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METHODS FOR SCREENING AND FOR THE RAPID SELECTION OF ELMS FOR RESISTANCE TO DUTCH ELM DISEASE

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CHAPTER 1
INTRODUCTION

The Dutch elm disease has ravaged the elm plantings in the Netherlands ever since its discovery some 45 years ago. Research has been done in this country to understand the nature of the disease and to control it (SCHWARZ, 1922; BUISMAN, 1928, 1929). The most promising approach to control appeared to be the search for resistant clones of the European elm. It was to the credit of Dr. JOH. WESTERDIJK, that as early as 1928 she draw attention to the possibility of growing elms resistant to *Ceratocystis ulmi* (Buisman) C. Moreau, the causal factor of the disease (WESTERDIJK, LEDEBOER and WENT, 1931). Originally, trees showing some resistance were selected from the existing elm population and from seedlings imported from different parts of the world (BUISMAN, 1931; WENT, 1938). In 1937, hybridisation was started in order to combine factors for a desirable rate of growth and shape with those for resistance (WENT, 1954; HEYBROEK, 1957). In 1936 and 1947 respectively the clones *Ulmus carpinifolia* Gled. cl. 'Christine Buisman' and *Ulmus hollandica* Mill. cl. 'Bea Schwarz' were released. Of these clones 'Ch. Buisman' was prone to *Nectria cinnabarina* Tode, and the 'B. Schwarz' elm had a poor shape. None of the clones available up to then, though fairly resistant to *C. ulmi*, could be generally recommended as suitable substitutes for the Belgian elm *Ulmus hollandica* Mill. cl. 'Belgica'. More recently the *U. hollandica* cl. 'Commelin' (HEYBROEK, 1961) and the *U. hollandica* cl. 'Groeneveld' (HEYBROEK, 1963) have been released.

Breeding of resistant fast growing elms is still being continued in the Netherlands. The work is carried out by H. M. Heybroek at Baarn as a part of the program of the Forest Research Station "De Dorschkamp" at Wageningen. Every year thousands of hybrid seedlings are grown. During their first year they are kept in a cold frame. In spring, when entering their second year, the seedlings are rogued and transplanted to a nursery, where they are left undisturbed for another year.

In the 3rd year they are tested for resistance by inoculating them with a spore suspension of *C. ulmi*. The inoculation is repeated the next year and those plants which have not shown symptoms are selected and sometimes transplanted again. After a period of rest, testing is repeated and the rate of growth and the habit of the plant are critically observed. Trees without external symptoms are propagated by means of grafting. These clonal progenies are again tested for resistance and finally the shape and rate of growth of the selected clones are estimated. The complete process of selection may take as long as 22 years before a clone can be released. Many difficulties have been encountered. Early investigators have referred to the difficulty of inducing infected young seedlings to show symptoms of disease. Though they were susceptible, as appeared at a later developmental stage, they did not show symptoms because of their unsuitable condition.

In the past only sporadic attention has been paid to the influence of environmental and other factors on host resistance. The empirical methods applied in selection appeared to be inadequate to decide whether an elm was really resistant or whether it was susceptible though it did not show symptoms after inoculation.

As well as on the condition of the host, the result of an inoculation may depend on:

a. The inoculation procedure, including the kind and the amount of inoculum and the way the tree is wounded in order to insert the inoculum.
b. The time of inoculation and the assessment of the results.

A critical study of the methods used in the process of selection seemed desirable. Further information was needed on the variation in morphology and pathogenicity of the causal fungus. The environmental conditions before and after inoculation had also to be studied more thoroughly. Attention had to be paid to the best time of year for inoculation and the method of propagating promising seedlings. Only when it was based on knowledge of these factors, could a rapid and accurate testing method for selecting resistant elm clones be developed.

The study was carried out at the Phytopathological Laboratory "Willie Commelin Scholten", Baarn, with the intention of trying to shorten the time needed for recognizing among hybrids the individuals that possessed a high grade of resistance, and to develop a test suitable for determining the degree to which the resistance of clones was under genetic control.

CHAPTER 2

MATERIALS AND METHODS

THE TREES

Various European elm clones of different degree of resistance have been used in the glasshouse as well as under field conditions.

Plant material was obtained from H. M. Heybroek, Forest Research Station, Wageningen. Seedlings originating from crosses between good parents were grown in the experimental nursery. Seedlings and clones have been propagated by means of "callus cuttings", produced by the "double propagation method" described by TCHERNOFF (1963). Callus cuttings have been widely used to advantage in pathological studies, as this kind of material offers maximal uniformity and the best possibility of obtaining uniform results in testing elm clones.

Most of the work reported here was conducted with relatively small trees of 1, 2 or 3 years old. The plants were raised in 16-cm pots, in wooden boxes in the glasshouse, under a glass-shed and in the nursery. In some experiments, containers of cement with a perforated bottom and a size of 75 × 75 × 100 cm were used, in each of which a group of 10 plants could be raised.

Trees were maintained in good growing condition by fertilizing and adequate pruning.

At least five plants were used in each treatment, though in many cases the number of plants treated in a similar way was 10, 20 or even more.

The pathogen

Pure cultures of *Ceratocystis ulmi* on cherry-agar were obtained from chips cut from the wood of naturally infected elms. The fungus was further cultivated on cherry- and oatmeal-agar at a temperature of 23° C.

From five current strains monoconidial cultures were obtained and used for inoculation in successive years. In other experiments ascospore cultures derived from ascospores out of one perithecium were used, the latter having been developed by crossing two native *Ceratocystis* strains.

Single-ascospore cultures were obtained by the dilution technique: the perithecia were disinfected with 50 % ethanol, followed by treatment with 3 % Na-hypochlorite for ten seconds, in order to kill any conidia adhering to the perithecium wall. The perithecia were carefully opened in a drop of sterile water. The spore suspension thus obtained was diluted with sterile water until only one ascospore appeared to be present in a small droplet. Drops of this suspension were transferred to 2 %-agar. After eighteen hours colonies became visible and could be subcultured on cherry-agar.

To maintain virulence unchanged, strains were not only kept under paraffin-oil, but a number of them, differing in morphological features, were preserved in lyophilized condition. Some strains were also kept in elms. In early summer a tree was inoculated with a strain which could be isolated from it in the next year.

The inoculum

Generally spore suspensions, consisting of one or more isolates of *C. ulmi*, were used for inoculation. Inocula were produced by shaking a piece of an agar culture in 100 ml of a liquid medium in flasks of 250 ml capacity for two days at 20° C. A modification of Zentmyer's nutrient medium for *C. ulmi* was found to be satisfactory: 20 g glucose, 2 g L-asparagine, 1.5 g KH_2PO_4, 1 g $MgSO_4.7\ H_2O$, 20 mg $ZnSO_4$, 10 mg $FeCl_3$, 1 mg vitamin B_1 (thiamine), 1 mg vitamin B_6 (pyridoxine) in 1 liter distilled water. After incubation the inoculum consisted of a suspension of yeast-like spores with a density of over 10^6 spores/ml.

Incisions in the stems of young trees (one to two years old) were made about two–three cm above the soil using a surgical chisel with a two mm wide point. This point was placed horizontally against the stem, the spore drop was placed on the point and the cut made into the xylem. Both sides of the stem were inoculated.

Older trees were wounded with a Stanley utility trimknife as used by Heybroek. With the knife still in the incision, the blade was flooded with a suspension of bud cells. Inoculum was added from a fine pipette until at least four drops were sucked in by the plant. For this to occur the incision had to be made into the newly formed vessels of the spring wood.

The temperature in the compartments of the glasshouse, where some of the experiments were performed, varied from 15° to 23° C.

Numerical evaluation of disease severity after inoculation

Numerical evaluations were based on the external disease effects. The simplest statistic was the percentage of trees affected in any treatment. Since the very young, about one-year-old trees, showed two manifestations of the disease, an acute one and a chronic one, the sum of the numbers showing each type of symptoms was considered as the total number of affected individuals.

The effect of the disease on the plants was expressed as a "disease index", which was determined as follows:

Two to three weeks and again 1 to 1½ month after inoculation the plants were examined, each one being assigned to one of the following classes and given a score equal to the class number:

class 1 – healthy

class 2 – a few leaves flaccid or yellow, doubtful whether diseased

class 3 – many leaves wilted, wilting irreversible, tree clearly diseased

class 4 – many leaves fallen and/or brown, more than one shoot or branch tip dead and crooked

class 5 – top of the stem drying; in the case of trees older than two years, the second-year wood is infected.

For each group of plants the disease index was calculated as follows: the score of each tree in the first class was multiplied by 0, that in the second class by 1, in the third class by 2, in the fourth class by 3, and in the fifth class by 4, so that the categories in this rating scale received the numerical values resp. of 0, 2, 6, 12, 20. The number of plants of each category was multiplied by these converted data. The sum of the products multiplied by ten was divided by 2 × the total number of plants, making the maximum possible disease index 100.

Holmes (1965), who analysed in this way the data of his experiment on virulence in *C. ulmi*, reported that the difference between the raw and the converted data, as mentioned above, did not affect the outcome of the statistical analysis of the data.

The accuracy of assigning numerical values depends upon familiarity with the expressions of the disease and, as a matter of course, with care in observation. The numerical classification made one month after inoculation was, however, not difficult to use after some experience.

CHAPTER 3

PHYTOPATHOLOGICAL APPROACHES TO THE PROBLEM OF A RELIABLE SCREENING OF ELMS FOR RESISTANCE

3.1. The cultural types of the fungus

The fungus, *Ceratocystis ulmi* (Buisman) C. Moreau, the causal organism of the Dutch elm disease, has been the subject of many varied investigations and experiments in Europe as well as in America and Canada. Life history studies of this fungus have shown that it is an organism living almost exclusively in xylem tissue of the living host, except during the period when it grows in dead wood and in the galleries bored by bark beetles and when it is carried on or in the body of this vector. The disease is a typical vascular wilt.

The conidial stage, which was discovered by Schwarz in 1922 in the Netherlands, is *Graphium ulmi* (Schwarz, 1922). This fungus has been found to possess a perfect form named *Ceratostomella ulmi* by Buisman (1932a). It was later transferred to the genus *Ophiostoma*, which was renamed *Ceratocystis* in 1952 (Moreau, 1952; Hunt, 1956).

Numerous isolates of *C. ulmi* were made from diseased elms in this country. In the present study it was noticed that there was noticable variation among the strains obtained. A classification system, based on the kind of spore formation, is given by many investigators (Schwarz, 1922; Wollenweber, 1927; Clinton and McCormick, 1936; Walter, 1937; Georgescu and Orenski, 1957). As a correlation between the metagenetic forms and virulence has not been detected, it was asked whether such a correlation might exist between certain cultural types and pathogenicity. The isolates were therefore divided into three classes based on differences in growth characters macroscopically observed in cultures on cherry- and oatmeal-agar. They are designated as follows:

class 1 – mycelium appressed forming slimy yeast-like colonies without aerial hyphae, distinctly concentrically zonate (Plate 1A); spore formation of the *Pionnôtes* type (Wollenweber)

class 2 – mycelium raised, forming a cottony, white to dirty grey mat, which spreads practically non-zonate (Plate 1B); conidia of the *Rhinotrichum* or the *Hyalodendron* type (Georgescu and Orenski)

class 3 – mycelium raised, with numerous coremia, culture usually dark coloured (Plate 1D); in some cases coremia-like structures may appear as long, black, tufted stalks lacking the ball of spores on the top and called "staghorn growth" by Clinton and McCormick.

It is recognized that there are no sharp boundaries between these cultural classes, since intermediate forms can always be found (Plate 1C), and for this reason it is probably not worthwhile to attempt to make subdivisions of the classes.

Isolates retained, however, the character of the class to which they originally belonged, when transferred to new slants, if the same nutrient medium was used. Transferring fifteen times did not change the cultural characteristics.

The cultural characters can, however, be changed by different factors (BOUDRU, 1933; TAYLOR, 1945a, and TAYLOR and PARKER, 1945b).

Evidently the metabolic pattern of the fungus can be modified by nutrients, oxygen tension, temperature and other environmental changes.

