U. japonica* in relation to their geographical origin. Wiersma value = geographical latitude in degrees + height a.s.l. in m/100.



SUSCEPTIBILITY AND POSSIBLE MECHANISMS OF RESISTANCE
TO DUTCH ELM DISEASE

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In trees resistant to Dutch elm disease the spread of the fungus through the vascular system is impeded. Data indicate that the diameter and size of vessel groups when decreased, may reduce the spread of translocation of conidia. A faster tylose formation may also limit the spread of the pathogen in resistant trees.

For more than 60 years we have been faced by the highly destructive Dutch elm disease (DED), caused by *Ophiostoma ulmi* (Buisman) Nannf. Intensive efforts have been made during this period to understand and to combat this disease.

Recently data have been reviewed by Sinclair and Campana (31) and by Mace *et al.* (19). Nevertheless, many questions pertaining to the physiology of disease development and even more on mechanisms of disease resistance remain open.

Host-parasite interactions in vascular wilts are so complex that studying each process or factor separately seems impossible. A prerequisite for disease development is a satisfactory growth of the pathogen in the tree and spread throughout the vascular system of the host. A complex of metabolites produced by the pathogen such as cell wall-degrading enzymes, growth substances, toxins, etc., or pH changes in the vessels may induce tyloses and gums. The products interfere directly or indirectly with the water movement in the vascular system, causing desiccation of the leaves and ultimately death of the tree. The individual role of each factor, however, is not well understood. The degree of symptom expression and rate of disease development, aside from the genetic makeup of the tree is influenced by environmental factors. In general, vigorously growing trees are more susceptible than slow growing ones (13, 40). Temperature, soil moisture, nutrients and especially the season in which infection takes place can be factors important for symptom expression (38, 37).

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In the late 1920's a breeding program for selecting resistant elms was begun in the Netherlands and several resistant elm clones have been released since (13, 15). After introduction of the disease in North America, probably by the import of elm logs for veneer wood from France, instead of the Netherlands as was erroneously stated (17), elm breeding programs were also initiated here (30). Gradually it became obvious that resistance to DED was polygenetically controlled (14, 16) and indeed up till now no race-specific effects have been found. As probably many genes are involved in resistance, we might expect many relatively small effects and therefore difficult to distinguish and to determine. This probably explains why so much disagreement exists over proposed mechanisms of resistance. This polygenetic and probably quantitative nature of resistance will also make determination difficult if correlated phenomena also segregate in absolute association after crossings (12).

In the early 1930's Christine Buisman suggested that differences between susceptible and resistant trees might be due to differences in their capacity for water transport (2). She assumed that blockage in trees with narrow vessels would cause more damage than in trees with large vessels. From her experiments, however, in measuring transpiration rate and conductivity of water or spore suspensions through the vessels, no further conclusions could be drawn (2, 3, 4). Pope (24) also studied the relation between wood structure and wilting. He found that the diameter of spring wood vessels in the resistant elm, *Ulmus pumila*, was noticeably smaller than that of vessels of the susceptible *U. americana*. Frequently only one row of such vessels was formed after which summer wood started to develop, in contrast to *U. americana*.

Since summer wood of both species is characterized by smaller, more scattered and less contiguous vessels, the distribution of the fungus in this wood is hampered and therefore sufficient non-infected vessels are left open for water transport. According to Pope (24) the early formation of summer wood in *U. pumila* renders this tree susceptible only during a very short period.

Later research has indeed shown a strong correlation between certain anatomical factors and resistance. Elgersma (6) showed that the average diameter of xylem vessels of a resistant Dutch clone was smaller than those of a susceptible clone. This was also observed by Sinclair *et al.* (32) in American elms. In addition, McNabb *et al.* (20) also found xylem vessels in resistant elms to be more widely spaced in smaller groups than in susceptible elms. These differences in anatomical structure makes the spread of the fungus through the

vascular system of the resistant trees more difficult than in susceptible ones. Obviously, anatomical factors alone do not suffice to confine the pathogen. Impeding the progress, however, may allow the tree enough time to bring some defence mechanisms into action, i.e., Tylose formation and accumulation of phytoalexins.