It was found that forms which were respectively yeast-like and mycelial when grown on agar both may produce the typical *Graphium*-coremia when grown on chips of elm wood. Since these isolates belonging originally to the classes 1 and 2 could thus be converted into the coremial form belonging to the class 3, it seems that the types are not fundamentally different.

Some physiological variability was found also. It was, for instance, found that one of the above mentioned yeast-like strains was unable to assimilate lactose as a carbon source or potassium nitrate as a nitrogen source, in contrast to a coremial strain, which assimilated these compounds readily.

Our observations did not prove that the mycelial forms are always less virulent than the strains of the yeast-like types or the *Graphium*-type. A mycelial form was as virulent as a yeast-like one (p. 418), which is remarkable, since hyphae of *C. ulmi* have seldom been found in the vessels of diseased elms, whatever type of culture had been used for inoculation. This scarcity of mycelium, and the rapidity of the progress of the fungus in the tree, suggests that it is present as yeast-like spores in the vessels, as all *Ceratocystis* spores are able to multiply by budding irrespective of the class to which the cultures belong. Conditions in the spring vessels of the elm tree may thus favour the development of the yeast-like form.

The small isolated yeast-like bodies were often observed by the writer in sections of wood of inoculated trees that were stained following the technique of GRAM and JØRGENSEN (1952).

It seems to us that the research on the yeast-like form would be more fruitful for investigators seeking an explanation of the disease mechanism than work performed with the mycelial forms.

It is, however, recommended that a mixture of spore suspensions derived from each of the three types should be used for screening an elm population or to test elm clones for resistance to the elm disease. If different types of the fungus are present in the inoculum, the chance of escape of hybrids or clones susceptible to certain specialized strains will be reduced.

3.2. Virulence in Ceratocystis ulmi

The pathogenic behaviour of an isolate of *C. ulmi* to different elm varieties or clones suggests that the same isolate can be highly virulent for one elm and less virulent to another. Variation in pathogenicity among isolates of this fungus has been little studied in the Netherlands. In 1932 Buisman (1932c) stated that strains of *C. ulmi* differ in virulence but the extent and constancy of these differences was not indicated. Went (1938) found no differences among the strains she used, but observed the occurence of more and less virulent ones in a few cases.

In America, Tyler and Parker (1945c) demonstrated, however, significant differences in the capacity of different races to induce disease, and the authors' data show the constancy of these characters in each strain.

Experiments were undertaken at the laboratory "Willie Commelin Scholten" to study variation in pathogenicity of some Netherlands' strains of *C. ulmi*. In studying differences in pathogenicity, the elm material to be used for testing has to be genetically uniform and moreover the plants have to be of the same age and raised under the same conditions.

The pathogenicity studies were divided into three groups:

a. to compare the degree of the pathogenicity of different isolates of *C. ulmi*
b. to determine comparative virulence of various cultural types of the fungus
c. to investigate the occurrence of physiological specialization of strains of *C. ulmi*.

The experiments were carried out in different years between 1958 and 1965.

Pathogenicity of some current strains

The first experiment was conducted with three-year-old susceptible plants grown from layers of the Belgian elm. The trees were raised outdoors and they were watered periodically. They showed only slight variations in vigour. Each time nine plants were inoculated with one of four strains obtained from the "Centraal Bureau voor Schimmelcultures" at Baarn, nine other plants with a spore suspension consisting of a mixture of these strains, and some other trees were injected with sterile water for control (Table 1, experiment I).

Two years later, when we had succeeded in growing root "callus cuttings", this material seemed to be most appropriate for testing the virulence of *C. ulmi* strains, since the cuttings are highly uniform, being derived from one tree and raised under the same conditions. Cuttings of the moderately resistant clones No's 1 and 148 were raised in the glasshouse and grown in pots; later they were kept in a glass-shed. At the age of eighteen months they were inoculated. Each time ten plants were treated with one of five strains to be

tested for virulence. All strains were monoconidial cultures except TX 59, which was a mass-transfer-isolate (Table 1, experiment II).

TABLE 1

Pathogenicity of various strains of *C. ulmi* to European elms

material	strains	disease indices after inoculation:	
		2 weeks	4 weeks
experiment I:			
Three-year-old layers of the Belgian elm	S 9	5.0 [1]	6.2 [1]
	V 5	35.5	79.0
	S 13	41.7	85.0
	I 10	56.2	86.2
	mixture	40.0	95.0
	control	0.0	0.0
experiment II:			
18-month-old callus cuttings of the clones No's 1 and 148	TX 10/8	—	8.2 [2]
	TX M/3	—	20.7
	TX 50/1	—	31.7
	TX 59	—	36.5
	TX 21/4	—	41.0
	control	—	0.0

[1] Indices of groups of nine plants.
[2] Indices of groups of ten plants.

All strains of *C. ulmi* used in both experiments were able to infect the test plants and hence all of them were considered to be potentially pathogenic to elm. Their virulence differed considerably, however. The single spore culture TX 10/8 and the mass-isolate S 9 proved to be only weakly pathogenic, whereas the strain TX M/3 had to be considered as mildly pathogenic. The inoculum consisting of a mixture of spores of four strains was equally virulent to that of the most strongly pathogenic strain. It was concluded that the association of spores of weaker strains with those of stronger ones exerted no inhibitive effect upon the effectiveness of the latter.

Virulence of different cultural types of C. ulmi

It may be asked, whether a correlation might exist between different cultural types of the parasite and their virulence.

For an experiment three mass-isolates were chosen, each of them belonging to one of the three cultural types. From three other mass-isolates also belonging to one of these types, single-spore cultures were derived, which were also used in the experiment. In an earlier experiment the pathogenic character of these strains had been proved. As test plants two-year-old layers of the Belgian elm were used. The trees grown in containers in the garden of the laboratory. Each time

nine trees were inoculated with one of the six strains. After the treatment the weather conditions were very favourable for incidence of disease. The plants were examined weekly. They all showed symptoms of nearly the same degree of severity and there was no marked difference between the effect of the three cultural types (Table 2).

TABLE 2

Effect of inoculation of groups of nine trees with spores of three cultural types of *C. ulmi*

strains	cultural type	disease indices after inoculation:	
		3 weeks	6 weeks
	yeast-like form:		
TX 21/4	monospore culture	61.0	64.4
TX 6	mass-isolate	68.0	70.0
	mycelial form:		
TX 10/6	monospore culture	50.0	60.0
TX 50	mass-isolate	53.0	68.0
	coremial form:		
TX D/3	monospore culture	52.2	59.0
TX 36	mass-isolate	50.0	56.7
mixture of six strains		59.0	69.0

Results of inoculations with a spore suspension containing a mixture of spores of all six strains were similar to those obtained by the other treatments.

From the results of the first experiments (p. 417) on the pathogenicity of different strains of *C. ulmi* it could be concluded that the strains varied greatly in virulence. No consistent correlation was found, however, between a cultural type of parasite and pathogenicity.

Physiological specialisation of C. ulmi

In 1960 PEACE (1960) remarked that "there is at present no evidence that different elms vary in resistance to different strains of the fungus". Shortly after the appearance of this paper, it became evident from a preliminary experiment carried out at Baarn, that specialized strains might exist (SMITH, 1962, not published). Probably the pathogenicity of different isolates of *C. ulmi* is to some extent dependent on the variety or *Ulmus*-clone inoculated. A strain that is virulent to one clone might not be equally virulent to another one.

A more clear concept of terms used in this respect is necessary. ZADOKS (1961) discussed this terminology in a general sense. Pathogenicity is a character of the fungus and the analogue of resistance is the host. In pathogenicity of the fungus two forms may be dis-

tinguished: variety-non-specific and variety-specific. Non-specific pathogenicity was up to now considered as occurring generally in the interaction between the isolates of *C. ulmi* and the elm clones. Specific pathogenicity would be a form which is only active against one or a few elm varieties or elm clones. The reactions of different varieties or clones together form a reaction spectrum which characterizes the strain. A strain, which is virulent to many varieties or clones is more versatile than a strain which is virulent to a few of them only.

Some experiments have been undertaken at the laboratory "Willie Commelin Scholten" to examine the possibility of such physiological specialization among the Netherlands' strains of *C. ulmi.*

I. A preliminary experiment concerning this question has been performed with potted plants of Belgian elm (*U.h.* 'Belgica') and of field elm (*U. carpinifolia*). Both varieties are known to be entirely susceptible to the Dutch elm disease. Fifty callus cuttings of each clone were grown and inoculated in the glass-shed with spores of the strains TX 21/4, TX 50/1, TX 59, TX 36 and TX M/3 (Table 3).

TABLE 3

Effect of inoculation of groups of ten trees of two susceptible *Ulmus*-varieties with spores of different strains of *C. ulmi*

strains / varieties	disease indices six weeks after inoculation with strains:				
	TX 21/4	TX 50/1	TX 59	TX 36	TX M/3
field elm (*U. carpinifolia*)	68.0	67.0	59.0	*41.0*	*18.0*
Belgian elm (*U.h.* 'Belgica')	60.0	63.0	55.0	*20.0*	*40.0*

From the results given in the table it appears that the strains TX 21/4, TX 50/1 and TX 59 only show a limited variation in virulence. The peculiar effect after inoculation of the strains TX 36 and TX M/3 is, however, striking. TX 36 affected the Belgian elm to a much lower degree than the susceptible field elm, which is generally not less resistant than the Belgian elm. The reverse happened after inoculation with the strain TX M/3, which attacked the Belgian elm rather heavily but induced only a weak disease syndrome in the field elm.

In this case specialization of the strains TX 36 and TX M/3 in relation to both elm varieties used, was a possible explanation of the facts observed, since the reaction spectrum was different.

Following this preliminary experiment two other experiments were made in which more clones were involved.

II. The second experiment was also conducted in the glass-shed. The pathogenicity of five strains TX 21/4, TX 50/1, TX 59, TX M/3 and TX 10/8 was tested on four clones, i.e. Belgian elm, American elm, clone 1 and clone 148. One-year-old callus cuttings of these varieties in pots were used. There were fifty similar plants of each clone ten callus cuttings being inoculated with each strain (Table 4).

TABLE 4

Effect of inoculation of groups of ten trees of four *Ulmus*-clones with spores of five different strains of *C. ulmi*

clones / strains	disease indices six weeks after inoculation of the clones:			
	Belgian elm	American elm	clone No. 1	clone No. 148
TX 21/4	72.0	55.0	10.0	27.0
TX 59	66.0	52.0	22.0	*6.0*
TX 50/1	38.0	29.0	21.0	39.0
TX M/3	*39.0*	17.0	11.0	16.0
TX 10/8	10.0	20.0	3.0	0.0

Also in this experiment the pathogenicity of different isolates of *C. ulmi* depended to some extent on the variety or clone inoculated.

The strain TX 59 appeared to be virulent for three of the clones but it was hardly able to induce disease symptoms in clone 148. TX M/3 was highly virulent to the Belgian elm, but to the other three clones its virulence was only slight. The pathogenicity of TX 10/8 was weak for all clones tested.

It can be concluded that among these strains of *C. ulmi* some host selectivity exists but there was obviously no complete specialization of the strains.

This difference in reaction spectrum between some strains is of importance in testing for resistance. If only the strains TX 10/8 or TX 59 or both of them had been used as inoculum, the clone 148 would have been assessed as practically resistant. This clone, which became moderately diseased after inoculation with TX 21/4 and TX 50/1 can hardly be considered as resistant.

III. A third experiment was carried out in the glasshouse under conditions which were kept as uniform as possible. The pathogenicity of five single-ascospore cultures was tested on one-year-old callus cuttings of different already known degrees of resistance: entirely susceptible (*U.h.* 'Belgica' and *U. carpinifolia*), moderately resistant ('Ch. Buisman' elm and clone 248) and resistant (clones 296 and 390). From each clone twenty callus cuttings were available and four plants could be inoculated with each strain.

Table 5 shows the disease index of each group of four plants, six weeks after inoculation. The variability among the individuals of the groups was only small, presumably because of the homogeneity of the plant material raised in the glasshouse.

TABLE 5

Effect of inoculation of groups of four trees of six *Ulmus*-clones with single-ascospore cultures of five *C. ulmi* strains

clones / strains	disease indices six weeks after inoculation of the elm clones:					
	U. carpinifolia	*U. holl.* 'Belgica'	clone 248	'Buisman' elm	clone 296	clone 390
K 5021/4	60.0	55.0	12.5	17.5	2.5	0.0
K 2155/a	67.5	62.5	12.5	*5.0*	0.0	0.0
K 2110/8	70.0	32.5	27.5	10.0	0.0	0.0
K 5936/1	*30.0*	40.0	12.5	*32.5*	2.5	0.0
K 21D/1	*27.5*	47.5	*20.0*	10.0	5.0	0.0

Also among the single-ascospore cultures of *C. ulmi* used in this experiment some host selectivity existed. *U. carpinifolia* and the 'Buisman' elm were infected to the same degree by strain K 5936/1, though the 'Buisman' elm behaves as a resistant clone towards strain K 2155/a. If only this strain had been used for testing the 'Buisman' elm, this clone would have been considered as resistant, just as it was in former years. In the thirties, however, BUISMAN (1936a, 1936b) noted, that in a few cases this elm could be slightly attacked by the fungus. This fact can be explained by use of a specialized strain of *C. ulmi*. A similar case of specialization, although less pronounced, occurred with the strain K 21D/1. Nearly equal disease indices were rated for the entirely susceptible *U. carpinifolia* and for the moderately resistant clone 248.

From the data from the three above-mentioned experiments it may be concluded that in some cases strains of *C. ulmi*, having the same degree of pathogenicity for one clone, were not equally virulent in their attack on another host-clone. Up to now we were, however, not able to note a complete physiological specialization of our current *C. ulmi* strains. Whether the differences in virulence observed were significant was not calculated.

More experiments will have to be performed with a greater number of clones and isolates. A differential set of *Ulmus*-clones will have to be built up, in order to test the reaction spectrum of current isolates of *C. ulmi*.

We should prefer to use non-specific strains of moderate virulence for screening rather than highly virulent ones with a trend to physiological specialization and possessing only a narrow reaction spectrum of *Ulmus*-clones.

3.3. Retention of pathogenicity of C. ulmi in culture

For inoculation purposes a simple and efficient method of preservation of cultures had to be developed to keep the isolates of *C. ulmi* in their original state of virulence. Only by use of strains with a constant level of pathogenicity throughout all phases of the selection process would the results of assessment of elms be comparable.

It was observed that after semi-annual transfers to fresh cherry- or oatmeal-agar, the sporulating ability of some isolates deteriorated, especially that of the coremial form of the fungus. To keep virulence at a constant level several methods of preservation were tried out. It is known that stock cultures of several fungi can be kept under mineral oil or in lyophilized condition without any alteration of their viability and their morphological characters for an extended period. Nothing was known, however, about the retention of pathogenicity of *C. ulmi* under these conditions.

A third method consists in introducing a strain in an elm, from which it can be readily isolated when inoculum is needed. This method appeared to be laborious and expensive, however, when many strains had to be preserved and consequently many elms were needed, one for each strain. Moreover after isolation, each strain has to be checked for purity.

There was evidence that the fungus would die in infected trees. This may be correlated with the fact that many trees apparently recover completely from the disease (Peace, 1960).

To test the effects of different methods of storing cultures the pathogenicity was compared of subcultures of four strains of different virulence, marked TX I to TX IV, from which the stock cultures had been preserved in the following ways:

a. under mineral oil for one year
b. in lyophilized condition for one year
c. in elms into which the culture had been introduced one year before.

Sixty callus cuttings of the Belgian elm had been raised in wooden boxes for two years. In their third growing season they were inoculated with a conidial suspension prepared from ten-day-old subcultures of the stock cultures.

Batches of five individuals received the same type of inoculum. All treatments were performed in the glasshouse on May 26th, about 35 days after bud break.

Within one week after treatment all trees inoculated with spores provided from lyophilized cultures exhibited incipient wilt symptoms. Three days later all other test plants became diseased. Final results, based on the disease index of groups of five inoculated plants, are shown in Table 6.

Preservation of stock cultures under mineral oil, in lyophilized condition or in elms appeared to have hardly any influence on the virulence of *C. ulmi*.

TABLE 6

Comparison of the pathogenicity of four *C. ulmi* strains, preserved for one year in different ways, on groups of five elms

strains of *C. ulmi*	disease indices after inoculation with suspensions obtained from stock cultures preserved:					
	under mineral oil		lyophilized		in elms	
	assessment after		assessment after		assessment after	
	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
TX I	60.0	100.–	54.0	100.–	30.0	100.–
TX II	44.0	83.0	48.0	78.0	44.0	78.0
TX III	44.0	54.0	22.0	32.0	38.0	38.0
TX IV	23.0	36.0	23.0	36.0	23.0	36.0
average	43.0	*68.0*	36.8	*61.5*	33.7	*63.0*

3.4. SEXUAL REPRODUCTION OF CERATOCYSTIS ULMI

As early as 1932 it was found by BUISMAN (1932a) that the causal fungus of the Dutch elm disease was heterothallic and that mating of a plus and minus strain in vitro resulted in the production of the perithecia of *C. ulmi*. Later BUISMAN (1932b) found perithecia on elms growing in the Netherlands.

Following Buisman's work various investigations were carried out by American workers to study the sexuality in *C. ulmi* and to determine the conditions that would promote the production of fruiting bodies (SWINGLE, 1936; CLINTON and MCCORMICK, 1936; SHAFER and LIMING, 1950; ROSINSKI, 1958, 1961).

It has been possible to differentiate our strains of *C. ulmi* on the basis of their sexual behaviour. The terminology of "A" and "B" types, as introduced by Shafer and Liming, has been used to determine the compatibility of the Dutch strains. American strains of known compatibility were kindly put at our disposal by Dr. Holmes, who stayed in our laboratory in 1962. Crossings between these strains and our TX 50/1 and TX 21/4 revealed that TX 50/1 belonged to the "A" and TX 21/4 to the "B" type. These strains were used as standard types in further matings. It was not possible to correlate the American terminology with that used by Buisman as the strains used by her were no longer available.

Using a modified Buisman's technique, we observed that perithecia were readily formed on autoclaved, split and peeled elm twigs in test tubes. Pieces of the parent cultures were placed on the wood. About 1 ml sterile tapwater was added and the tubes were kept at 20° C. Perithecia developed on the twigs, regardless of the variety of elm, when they were incubated in darkness or in daylight. It was

concluded that high moisture was necessary for the initiation and the production of mature fruiting bodies. Cherry-agar and other synthetic media appeared to be unsuitable for perithecia production; only in a few cases, such as when our strains TX 21/4 and TX 50/1 were paired on oatmeal-agar, abundant mycelial growth was followed by development of mature perithecia.

Only combinations of fresh isolates of *C. ulmi* readily produced fruiting bodies. Two-years-old cultures never developed perithecia, which suggests that the fruiting ability is lost after a series of transfers or preservation under paraffin-oil.

At present growing of the ascomycetous stage of *C. ulmi* in vitro does not seem to be difficult. When a compatible pair of strains is used, perithecia develop on the split elm twigs, though some crosses may produce abundant perithecia and other only few. Of thirty-six Dutch strains, which were paired with our standard types successively, only ten appeared to belong to the "A" type and twenty-three to the "B" type. Three strains produced perithecia when paired either with the "A" or the "B" type. These strains might be considered as bisexual, or as a mixture of "A" and "B". They did, however, not produce perithecia by themselves.

From one of these strains twenty monoconidial cultures were grown, which were tested for compatibility. It appeared that five of these cultures belonged to the "B" type, only one to the "A" type and fourteen cultures did not produce perithecia when mated with the standard types. In this way it was determined that both "A" and "B" races were present in the original isolate.

Cultural and morphological differences between the sexual races have not so far been observed.

Mating also occurred when a young elm tree was inoculated simultaneously with inoculum of two compatible types. After the tree became diseased, a piece of stem was cut and split and incubated in the usual way. On the wood, that had died in the meantime, perithecia of *C. ulmi* appeared after one month.

Since *C. ulmi* is heterothallic, it can be expected that through the sexual process occurring in nature, new strains might arise, which might alter the genetic status. To obtain more information on this matter, an experiment on a limited scale was undertaken. The strains TX 50/1, TX 21/4, TX 36 and TX 59 were paired. From the drop containing ascospores expelled from the pore of a perithecium new cultures were grown:

parent cultures:	TX 21/4 × TX 50/1	TX 50/1 × TX 36	TX 50/1 × TX 59
F_1 cultures	K 5021	K 5036	K 5059

The virulence of the three F_1 cultures and their parents were tested on two-year-old *U.h.* 'Vegeta' grafts in the nursery. Each group of five similar plants was inoculated with one strain. With exception of five control plants treated with nutrient medium, all elms became diseased. Disease indices were calculated for groups of five plants.

disease indices of 5 trees inoculated with the parent cultures	disease indices of 5 trees inoculated with the F_1 cultures
strain TX 21/4 – 70.0	cross-culture K 5021 – 91.0
strain TX 50/1 – 60.0	cross-culture K 5059 – 84.0
strain TX 36 – 54.0	cross-culture K 5036 – 78.0
strain TX 59 – 48.0	

The F_1 cultures appeared to be more virulent than their parent strains. The symptoms induced by the former were not only more severe than those incited by the parent strains, but they also appeared at an earlier date.

These results suggest that if a fungus with a new genetic structure arises in the field, it might be more virulent than the parent strains.

HOLMES (1965) obtained perithecia by crossing European with American isolates during his stay at Baarn. He inoculated young susceptible and moderately resistant elm clones in our glasshouse with several single-ascospore cultures originated from these perithecia. A few of the ascospore cultures did not incite symptoms at all and have to be considered as wholly non-pathogenic; a few, however, induced severe disease symptoms in the 'Buisman' elm. The fact that this highly esteemed elm showed disease symptoms may be an indication that these cross-cultures were highly virulent.

From the results of our experiment and that of Holmes it can be concluded, that combination and segregation of nuclei may give rise to strains that are more or less virulent than the parent cultures.

When breeding elms resistant to the Dutch elm disease, one has among other things, to pay close attention to changes in the genetic status of the fungus. New physiological strains of *C. ulmi* may appear, which might be able to attack previously resistant elm clones.

3.5. METHODS OF INOCULATION

For screening of seedlings for resistance to the fungus and as an adjunct to field testing of clones we rely upon artificial inoculation carried out under conditions favorable to the disease.

Up to 1958 the early investigators in this country inoculated elms by introducing the spore suspension by means of a syringe with a short needle. This method was laborious to carry out on a large scale and it gave somewhat erratic results. It was specially unsatisfactory when applied to young, one to two-year-old elms. The woody parts of the stems of these plants, being very tough, prevented the needle from penetrating and moreover the inoculum was not or only sparingly absorbed.

Meanwhile for a quicker inoculation of older elms, Heybroek introduced a Stanley trimknife with a triangular exposed blade. The results of inoculation were at least not less effective than the syringe-infection, but the knife was too large for inoculation of very young plants.

To develop a reliable procedure for inoculation, which would result in consequent and uniform disease development in *young* elms susceptible to the fungus, many small scale experiments have been performed:

Each time a group of five plants of the susceptible 'glabra' type (*U. glabra*) and of the moderately resistant clone No. 248 was inoculated with a spore suspension of virulent *C. ulmi* strains.

The methods applied were as follows:

1. a spore suspension was injected into the main vein of a leaf. Injections were repeated at different times during the season
2. a spore suspension was sprayed on the leaves
3. a spore suspension was put into a wound made by cutting into the axil of a young twig
4. drops of suspension were placed on the wound made by cutting off the tip of the stem
5. the suspension was injected by means of a syringe
6. a curved surgical chisel was held with its point against the stem. A drop of suspension was placed on the point. A cut was made into the xylem and the drop was immediately sucked up by the vessels.