Elgersma (7) observed a faster tylose formation in resistant than in susceptible elms, which may seal off the vessels, before conidia have spread especially when the vessels are of a small diameter. On the contrary, MacDonald and McNabb (18) found no difference in the rate of tylose formation between resistant and susceptible elm species. In addition, phytoalexins, another factor which may contribute to resistance, are also produced in diseased elms. Overeem and Elgersma (23) isolated fungitoxic naphthoquinones mansonone E and F from diseased wood. *O. ulmi*, however, can tolerate a relatively high concentration of these compounds and the resistant clone 390 accumulated no more of these compounds than the susceptible clone Belgica (11). Recently the accumulation of these compounds in diseased elms was questioned by Bell and Mace (1). Boiling ethanol (80%) was used for extraction, which, according to these authors might cause the sole transformation of mansonone E and F from mansonone C or even from precursors of mansonone C. This seems highly unlikely. Mansonone E and F can be extracted with cold chloroform or cold ethanol from infected elm tissue as well. It was, nevertheless, extracted in 80% hot ethanol at that time to inhibit any further enzymatic reaction during the extraction procedure.

These mansonones probably accumulate in infected blocked vessels and so aid in arresting the growth and confinement of the pathogen. Their importance in resistance, however, is still debatable.

The role of toxins produced by *O. ulmi* in disease development has recently been discussed by Van Alfen and MacHardy (39). *O. ulmi* produces a glycopeptide in shake culture (25, 35, and 22), which has also recently been detected in infected trees by means of an immunological technique (ELISA) (26).

Pure glycopeptide added to wood sap from infected susceptible elms could be quantitatively detected, indicating that there was no important interaction between the glycopeptide and the wood sap. In the resistant clone 390, however, with increasing time after inoculation the glycopeptide could only be partially detected. Possibly a substance produced in inoculated trees reacts with the fungal peptide and plays a role in disease resistance or interferes with one of the steps in the ELISA (27).

Nutrient levels for fungal growth in the xylem sap seem to play no direct role in resistance (5, 9, 33, 34).

After the discovery of a highly aggressive strain of *O. ulmi* in England we focused our attention on the mechanisms of this strain which affects our so-called resistant Dutch clones. Scanning electron microscopy showed that the aggressive strain develops more rapidly in a resistant clone than does the non-aggressive one (21). Apparently the resistance mechanisms as proposed earlier (6, 7) do not function effectively enough to limit the spread of the pathogen.

Studies by Elgersma and Heybroek (10) have shown that when a conidia mixture of aggressive and non-aggressive strains was used for inoculation, survival of the aggressive strain was better than that of a non-aggressive strain *in vivo*. When trees resistant to the non-aggressive strain such as clone 390 and the Christine Buisman elm were inoculated with a mixture of conidia of both strains, progress of the non-aggressive strain was limited and survival of the aggressive strain was favored. Obviously the chance of contamination by the aggressive strain over the non-aggressive one by beetles is also enhanced by the difference between their growth and survival in the tree. Disease symptom expression was significantly reduced in a resistant clone when a mixture of both strains was used, compared to inoculation with the aggressive strain alone. It seems that the non-aggressive strain induces a mechanism in the tree which counteracts, at least partially, the induction or expression of disease symptoms provoked by the aggressive strain (29).

The superior growth and spread of the aggressive strain compared to the non-aggressive one in resistant trees (21, 10) might be explained by a higher production of cell wall degrading enzymes. Nevertheless no correlation was found between polygalacturonase, cellulase (Cx) production and aggressiveness, when the fungus was grown on pulverized elm wood in shake culture (8). In a recent study by Svaldi and Elgersma (36) glycosidase and exo-glycanase activity of several aggressive and non-aggressive isolates was determined. These enzymes can play a role in cell wall degradation on one hand and can stimulate growth and toxin production on the other by releasing monosugars from host cell wall polysaccharides. Enzyme preparation from aggressive isolates released more arabinose and xylose from cell walls of elm wood than those from non-aggressive isolates. Significantly more rhamnose too, was released by aggressive isolates, although some of the non-aggressive isolate released comparable amounts. The higher cell wall degrading activity by enzyme preparations from aggressive isolates is in

agreement with the results of scanning electron microscopical studies on vessel wall degradation by aggressive and non-aggressive isolates (28), and with the observation of a more active penetration through pits by the aggressive strain (21).

Data on mechanisms of resistance to DED are scant and sometimes contradictory. Also, our knowledge of mechanisms of pathogenicity in *O. ulmi* is quite limited. Let us hope that this only stimulates further research in this field.

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A PHYTOTOXIC GLYCOPEPTIDE PRODUCED BY *OPHIOSTOMA ULMI*
IN SEVERAL ELM SPECIES AND CLONES

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An ELISA was developed for a pure glycopeptide from Ophiostoma ulmi culture filtrates. In wood sap from resistant elms, showing no symptoms after inoculation with an aggressive strain of O. ulmi, this glycopeptide could not be detected. In several susceptible elm species, however, the glycopeptide could readily be detected after inoculation. The relation of the glycopeptide to other toxins and use of the term vivotoxin is discussed. A supplement to the criteria for defining a vivotoxin is proposed.