In other experiments on a large scale other inoculation methods have been tried:

7. young plants from which the roots were partly cut were placed into a spore suspension, where they remained for 24 hours. After that period they were replanted
8. a spore suspension was added to the soil of potted plants, the roots of which were cut on several occasions, at each time before watering
9. inoculum was injected into the main root of potted plants
10. a number of seeds were kept between blotting paper moistened several times with a spore suspension. After germination the seedlings were potted.

The only successful methods were No's 5 and 6. After all other treatments the plants remained healthy without even any discoloration of the xylem. At most one short dark marking could be observed, originating from the point of infection. There was never evidence of extensive discoloration of the wood.

Positive results were only obtained when the xylem of the stem had been cut followed by the active adsorption of the inoculum by the plant. After applying the chisel or knife 80 to 90 % of the plants became diseased. If a dry and clean surface of the stem is ensured, it was easy to state whether the inoculum was absorbed. This phenomenon coincided with a "sipping sound" in old trees. That an active sucking by the trees is an important factor in establishing an infection, was shown in the following experiment: those plants were marked,

which absorbed the inoculum well and also those ones which absorbed it badly or not at all. From 92 seedlings into which the entrance of the inoculum was rapid, 77 % became diseased. From 26 seedlings estimated as bad suckers only 36 % became diseased.

Contrary to the results of OZOLIN (1958), covering the incisions with masking tape to prevent desiccation improved the percentage of affected trees only insignificantly.

All studies hitherto performed indicate that the most convenient method of artificial inoculation of European elms is the introduction of inoculum directly into cut xylem vessels.

Similar results have been obtained by SMUCKER (1937) with the American elm.

3.6. EFFECT OF AGE AND NUMBER OF SPORES ON THE EFFECTIVENESS OF INOCULA OF CERATOCYSTIS ULMI

The results of inoculation of elm seedlings in the field suggested that spores derived from old cultures of *C. ulmi* were only little effective. Following a prelimenary test on the influence of age of the spores on the disease index of seedlings in the field in 1960, a more complete experiment was performed under controlled conditions in the glasshouse in 1961.

On April 26 groups of five one-year-old callus cuttings of the Belgian elm were inoculated at the stem base with an aqueous suspension containing 4, 7, 10, 14, 28, and 42-days-old spores respectively. The percentage germination of the spores appeared to be independent of their age. All spores originated from sub-cultures of a strain representing a virulent coremial form of *C. ulmi*. The number of spores in the inoculum has been adjusted to about 250 000 spores/ml. In the glasshouse the relative humidity was kept at about 90 %, and the day temperature varied between 20° and 24° C. The foliar symptoms were evaluated twelve days and 28 days after inoculation. Thereafter the symptoms remained stationary (Table 7).

TABLE 7

Effect of the age of spores of *C. ulmi* on the disease indices of groups of five Belgian elms

date of sub-culturing of the strain	age of spores in days	disease indices on	
		May 9	May 28
April 22	4	27.0	60.0
,, 19	7	27.0	40.0
,, 16	10	35.0	50.0
,, 12	14	20.0	27.0
March 30	28	23.0	33.0
,, 14	42	15.0	27.0

All trees became more or less diseased, but the severity of the infection was less with trees which received spores older than ten days. This response may be the result of decreased viability of the spores with increasing age, but it may be doubtful if this factor only accounts for the variation observed, as the number of spores absorbed by the different trees may have differed. This factor may also be of influence on the data obtained. It was, therefore, of interest to measure the effect of spore loads on development of disease.

An inoculum containing over 10^6 ten-days-old spores/ml was partly diluted with sterile water, after which a spore count by a haemocytometer revealed about 10^3 spores/ml in this suspension. Both suspensions were used to inoculate similar elm material as in the previous experiment. As each of the twenty callus cuttings tested absorbed four droplets of inoculum (i.e. 1/5 ml), each of the plants of one batch of ten plants received approximately 200 000 spores, and each individual of the other batch took up only about 200 spores. The disease index of each group of ten plants was estimated at several occasions during a period of two months after inoculation. No marked difference in severity of the symptoms could be observed. With a load of about 200 spores per tree fairly good infection was obtained.

This experiment confirmed that the number of spores present in a suspension between the limits of 10^6 and 10^3 ml was of minor importance for establishing the disease in susceptible elms.

ZENTMYER *et al.* (1946), using young American elm seedlings, found that even as few as 100 spores were sufficient to produce some disease symptoms in all inoculated trees and only after inoculation with ten spores not every tree became visibly infected. These investigators reported also that disease symptoms only appeared in trees receiving two- and four-days-old spores.

Our own studies and those of Zentmyer have indicated that the age of the spores has a greater effect on the incidence of disease than the number of spores in the inocula.

It is advisable to use only spores not older than ten days in all comparative trials in testing European elms for resistance to *C. ulmi*. If the inoculum is prepared as described in the chapter "Materials and methods", it will contain for the greater part young spores.

3.7. PERIOD OF SUSCEPTIBILITY OF ELMS TO THE DUTCH ELM DISEASE

It is still unknown when in the season a European elm starts to be susceptible to Dutch elm disease.

Inoculation of young European elms at various times during the growing season of 1960 and 1961 has provided us with information on the time of the year at which elms are most susceptible to the disease and the length of this susceptible period.

The experiments were performed with elm seedlings of moderate resistance in their second season of growth, raised outside in wooden boxes in groups of ten plants per box. Beginning soon after bud break,

groups of ten trees were inoculated every week. As usual a suspension consisting of a mixture of spores from five current strains was applied. One month after each inoculation the plants were rated for external symptoms and disease indices were estimated for groups of ten plants. At the same time two uninoculated plants were cut off and the development of the spring wood was microscopically examined.

Climatological conditions in the rather normal year 1960 favoured the development of the disease symptoms. On the contrary the chilly and rainy weather in the spring of 1961 influenced the development of disease in such a way that only a few trees showed incipient wilt after early inoculation. The data of the experiments in 1960 and 1961 are shown graphically (Fig. 1).

The period in which the trees were most susceptible and disease indices of 35 or more were obtained, is indicated by hatching. In both years susceptibility increased rapidly after bud break to an optimum in June. After the period of extreme susceptibility the effectiveness of inoculation fell off rapidly. Inoculation in late July produced no external symptoms and only a few plants developed any vascular discoloration.

All plants inoculated in the beginning of April 1960, 45 days after bud break, showed external symptoms in various degrees of severity. A period of extreme susceptibility began on May 20 and extended for 30 days (Fig. 1a). The increase of susceptibility in 1961 has been observed 52 days after bud break, but the period of extreme susceptibility extended only for thirteen days (Fig. 1b). It began on June 13 i.e. more than three weeks later than in 1960. A period of extreme susceptibility between June 13 and 19 was common in both years (Fig. 1c). Only during that week one would have been sure of a successful inoculation. Based on these dates mid-June can be considered as the most suitable time for inoculation of European elms in the Netherlands.

The results of these experimental inoculations were supported by observations in the nursery during the succeeding years. The period of extreme susceptibility coincided with the rapid development of spring wood. The highest susceptibility was observed in both years at the moment that the cambium was active and a wide zone of spring vessels had been formed. From a microscopical study of the spring wood it could be concluded that the decline in susceptibility coincided with the beginning of the formation of summer wood. The early investigations with American elm lead to similar conclusions (SMUCKER, 1937; ZENTMYER, 1946).

Recently SMALLEY (1963), studying the seasonal fluctuation of the disease in young American seedlings, found a correlation between decline in susceptibility and the declining rate of terminal growth and subsequent abortion of the apical bud.

Marked differences have been observed by Smalley between European elm (*U. carpinifolia* Gled.) and American elm (*U. americana* L.) in the length of the susceptible period and the time of highest susceptibility.

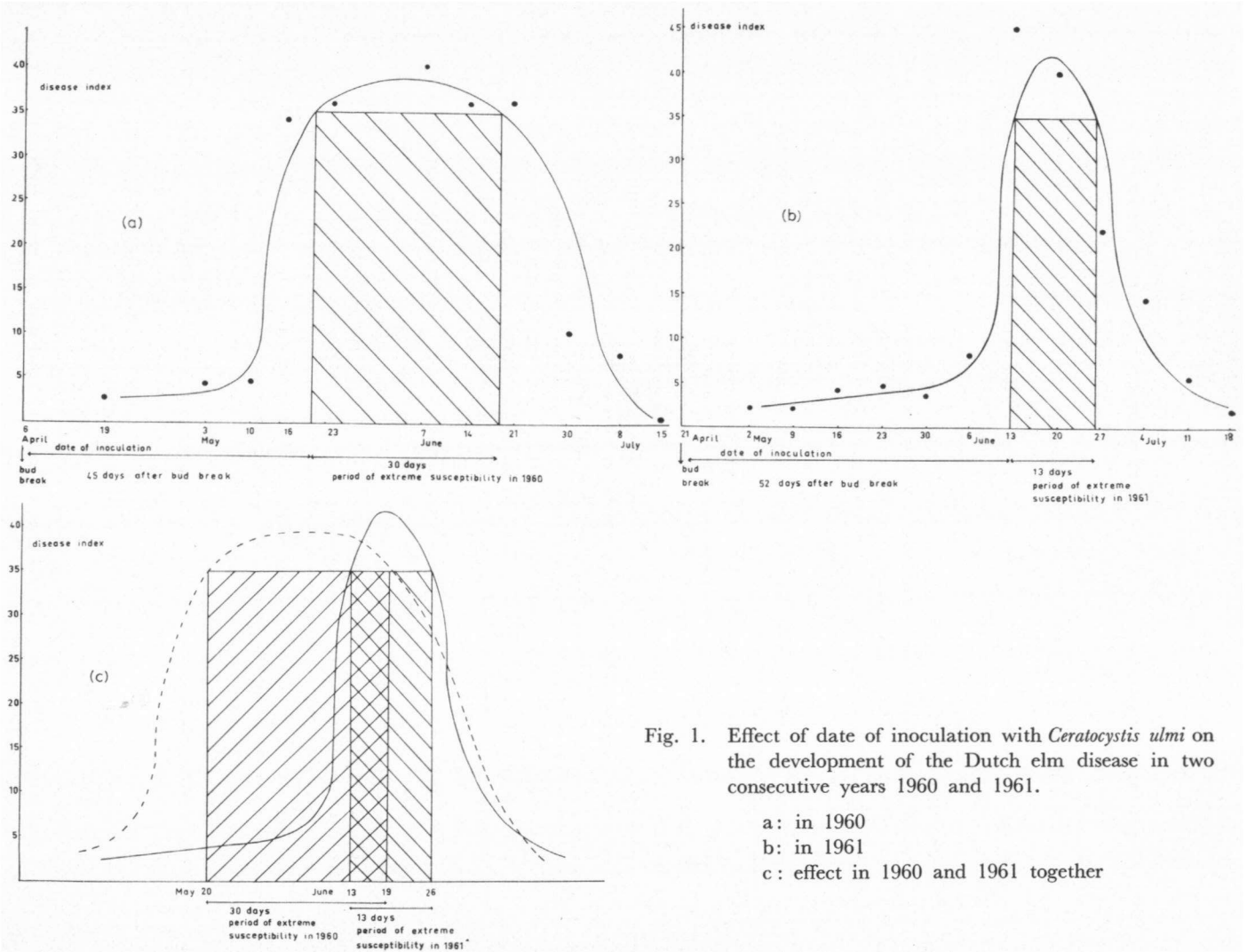

Fig. 1. Effect of date of inoculation with *Ceratocystis ulmi* on the development of the Dutch elm disease in two consecutive years 1960 and 1961.

a: in 1960
b: in 1961
c: effect in 1960 and 1961 together

In connection with these results the importance of performing inoculations in the right period has to be emphazised. This period starts approximately 40 to 50 days after bud break. At that time trees still show elongation, and all inoculations have to be performed with the least delay.

3.8. Tolerance of susceptible elms to C. ulmi in relation to their physiological condition

Various investigators have noted, that the physiological condition of the European elm is of importance in relation to the development of external visible symptoms of the Dutch elm disease after inoculation.

It is generally considered that vigorously growing trees are more affected by the disease than those with a poorer growth (Buisman, 1929, 1935; Went, 1954; Heybroek, 1957; Peace, 1960). As early as 1939 Krijthe and Went (1939) screened 434 seedlings of the same origin, planted out in a nursery partly on fertile and partly on poor soil. It became obvious that after inoculation the symptoms of trees on the fertile soil were more severe than those on the poor soil. From the first group 97.5 % became diseased against 57 % from the latter. It will be clear, that the behaviour of the trees of the last group was misleading, since many of them might erroneously have been taken for resistant when tested for susceptibility.

Zentmyer *et al.* (1946) came to a different conclusion. They reported that the appearance of symptoms in American elms following inoculation, tended to become more erratic as the size of the trees increased. When fertilizer was applied to some plants, however, it was found that the progress of the disease in vigorously growing trees was markedly less than in those of poor vigour. Criteria of "vigour" included size and colour of foliage, diameter increment and shoot growth. These results suggest that the use of fertilizers had some beneficial effect in enabling the trees to withstand infection by the fungus. In contrast, Ouellet and Pomerleau (1965), also experimenting with the American elm, reported that in the same seedling population the larger trees reacted more severely on inoculation (87.9 % diseased), than the shorter, less vigorously growing trees (53.6 % diseased). They noted that the development of external disease symptoms of elms was stimulated by maintaining moist soil conditions by regular watering. These results of the Canadian investigators are in agreement with those from early work in the Netherlands on the European elm.

Also Kais, Smalley and Riker (1962) reported that inoculated plants, grown on soils having low water holding capacities, were less sensitive to the disease than plants grown on soils with greater moisture holding capacities. Vascular discoloration was greatest in plants watered daily and least in plants watered every fifth day. The growth of both groups of plants was similar.

It may be postulated that any factor which tends to increase the growth of a susceptible elm, also encourages the development of disease symptoms after inoculation. This is in accordance with the finding of BECKMAN (1958) who has shown, that symptom expression in Dutch elm disease can be delayed by treatments that retard tree growth. Susceptible elms artificially inhibited in their growth, became only 16 % diseased after inoculation.

We studied the reactions of two groups of seedlings grown and inoculated in the glasshouse. Twenty of them had a circumference at the stem base of 3.8 to 5.5 cm and twenty had a circumference of 2.6 to 3.7 cm. Both groups developed symptoms to the same extent and had similar disease indices. Cross-sections of the stems revealed that in trees of both groups the spring wood was equally well developed. Probably the appearance of symptoms is not determined by the circumference of the stem at a certain developmental stage. It is considered probable that it is correlated with the degree of activity of the cambial tissue in the production of secondary xylem elements, specially in the formation of xylem vessels. Further study is in progress on the development of spring wood during the period during which the trees show the greatest development of symptoms after inoculation.

The effect of *transplanting* may also be reckoned among the factors influencing the "vigour" of an elm and consequently the appearance of external disease symptoms after inoculation. The unavoidable damage to roots caused by transplanting may inhibit active growth in length as well as the development of secondary tissues of a tree. After inoculation such an elm does not show symptoms but it can not be considered as resistant. Early investigators in the Netherlands were already aware of this fact (BUISMAN, 1929; BROEKHUIZEN, 1929; WENT, 1954).

SMALLEY (1963), also found in glasshouse experiments that young seedlings of American elm inoculated shortly after transplanting, remained symptomless. They showed only a limited discoloration of the wood.

The results of our early studies during the years 1959 to 1962 were in agreement with those of Smalley. One-year-old grafts of *U. americana*, *U. hollandica* 'Belgica', *U. hollandica* 'Vegeta', 'Bea Schwarz' elm and the clone No. 248 were transplanted into the nursery of the laboratory. It was our experience that none of these trees showed symptoms after inoculation for at least one year after transplanting. If the environmental conditions were unfavourable for further growth, the period of tolerance or non-sensitivity was even longer.

Presumedly, desirable conditions of the trees during the selection and breeding of elms resistant to *C. ulmi* would be: adequate manuring, watering if necessary to keep the soil at an adequate moisture level and, especially, avoidance of transplanting during the period of screening of young plants.

3.9. Pathological anatomy

Reactions of elm wood to infection by the fungus *C. ulmi* have been described by many investigators. The symptoms are briefly recapitulated here.

In cross-section, diseased twigs or branches usually show one or more rings of brown spots. They occur in the spring wood of the annual ring. In many cases the spots may coalesce to form a more or less continuous circle round the stem. If the bark is peeled away and the sap wood exposed, longitudinal discontinuous brown streaks of varying length will be observed, which correspond to the ring of spots in cross-section. The brown spots are composed of vessels blocked by tyloses and gum. Discoloration of the outer xylem and the formation of tyloses and gum in the vessels of European elms have been observed by Broekhuizen (1929) and Buisman (1933). Adjacent living cells may also be filled with a dark sustance. In a more recent study of vascular discoloration Kerling (1955) reported that the first alteration in infected tissue of European elm is the discoloration of vessels' walls, followed by disintegration and browning of the contents of the living cells. Gum droplets were exuded through pits of parenchyma and medullary ray cells into the lumina of vessels followed by the formation of tyloses in the vessels.

The fungus thus stimulates the production of tyloses and gums which block the vessels and induce the characteristic markings. It seemed unlikely that the comparatively restricted formation of tyloses would block a large trunk completely. The rapidity with which the fungus often acts lends support to the conception of Zentmyer (1942) and of Dimond (1947) that the toxin formed by the fungus is the causal factor of the abnormalities. The experiments of Kerling with the European elm confirmed that the effect of toxin is comparable to that of a spore suspension introduced into a tree.

The purpose of our study was to compare the behaviour of living cells adjacent to the vessels in more resistant elms with that of less resistant ones.

In a preliminary experiment, the transport of small particles in a healthy tree was studied using the chemically inactive Indian ink. A dilute suspension was introduced into the stem of young elms of thirteen various *Ulmus*-species and clones of different grade of resistance. The technique of treatment was based on that used by Banfield (1941): cones made from plasticine "Jumbo" were fixed around the stems and filled with the diluted ink. Two chisel cuts were made into the xylem below the level of the fluid. Two days later cross-sections of the stems were studied. The pattern of movement of the ink-particles was most evident in the outer growth layer of the sap wood. In all species studied, susceptible as well as more resistant ones, similar patterns were found.

The phenomenon of lateral transfer of the ink-suspension in these woody stems was observed just as described by Greenidge (1955). The ink-spots coalesced to form a continuous circle around the stem,

at the time the ink-suspension continued to move quickly into the apices of treated trees. This spreading ink-pattern most probably reflects the path of the inoculum after inoculation.

Contrary to the absence of reaction of the host to the presence of the neutral ink-particles, reactions do occur to the presence of the fungus, resulting in the typical vascular discoloration of the wood centered around the site of the living spores of the parasite.

The reaction pattern of the host induced by the fungus was studied in several wood samples: Belgian elm (extremely susceptible), clone No. 248 (moderately resistant) and 'Bea Schwarz' elm (resistant). The wood pieces were taken at intervals of 2, 10, 20, and 30 days after inoculation during the growing season. These samples and also those taken from untreated trees were fixed in 1 % chromic acid, to which few drops of strong acetic acid was added. The samples were kept in a vacuum for 18 hours. After fixation they were washed out in running water for 24 hours and microtome-sections were made. In this fixation medium dead and disintegrated cells became plain brown and the patterns of discoloration became more accentuated. In the healthy wood the discolorations were limited to only few cells of the parenchyma which had collapsed.

The discoloration always started in the vessels and spread from there to the living cells of the wood and the medullary rays, the cells of which were sometimes darkened as far as into the pith or up into the cortex.

The process of discoloration in the Belgian elm was not confined to a restricted part of the xylem situated above the incision. It advanced in tangential directions, including more and more of the spring wood until a complete xylem discoloration encircled the stem. The walls of the vessels darkened, tyloses and gum masses occurred throughout the xylem. The discoloration pattern became *spreading* (Plate 2A).

The resistant, inoculated 'Bea Schwarz' elm, however, contained sharply localized discolored patches consisting of one or a group of vessels, plugged by tyloses and gums, surrounded by woody elements. In a cross-section several of these spots were observed, giving rise to a *spotted* pattern.

A study of the discoloration pattern of the wood of various elm species and clones revealed that a spotted pattern was only found in elms with high resistance to the fungus as assessed by considering the external disease symptoms after inoculation. As well as the 'Bea Schwarz' elm, the clone No. 296 and the clone No. 390 also showed this pattern (Plate 2C and 2D). The latter clone is considered to be a useful addition to the assortment of valuable clones, and may be released for commercial use in future if other characters are equally favourable. Alternatively it might be used for further breeding.

Between the two clearly defined types of pattern a range of intermediate types was found in other elms. Many clones were examined, with varying degrees of susceptibility, as judged by the external symptoms. In all of them a complete or partially spreading pattern was perceptible.

One of the wood samples studied was cut from a *U. wallichiana* elm growing in the nursery of the laboratory "Willie Commelin Scholten", an offspring of the Himalaya elm, imported in former days. Although formerly this elm was considered to be resistant, it became severely diseased after inoculation. The spreading pattern of the cross-section confirmed its susceptibility to the disease (Plate 2B).

The cross-section of the moderately resistant clone No. 248 showed in one of the stems sectors with a spreading discoloration and also sectors with a spotted pattern. In sections of another individual of the same clone the pattern was completely spreading.

After a detailed study of the discoloration patterns of many elms it was concluded that no strict correlation between patterns and the degree of susceptibility could be stated. Only an *extreme* degree of resistance against the fungus appeared to be correlated with the spotted pattern. No correlation could be found between the width of the vessels, the amount of parenchyma cells in the wood, the number of rows in the medullary rays on one hand and the pattern of discoloration on the other. Though the wood of the resistant 'Bea Schwarz' elm showed wide vessels and multiseriate medullary rays, these characters did not correspond with resistance or susceptibility in other elms.

After observing the reaction of the wood of several clones of which the degree of resistance was known, the internal symptoms of inoculated *seedlings* were studied. Among individuals showing clear external symptoms of disease a spreading pattern of discoloration was found in all cases. Plants which remained without symptoms in many cases showed localized discoloration, which remained restricted to patches of one or a few vessels with the surrounding tissue (spotted pattern). The two types of discoloration patterns are shown by photographs (Plate 3A and 3B).

A correlation between the absence or presence of external symptoms and the pattern of discoloration was, however, not always found. It was therefore desirable to examine the frequency of exceptions. Sixty seedlings of the cross "202 × 336", growing in the nursery, were inoculated. After two months they were divided in two groups: one group comprised 23 plants showing symptoms and the other 37 symptomless elms. The stems of all the plants were cut. The plants of the first group showed a spreading pattern, whilst nearly all plants of the second group showed a spotted pattern of discoloration. Three plants of this last group revealed, however, a spreading pattern. Without further study they would have been considered as "tolerant", being heavily discolored but externally symptomless. Later on it became evident, however, that the vegetative offspring, grown as callus cuttings from one of those apparently resistant trees were susceptible to the fungus. In this case the internal symptoms gave the correct indication about the genetic constitution of the mother tree. The mother tree had only an apparent resistance.

The screening for resistance is rendered difficult by this occasional lack of external symptoms, which can be attributed to environmental conditions such as the position of the tree in relation to light or to the local condition of the soil.

If the screening of seedlings is based only on external disease symptoms it *may* lead to escapes. In cases of doubt in selection for resistance it is recommended that next to the external symptoms the distribution of xylem discoloration should be studied in a cut branch or even a few branches.

3.10. Conditions which have to be fulfilled for an effective testing of elms for resistance to C. ulmi

Each of the predisposing factors studied may affect the incidence of symptoms after inoculation. Our study has indicated the following recommendations for a reliable method for testing for resistance:

A. Concerning the plant

1. The way in which the test plants are raised may determine their reaction after inoculation. If growth is inhibited, the trees may react to a far lesser degree than when growing vigorously or they may not show external symptoms at all.
2. After transplanting, the trees are not in a suitable condition to show symptoms following inoculation for at least a year.
3. The elm is sensitive to the fungus only during the time spring wood is formed. The period of highest sensitivity starts about 40 to 50 days after bud break. Inoculations performed in mid-June are likely to give the most reliable results.