The involvement of toxins in Dutch elm disease has been the subject of heated debates of decennia. Compounds, shown to produce phytotoxic effects, have been isolated from shake cultures of *Ophiostoma ulmi* (Buisman) Nannf. and purified with varying success over the years. The increasing number of purified toxins did not settle the dispute concerning their importance in pathogenesis, however.

In this article attention will be focused mainly on non-enzymatic toxins of which four groups are known: phenolics, polysaccharides, cerato-ulmin and a glycopeptide.

PHENOLICS

The phenolic metabolites of *O. ulmi*, investigated by Claydon *et al.* (3, 2) express phytotoxicity in elm shoots. Attempts to reproduce phytotoxic effects by injecting these compounds into stems of four-year-old rooted elm saplings were unsuccessful, however, and no evidence was obtained pertaining to their *in vivo* formation.

POLYSACCHARIDES

A polysaccharide fraction can be precipitated from culture filtrates with 70% ethanol. In 1947 Dimond (4) observed some effects of this material on tomato and elm cuttings already,

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probably due to impediment of the water flow. The importance of the polysaccharides *in vivo* is questionable. Apparently the viscosity of the xylem fluid is not affected after infection of the tree by *O. ulmi* (7). Thus, polysaccharide production should be low in comparison to production in shake cultures where viscosity rises rapidly due to polysaccharide production. Both Feldman *et al.* (11) and Rebel (16) considered this fraction to be of minor or no importance as concentrations necessary to induce wilting were even higher than that occurring in shake cultures.

CERATO-ULMIN

A small protein possessing phytotoxic properties was isolated by Takai and co-workers and named 'cerato-ulmin' (17, 27, 30). Production of cerato-ulmin in shake cultures is reported to be correlated with aggressiveness of the fungal strain in many cases (27, 28) but exceptions have been noted (1, 28). Administered to elm cuttings, cerato-ulmin induces wilting, chlorosis and necrosis (27). Wilting could be induced by placing the shoots in a solution containing 7.5 µg cerato-ulmin per ml water. Extreme concentrations, as low as 2 ng/ml, are claimed to be toxic also (31).

Takai and co-workers suggest that cerato-ulmin does not induce wilt in elms by 'plugging' the vessels, because concentrations of cerato-ulmin necessary to induce symptoms in shoots are probably too low to physically interfere with the water flow. Instead, they propose that cerato-ulmin triggers an unknown host response.

Russo *et al.* (19) however, found that the surface tension of a cerato-ulmin solution was still affected at a concentration of 30 ng/ml although only a fraction of a monolayer could be formed at that concentration, even if all the cerato-ulmin rose to the air-water surface. The solubility in water also proved to be extremely low, less than 10 µg/ml. When the concentration increases, turbidity is observed, due to small air bubbles coated with cerato-ulmin. These surface-active properties of cerato-ulmin make that recognition by the host may not be necessary but that simply plugging or waterproofing of the pit membranes may account for the results of bio-assays.

Antiserum against cerato-ulmin was produced (13) and double diffusion tests were performed against extracts obtained from discolored xylem wood from elms infected by *O. ulmi*. Preliminary results indicate that the cerato-ulmin may be present in such extracts (29).

GLYCOPEPTIDE

A partially purified glycopeptide fraction was obtained by Salemink *et al.* (20) and later by Rebel (16). Using affinity chromatography, Strobel *et al.* (25) obtained a polydisperse glycopeptide fraction which was apparently free from contaminants. Structural analysis by Nordin and Strobel (15) revealed that the material is a polydisperse peptidorhamnomannan with a molecular weight range of approximately 105,000 to 120,000. Phytotoxicity of the glycopeptide fraction in elm shoots has been reported by Salemink *et al.* (20), Rebel (16), Strobel *et al.* (25) and in more detail by Van Alfen and Turner (34). The latter found that as little as 4 μg of glycopeptide or high molecular weight dextran measurably decreased the stem and petiole conductance in elms. Their conclusion is that physical plugging of the pit membranes and possible cavitation is the most likely mode of action of the glycopeptide. In a later study, Van Alfen and Allard-Turner (32) showed that even lower amounts of dextrans could seriously reduce vascular conductance.