B. Concerning the fungus

4. The inoculum should consist of a mixture of spores of *C. ulmi* strains, of which the virulence must be checked before use.
5. Spores, not older than ten days, should be used for inoculation purposes.
6. For inoculations, spores should be suspended in a nutrient solution of the following composition: 20 g glucose, 2 g L-asparagine, 1.5 g KH_2PO_4, 1 g $MgSO_4.7\ H_2O$, 20 mg $ZnSO_4$, 10 mg $FeCl_3$, 1 mg vitamin B_1 (thiamine) and 1 mg vitamin B_6 (pyridoxine) in 1 litre aq. dist.

C. Concerning the inoculation technique

7. The spores have to be introduced into the vessels by means of an incision in the youngest vessels of the outer xylem.
8. The inoculum has to be sucked up by the plants. In the case of large trees a hissing sound may be heard. Entrance of the suspension into vessels is promoted by a slight twisting of the knife.

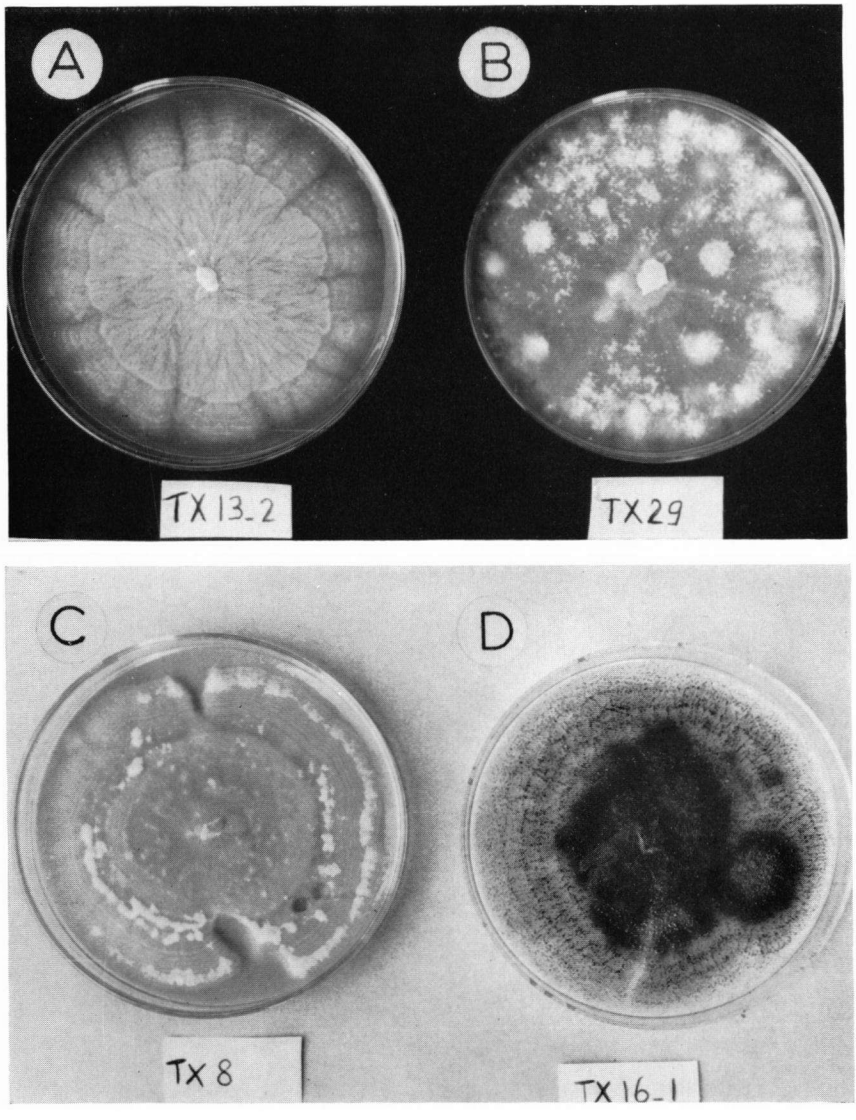

Plate 1. Variations observed in monoconidial strains of *Ceratocystis ulmi* in the Netherlands as shown by cultures on cherry-agar incubated in darkness at 23° C for ten days.

A: a typical zonate strain; a yeast-like slimy colony

B: a mycelial strain characterized by aerial white mycelium, spreading type, practically non-zonate

C: a mycelial strain, partly loosely aerial, partly resupinate, showing pronounced zonation

D: a dark coremial strain that produced numerous coremia on oatmeal-agar and a pronounced zonation

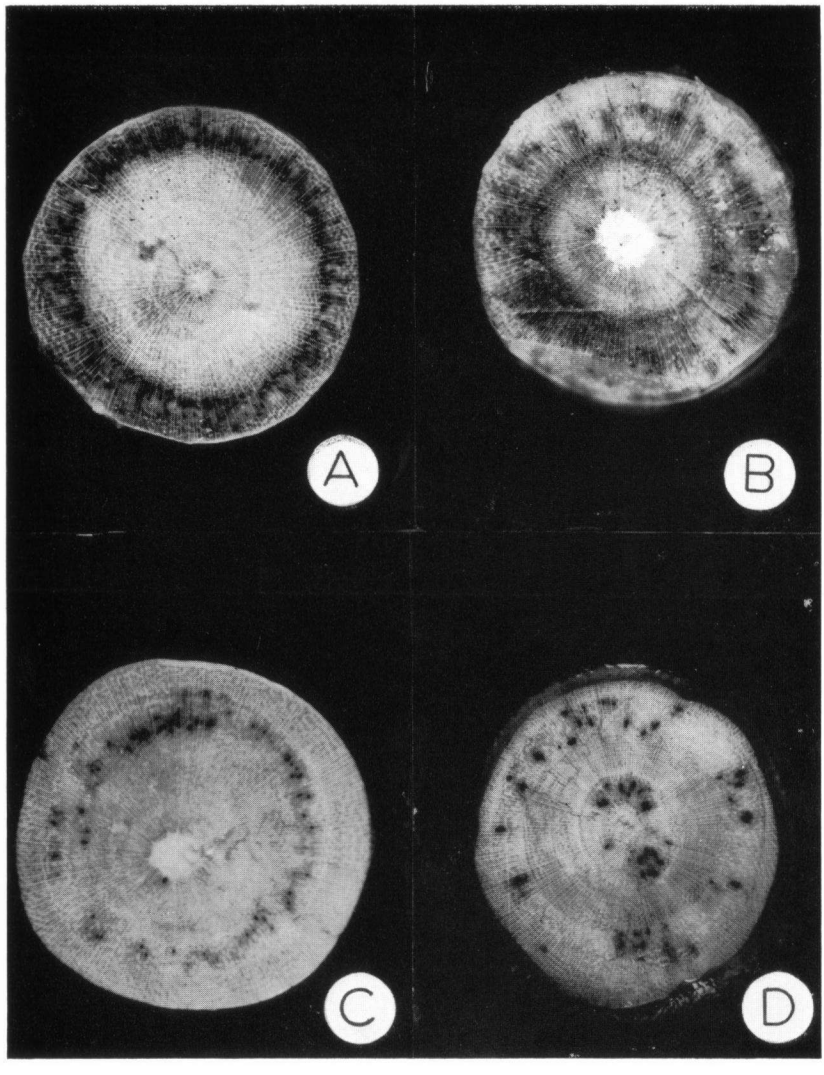

Plate 2. Cross-sections of infected branches of *Ulmus*-varieties showing discoloration of sapwood.

A: *Ulmus hollandica* 'Belgica'
B: *Ulmus wallichiana*

The heavy infection appears as a complete darkened ring in the sapwood ("spreading pattern"). Both varieties are entirely susceptible to the Dutch elm disease.

C: *Ulmus hollandica*, clone 296
D: *Ulmus hollandica*, clone 390

The discoloration of the sapwood shows as a broken circle of brown spots ("spotted pattern"). These newly raised clones are considered to be resistant to the Dutch elm disease.

Plate 3. Photomicrographs of transverse sections of stems of 16-month-old seedlings after inoculation with spores of *Ceratocystis ulmi*. Wood samples were fixed in 1 % chromic acid for 24 hours. Sections 18 μ thick mounted in glycerin-jelly.

A: Wood of a severely wilted hybrid *U. hollandica* 'Belgica' × *U. wallichiana*, showing a spreading pattern of vascular disoloration

B: Wood of a hybrid of the same population without external disease symptoms after inoculation, showing a spotted pattern of vascular discoloration.

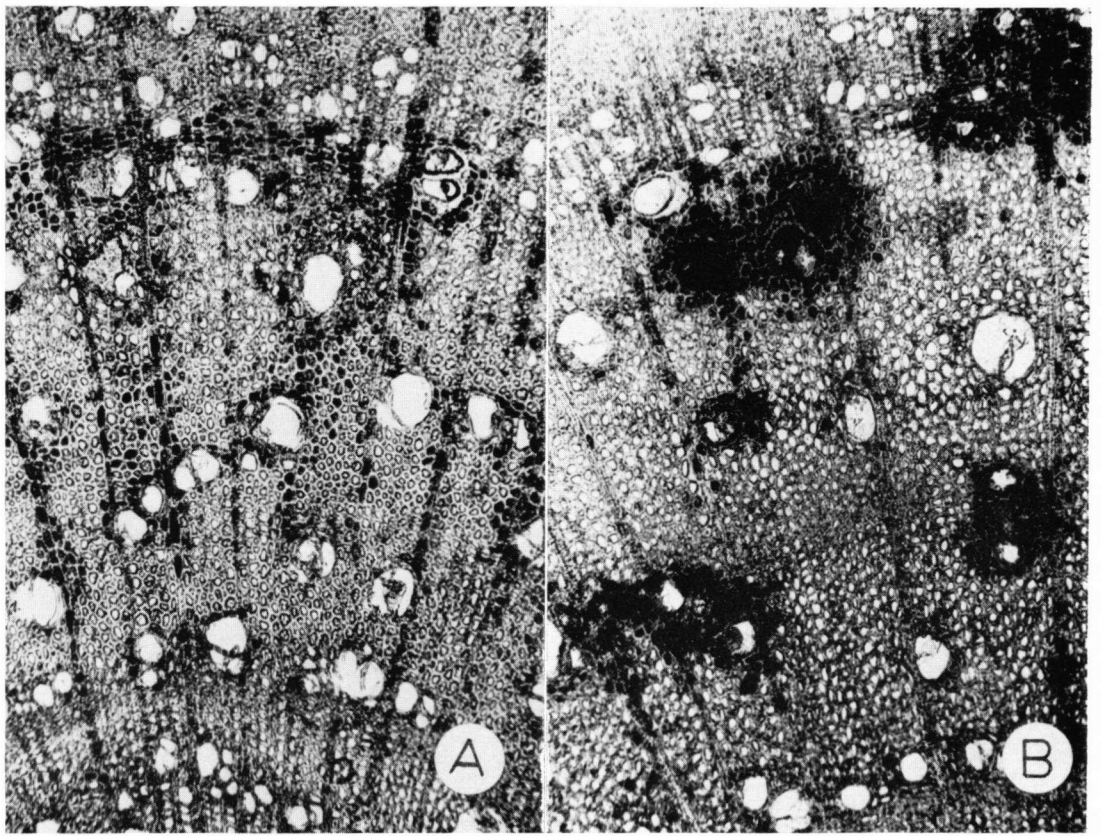

Plate 4. Effect of infection by *Ceratocystis ulmi* on young callus cuttings of European elms in their second year of growth in the glasshouse.

A: External symptoms of disease on Belgian elm two weeks after inoculation.

B: Symptomless callus cutting of the clone TA II about one month after inoculation.

D. Miscellaneous

9. Hot weather and direct sunlight during inoculation are favourable for the appearance of external symptoms. The time of the day at which the inoculations are performed, is also of importance: inoculation in the morning gives the best results.
10. Watering of the plants before or directly after inoculation is also favourable for obtaining good results.

These "ten commandments" should be considered as an effort to improve and standardize the testing technique leading to a reliable selection of elms resistant to the Dutch elm disease. This testing technique was found most satisfactory and was used throughout the research here reported.

CHAPTER 4

INVESTIGATIONS ON THE POSSIBILITY OF A RAPID SELECTION OF ELMS

4.1. Susceptibility of very young elms to the fungus

Attempts have often been made in the past to select young European elm seedlings resistant to *C. ulmi* by inoculating them with a spore suspension of the fungus. These experiments failed as none or only few of the plants reacted with external disease symptoms after inoculation. A higher percentage of the seedlings became diseased when testing was repeated at a later stage. It was assumed that European elms become progressively more susceptible while developing to the adult stage (Heybroek, 1957). For this reason a definitive judgment on the resistance of a clone to the disease did not seem to be possible as long as the juvenile stage continued. Seedlings were therefore screened from their fourth year on for several years. If trees of eight to nine years old still did not show external symptoms, the selection was continued within their vegetative progenies (Went, 1954).

Caroselli and Feldman (1951) have also referred to the difficulty of successfully inoculating young seedlings of the American elm with *C. ulmi*. They therefore advised a prolonged dark treatment of plants prior to inoculation in order to increase their sensitivity. Heybroek, however, was not able to duplicate their results, using European elm.

It seemed once again necessary to check the previous work and to investigate whether the hypothesis of youth-resistance could be confirmed. The following experiments were carried out to determine the susceptibility of young elms under controlled conditions in the glasshouse as well as in the open air.

Experiments on susceptibility of young seedlings

Ten one-year-old seedlings of the *U. glabra* type were kept and inoculated in the glasshouse. Ten others were also inoculated and kept outdoors. When inoculated the plants were in full growth and the other conditions favourable for a successful inoculation were also maintained.

Inoculations were performed at the beginning of the susceptible period as well as at the end of it. Inoculum consisted of a mixture of spores from ten strains of *C. ulmi*. Their virulence had been checked beforehand. All plants reacted with the typical disease syndrome and finally all seedlings were heavily affected. The isolation of the fungus from the discolored wood was proof that the disease symptoms were provoked by *C. ulmi*. Youth-resistance was not evident.

A number of experiments with moderately resistant one-year-old hybrids was performed in the glass-shed. In Table 8 the results of six experiments with this material are given.

TABLE 8

Effect of inoculation of one-year-old seedlings with a spore suspension of *C. ulmi*

material (hybrids)	date of inoculation	number of plants				diseased plants %	disease-index
		inoculated	with external symptoms				
			acute	chronic	none		
HB × *U. wall.*	May 25	15	9	3	3	80	57.0
HB × *U. wall.*	June 15	20	10	4	6	70	42.0
HB × 248	May 25	15	8	4	3	80	48.0
HB × 248	June 15	20	11	6	3	86	42.0
U. americana	May 29	24	17	6	1	96	42.0
U. americana	June 15	20	12	8	–	100	53.0

HB = *Ulmus hollandica* 'Belgica'; *U. wall.* = *Ulmus wallichiana*

It appeared that the one-year-old hybrids reacted after inoculation in a similar fashion to the susceptible *U. glabra* seedlings. The percentage of diseased plants varied between 70 % and 100 %. Two-year-old hybrids of the same origine grown under similar circumstances and treated in a similar way as the one-year-old hybrids also became diseased.

In view of the results of these experiments the hypothesis of youth-resistance could not be confirmed. It also appears from the recent experiments of ARISUMI and HIGGINS (1961) and SMALLEY (1963) with young American seedlings, that youth-resistance of *Ulmus* spp. is unlikely.

Experiments on the susceptibility of young vegetative progeny

The hypothesis of youth-resistance has been tested in another way. The susceptibility was compared of two groups of plants of the same age and belonging to the same clone, one group in the juvenile and the other in the adult stage of development. This possibility exists with the elm, as stem cuttings maintain the adult character, whereas one- to two-year-old callus cuttings from the same tree are in a juvenile stage (TCHERNOFF, 1963). Both groups are alike genetically.

Stem and callus cuttings of the Belgian elm, the 'Bea Schwarz' elm and the clone No. 248 were simultaneously raised in the glasshouse. At the age of eighteen months they were inoculated with a mixture of five current strains of *C. ulmi* (Table 9).

TABLE 9

Effect of inoculation of eighteen-month-old stem and callus cuttings with a spore suspension of *C. ulmi*

material (clones)	number of plants				disease index on		
	inoculated	with external symptoms			29/4	4/5	11/5
		acute	chronic	none			
HB stem cutting	5	5	–	–	28.0	78.0	78.0
HB callus cutting	5	5	–	–	8.0	72.0	72.0
248 stem cutting	5	2	2	1	14.0	20.0	26.0
248 callus cutting	5	2	1	2	10.0	20.0	30.0
BS stem cutting	5	1	1	3	0.0	6.0	10.0
BS callus cutting	5	1	1	3	0.0	8.0	12.0

HB = *U. hollandica* 'Belgica'; 248 = clone No. 248; BS = 'Bea Schwarz' elm

From the results of this experiment it could be concluded that the callus cuttings and the stem cuttings reacted with external symptoms of the Dutch elm disease and showed nearly the same grade of susceptibility after they were inoculated with a spore suspension of *C. ulmi*.

In another study on a larger scale, 200 callus cuttings of Belgian elm, American elm, clone No. 1 and clone No. 148, fifty individuals of each, were grown in 16-cm pots. At the age of sixteen months they were inoculated in the glass-shed with five current strains of *C. ulmi*. Six weeks after inoculation the plants were rated for symptom-development (Table 10).

It appeared again that young callus cuttings of different clones reacted positively on inoculation.

TABLE 10

Effect of inoculation of groups of 50 trees of four elm clones, in their juvenile stage of development, with a spore suspension of *C. ulmi*

material (callus cuttings)	number of plants infected in %	disease index	resistance
Belgian elm . . .	100	45.0	susceptible
American elm . .	84	34.0	susceptible
clone 248	52	17.6	moderately resistant
clone 1	60	13.4	moderately resistant

The abundant evidence in the past from other sources that there is a marked difference in susceptibility between American and Belgian elms on one side and clones No. 1 and No. 148 on the other, is confirmed by this trial using young callus cuttings as test material.

From the experiments with young elm material described above it can be concluded that the hypothesis of youth-resistance, in the sense as described above, was not confirmed. Young seedlings in their second year of growth as well as callus cuttings, both being in a juvenile stage, are able to react with disease symptoms after inoculation provided that the conditions for effective inoculation are fulfilled.

The first screening for resistance against the Dutch elm disease can, therefore, be carried out during the very first stages of development of elm material.

4.2. PROCEDURE LEADING TO A RAPID SELECTION FOR RESISTANCE

The purpose of selective breeding for resistance is to improve the genetic constitution of elms by hybridisation, i.e. by bringing together genes for resistance in one individual. The success of this method depends on the availability of sources of resistance and requires an appropriate crossing-program. Allied to these problems to be solved by the geneticist, the development of a rapid procedure to be used in selecting and raising of resistant and good growing trees is of the highest importance.

As the breeding of elms resistant to the Dutch elm disease is a time consuming work, it was asked whether the time needed to reach this purpose could not be shortened. Considering the present state of knowledge concerning the host-pathogen relationship of the Dutch elm disease, it seems appropriate to suggest a selection scheme, deviating in some respects from that applied in the Netherlands up to now (Fig. 2).

The following aspects of the selection procedure have to be emphasized:

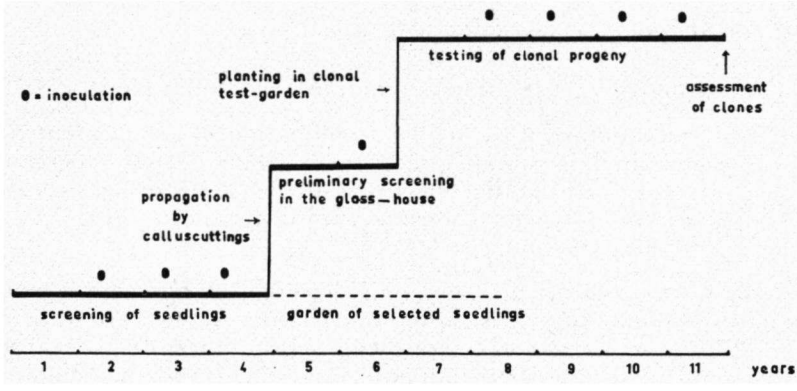

Fig. 2. Suggested scheme for selection of European elm for resistance to the Dutch elm disease.

a. It is of primary importance, that elms at all stages in which they are tested for resistance are vigorously growing, for only actively growing plants can be expected to show foliar wilt after inoculation.
b. Transplanting has to be avoided, as only plants from which the roots are left undisturbed react to inoculation within a short time.
c. A rigorous screening of the seedling-population has to be performed in a relative short time, in order to eliminate as soon as possible all plants that show disease symptoms or other unfavourable qualities.
d. Special stress has to be laid on screening of the vegetative progeny of selected seedlings.
e. Testing of callus cuttings is to be preferred to that of grafts, as grafted scions often show great variability in growth and there is also the possibility of a stock-scion effect. This influence of the rootstock is eliminated when callus cuttings are growing on their own roots.

Within the framework of the suggested new scheme three stages can be distinguished:

1. Screening of seedlings of a hybrid-population.
2. Testing of the vegetative progeny of resistant selections under controlled conditions in the glasshouse.
3. Testing of clones under field conditions.

These procedures have been tried out for six years in the nursery and the glasshouse of the laboratory "Willie Commelin Scholten" at Baarn. Our experiences with the methods of testing elms in their consecutive stages of selection are reported below.

4.3. Screening of seedlings for resistance

The testing of the seedling-population is as follows:

First year: Seed obtained by artificial pollination of good parent trees was germinated in wooden seed trays. Shortly after emergence, the saplings were potted in small "jiffy"-pots or in so-called "Nico"-plastic pots which were set out at distances of 25 × 30 cm in a well prepared nursery.

Second year: In May of the next year defective plants were removed and the remaining ones were inoculated, taking care that the conditions for an effective treatment were fulfilled. In the autumn the stems of the diseased individuals were cut and the remaining plants lightly pruned.

Third year: The second inoculation was performed. The plants that became diseased were removed and misshapen trees were discarded.

Fourth year: The third inoculation was performed. After a drastic selection only healthy looking and fast growing trees with a good shape were retained for further testing of their progeny.

Of the experimental nurseries established in 1959, 1960 and 1961 in the garden of the laboratory, that of 1960 provided the most complete data concerning the results of screening. The material grown therein and tested for resistance comprised seedlings from three lots of seed harvested early in the same year. They were inoculated for the first time in the second week of June 1961 and for the second time in the third week of June 1962. The process of inoculation was repeated for a third time in 1963 and the apparently healthy seedlings were classified as "tolerant" (Table 11).

Table 11

Number of seedlings which remained healthy after screening for resistance in the nursery 1960

year of inoculation	number of plants	backward and deformed plants	number of plants				total number of plants diseased in %
			inoculated	with external symptoms			
				acute	chronic	none	
1961 (1st inoc.)	233	44	189	54	29	106	44.0
1962 (2nd inoc.)	106	13	93	19	19	55	40.8
1963 (3rd inoc.)	55	—	55	26	21	8	83.6

During the three years of testing 57 plants (24.5 %) have been discarded on account of backwardness and deformity. Of the remaining 176 plants 168 individuals (95.45 %) became diseased and only 8 of the inoculated hybrids (4.5 %) eventually survived. These eight survivors were further screened by studying the pattern of discoloration on the transverse cut through the branches. The four plants that showed a spreading pattern of discoloration were discarded. The remaining four being undamaged have been marked as "resistant selections".

Though elms may show an inhibition of growth after being inoculated repeatedly, in the trial described here, two trees were present which showed fast growth although they were inoculated. Only the trees which showed this combination of tolerance and rapid growth were retained for further screening, in order to prevent selection for resistance resulting in the selection of trees with weak growth.