Using the enzyme-linked immunospecific assay (ELISA) we showed that in the susceptible Dutch elm, *Ulmus hollandica* cl. Belgica, accumulation of the glycopeptide began two weeks after inoculation and increased to approximately 5 $\mu\text{g}/\text{ml}$ wood sap four weeks after inoculation (21). Since the wood sap was collected by means of a hydraulic press, the sap was diluted by fluid from other tissues. In addition, part of the glycopeptide might have been trapped in the ultrapores of pit membranes or might possibly have reacted with host cell wall polysaccharides as recently was suggested by Nordin and Strobel (15) and therefore was not recovered. Thus, the concentration *in situ* must have been higher and beyond all doubt was high enough to expect phytotoxic effects.

In the 1980 field trials pure glycopeptide added to wood saps of the moderately resistant elm clone 390 proved to be quantitatively untraceable with increasing time after inoculation (22). Some interaction apparently took place during the ELISA. Gel filtration of the wood saps on Sephacryl S 300 (Pharmacia) proved to be a solution: the high molecular weight fractions do not exhibit this unexplained phenomenon.

Thereafter high molecular weight fractions were always collected from all wood sap samples. Experiments were performed as described earlier (21) with a few alterations.

The ELISA was essentially performed as described earlier (21) with two alterations:

- 1) Flexible polyvinyl chloride microtitration plates (Dynatech Laboratories, Inc., Alexandria, Virginia)

were used as these proved to increase sensitivity and decrease standard deviation between standard toxin samples. Absorbance measurements were performed after one hour in Immulon M 129 A plates (Dynatech) as optical properties of the flexible plates are poor.

2) The ELISA-substrate was prepared as follows:

80 mg 5-amino-salicylic acid was mixed at room temperature on a magnetic stirrer with 100 ml distilled water. Using 1 M NaOH the turbid liquid was titrated to pH 6.0; the solution will clear up during this procedure. Finally, 10 ml 0.1% H₂O₂ was added.

The results up to the present time are as follows: the highly resistant *Ulmus pumila* and the clones Lobel and 519 did not show external symptoms after inoculation with an aggressive isolate (H6) of *O. ulmi*. Also no glycopeptide could be detected.

The susceptible *Ulmus hollandica* cl. Belgica, *U. americana* and *U. gabra* developed heavy symptoms of which the first visible signs appeared after approximately one week (1981 field trials). Low levels of glycopeptide were detectable on the first sample date following the appearance of external symptoms. Later, glycopeptide increased to levels comparable to that reported earlier for *U. hollandica* cl. Belgica (21), between one and 10 µg/ml.

THE GLYCOPEPTIDE A VIVOTOXIN?

Criteria for vivotoxin were originally defined by Dimond and Waggoner (8), but amended by Dimond (5) and Graniti (12). They can be summarized as follows: (1) the substance must be isolated from the diseased host but not be present in the healthy plant; (2) it must be characterized chemically; (3) it must be produced by the causal organism(s) of the disease; (4) introduced in pure form into a healthy host, it must produce the symptoms of disease or a portion of the syndrome.

Although our work does not meet these criteria for a vivotoxin (or a vivoaggressin) (12) as we did not isolate and chemically characterize the fungal glycopeptide from the diseased plant, we suggest that the glycopeptide we detected in the elm should be regarded as a vivotoxin or -aggressin. The striking specificity of immunological detection methods and their high sensitivity, especially of the different enzyme-immunoassays (10, 35, 36, 37) and the radioimmunassays (24), gives an almost absolute proof of the identity of the detected antigen with the material used to raise the antibodies. Nevertheless, it is imperative that

many controls such as original antigens used for antibody production, extracts from healthy plants and sera from non-immunized animals should be included in the tests.

CONCLUSIONS

Due to the tremendous development of immunological methods new ways are clearly being created for detecting substances without elaborate purification. Therefore a supplement to the criteria for establishing the existence of a vivotoxin or -aggressin may be considered: instead of isolation and chemical characterization of the toxin from the diseased plant, immunological detection of the toxin in the diseased plant or in plant extracts should be sufficient as it proves the existence of a compound identical to that used for antibody production. The glycopeptide produced by *O. ulmi* meets these requirements and hence should be regarded as a vivotoxin.

Most evidence gained in studies on Dutch elm disease points towards impeding the water flow [a conclusion drawn generally for wilt diseases by Dimond (6)]; i.e., formation of tyloses and gums (33), plugging of pit membranes by degradation products of host cell walls (9, 23, 26) and by toxins, especially cerato-ulmin and the glycopeptide discussed above.

This does not explain, however, many phenomena observed after infection of an elm by *O. ulmi*, for instance, increased resistance of roots to water flow (18), increased respiration and increased electrolyte leakage (14).

Involvement of toxins in Dutch elm disease pathogenesis is obvious but their relative importance compared with other factors involved clearly needs more research.

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