The results of this experiment on the possibility of a drastic selection of elms in the *juvenile* stage of development have been wholly confirmed by those obtained in the next experiment performed in the nursery of 1961, where another lot of hybrids was planted. Here again a high number of susceptible plants could be traced within four years after germination. It is obvious that the time which has to be spent on screening of hybrids on resistance can be considerably shortened in comparison with the time needed in the past.

Seedlings selected in this way were planted out in a "seedling-mother-garden" in autumn of their fourth year. Here the plants can be kept growing for some years in order to observe their growth-rate, shape, resistance to *Nectria*, frost, and other qualities. To trace the origin of their progenies these trees were provided with a selection number.

4.4. Testing of the vegetative progeny of resistant selections

It was necessary to ascertain if such healthy good growing seedlings are really resistant to the Dutch elm disease or whether they are merely escapes. This is done by inoculation of the vegetative progenies of these plants in order to study the extent of their resistance to the disease.

4.4.1. *Preliminary screening of clones under controlled conditions*

As a preliminary check on the resistance to the fungus of the selected seedlings, callus cuttings from such trees have been raised. The cuttings were inoculated with virulent strains of the fungus. The methods described previously were applied. The use of this kind of vegetative progeny is obviously advantageous, as it makes it possible to test a large number of uniform replicate trees all of the same genetic constitution and all growing on their own roots.

The testing of the one-year-old callus cuttings should be done in the glasshouse, where the influence of climatological conditions can be largely eliminated. Moreover here the testing can be performed more precisely than in the field.

The following experiment confirmed that callus cuttings are highly suitable to be used as testing material. From each of six clones of a known degree of resistance, twenty callus cuttings were raised. The following clones were chosen:

a. *U.h.* 'Belgica' and *U. carpinifolia*: both extremely susceptible.
b. Clone 248 and 'Ch. Buisman' elm: both moderately resistant.
c. Clone 296 and clone 390: both resistant.

At the end of January 1963 roots of these clones were cut and planted. In May of that year all 120 cuttings in pots of 16-cm diameter were present in the glasshouse. In November they entered into a rest period and in December they shed their leaves. In March 1964 young leaves developed and at the end of April all the plants were inoculated, the treatment being given just 45 days after bud break of each clone. At that time the trees are considered to be most sensitive to the fungus. The plants were examined for disease symptoms three and six weeks after inoculation (Table 12).

TABLE 12

Disease indices of twenty 15-month-old callus cutting of each clone raised and inoculated in the glasshouse

material	disease indices		assessment of the clones
	3 weeks after inoculation	6 weeks after inoculation	
U. carpinifolia	28.0	51.0	very susceptible
U.h. 'Belgica'	33.5	47.5	very susceptible
clone 248	14.0	17.0	moderately resistant
'Ch. Buisman' elm .	11.5	15.0	moderately resistant
clone 296	0.0	2.0	resistant
clone 390	0.0	0.0	resistant

The abundant evidence already obtained from other sources, that there is a marked difference in susceptibility between the three groups of clones tested, was confirmed by the results of this experiment. Such tests with callus cuttings under controlled conditions offer not only the best conditions for maximal uniformity in the testing of young clones, but they also indicate the degree of susceptibility of the vegetative offspring of seedlings thought to be resistant.

Callus cuttings originated from four selected hybrids ex-nursery 1958, which received the clonal numbers TA I to TA IV were tested in the glasshouse in the same way. Two months after inoculation all cuttings of the Belgian elm and the clones TA I, TA III and TA IV appeared to be diseased to varying degrees of severity, whereas those of the clone TA II remained healthy (Plate 4A and 4B).

This inoculation test indicated again that the vegetative offspring from apparently resistant seedlings are not always really resistant to the fungus. In this case the offspring of only one of four selected seedlings could be retained for further testing. A first testing of a clone under glasshouse conditions may save the selectionist an elaborate and expensive testing in the field of a large number of clones which will be after all of no value for general planting on account of their susceptibility to the Dutch elm disease.

4.4.2. *Testing of clones under field conditions*

In order to establish whether the elm material screened on resistance in the glasshouse also maintained its supposed resistance when grown in the open air, the four TA-clones were raised and tested outdoors in big containers of cement buried in the soil. Layers of the Belgian elm were used as a control for the effectiveness of the inoculation, whereas the *U. hollandica* 'Vegeta' elm was tested at the same time to compare its degree of susceptibility with that of the TA-clones.

It was of interest to compare in the same experiment the behaviour of two kinds of vegetative progenies originating from the same clones: callus cuttings and grafts. For that purpose ten scions of the 'Vegeta' elm and of each of the TA-clones were grafted onto 1½-year-old rootstock. These grafts and one-year-old callus cuttings of each of the clones were planted at the same time in the spring of 1962. Ten grafts or ten callus cuttings were planted in each container. After one year, in spring 1963, all the plants were inoculated with a mixture of five virulent strains of *C. ulmi*. The scions not only showed variability in growth, but even similarly sized grafts became appreciably more diseased than others. The differences occured frequently enough with grafts to make it desirable to use preferably more than ten trees in comparative experiments. The callus cuttings of one clone showed a marked uniformity in growth as well as in the degree of disease symptoms.

The inoculation of all plants has been repeated in 1964 and the plants were rated again for severity of disease. The results of two years of testing are summarized in Table 13.

As expected the Belgian elm showed a rather high disease index and the 'Vegeta' elm appeared to be only slightly less susceptible. From the TA-numbers, the TA II clone showed again resistance in contradistinction to the others.

The disease indices of grafts and the callus cuttings of a same clone differed only slightly; the grafts showed a higher index with

TABLE 13

Comparison of the resistance to *C. ulmi* of ten callus cuttings and grafts of each clone grown outdoors

clones	kind of material	disease indices of ten tested individuals		assessments of the clones
		in 1963	in 1964	
TA I	grafts	16.0	15.0	moderately resistant
TA I	callus cuttings	22.5	12.5	moderately resistant
TA II	grafts	0.0	0.0	resistant
TA II	callus cuttings	0.0	0.0	resistant
TA III	grafts	21.5	28.5	moderately resistant
TA III	callus cuttings	16.2	12.5	moderately resistant
TA IV	grafts	11.2	22.5	moderately resistant
TA IV	callus cuttings	11.2	31.3	moderately resistant
'Vegeta'	grafts	20.0	32.8	susceptible
'Vegeta'	callus cuttings	27.0	46.7	susceptible
'Belgica'	layers	52.0	56.0	susceptible

the clone TA III and a lower index with the 'Vegeta' elm. On the assessments of clones based on their degree of susceptibility those differences were of no importance.

It was concluded that for a determination of the susceptibility of elms, callus cuttings as well as grafts could be used, though grafts react less regularly than callus cuttings. Still there is an other objection against the use of grafts in prolonged field trials. From the inoculated scion the fungus may penetrate into the rootstock and its roots. This phenomenon was observed on several occasions. The fungus could be isolated from the discolored parts of the roots of several grafts. If the fungus passes from an inoculated tolerant scion into the roots of the rootstock, the scion may be inhibited in its growth by the affected roots and evaluation of the scion would be too low.

The roots of the callus cuttings remained healthy with exception of those of the 'Vegeta' elm. Two cuttings of this clone showed a slight discoloration in the wood of the roots, out of which in one case *C. ulmi* could be isolated.

After 1964, testing for resistance of the clones, used in the experiment described above, should be continued for two years more. The TA II trees are of special interest since they unquestionably possess a high degree of resistance.

Another experiment was undertaken to test the value of the vegetative progeny of the resistant selections No's 433, 434, 435 and 436, which were selected by Heybroek from populations of hybrids made by Went in 1952. These hybrids had been tested for six to eight years, and still remained completely healthy. In our experiment,

which started in 1961, callus cuttings and grafts from those four seedlings were grown partly in containers of cement and partly in the nursery rows.

For comparison the highly esteemed clone 296, nowadays called the 'Groeneveld' elm (HEYBROEK, 1964), and layers of the Belgian elm, were used. Up to now the plants have been inoculated twice (Table 14).

TABLE 14

Comparison of the resistance to *C. ulmi* of groups of ten callus cuttings and grafts grown outdoors

clones	kind of material	disease indices of plants				assessment of the clones
		in containers		in the field		
		in 1963	in 1964	in 1963	in 1964	
433	grafts	14.3	6.6	28.0	10.0	moderately resistant
433	callus cuttings	11.0	1.3	—	0.0	moderately resistant
434	grafts	42.0	26.0	19.0	15.0	susceptible
434	callus cuttings	25.0	16.7	—	8.0	susceptible
435	grafts	0.0	10.0	34.0	10.0	moderately resistant
435	callus cuttings	0.0	8.8	—	6.0	moderately resistant
436	grafts	0.0	6.6	0.0	2.2	resistant
436	callus cuttings	0.0	12.5	—	1.4	resistant
296	grafts	0.0	1.2	0.0	0.0	highly resistant
296	callus cuttings	0.0	1.0	0.0	0.0	highly resistant
Belgian elm	layers	—	—	75.0	81.0	extremely susceptible

Of the four clones tested, the offspring of the number 433 and 435 appeared to be moderately and those of number 434 even highly susceptible despite the lack of reaction of the seedling-mother-tree after many inoculations.

This result of the experiment gives support to the previous conclusion concerning the assay in the glasshouse, that the vegetative progeny of apparently resistant seedlings, even after *many* years of testing of the mother trees still may be susceptible.

Next to the highly resistant clone 296, only clone 436 reached a reasonable level of resistance and should be observed for a longer period.

The disease indices after inoculation of the plants grown in the nursery rows were generally lower in the second year of testing than in the first year. This can be attributed to shading by large trees, growing in the vicinity of the test plants.

Two clones, originating from the seedlings TA II and 436 have resisted consecutive inoculations and did not take infection after two tests in the field. The time is approaching when both clones TA II and 436 can be subjected to further assessment. Tolerance to *C. ulmi*, as it has been shown by these clones in the trials up to now, is an important character, since other good qualities are only of value, if the trees' ability to withstand attacks of the fungus can be wholly confirmed. When the scheme (Fig. 2) is followed, a seedling and its vegetative progeny will have been screened altogether for eleven years, after the clones have withstood the last fourth inoculation in the test garden. The surviving clones may be planted out on a small scale at suitable locations in the country to test their resistance to natural infections and to evaluate their qualities under various field conditions.

CHAPTER 5
DISCUSSION

Selection of elms for resistance to Dutch elm disease started in 1928 as far as the Netherlands are concerned. The methods used, based as they were on earlier Dutch and German research, were tentative at first, but improved as experience increased and as subsequent research led to a better insight in the pecularities of the disease and the elm itself (Buisman, Broekhuizen, Franssen, Ledeboer, Went, Kerling, to mention Dutch names only). This does not imply, however, that the available methods left nothing to be desired in the year 1958, when this study started.

Its general aim was to shorten the time needed for selecting elms resistant to the disease, and to try to improve the methods in use up to then.

The observation that young seedlings of elm reacted less severely to inoculation than older ones had led to the hypothesis of youth-resistance (Heybroek, 1957), which had to be tested.

Another question to be studied arose from the fact, that the results of inoculation of elms, even if belonging to the same clone, tended to vary from year to year. This made it desirable to study in more detail a number of factors relating to the pathogen that might influence the outcome of inoculations. As such may be mentioned the variable virulence of different strains of the fungus, the preparation, age and concentration of the spore suspension, methods of storing of the fungus cultures etc.

In addition to these factors, the condition of the tree is of highest importance. It is also important for a successful inoculation to choose the right time of year. It was found that the susceptible period may vary widely from year to year, apparently under the influence of the weather and may be very short.

It was shown that even one-year-old elms in the juvenile stage reacted positively to inoculation, if they were growing vigorously and their roots were not damaged by transplanting. Thus after three

years of screening (following the proposed scheme, Fig. 2) the selected seedlings can be propagated by means of callus cuttings (TCHERNOFF, 1963), and the screening of the clones can be performed in the glasshouse for one year. Further testing has to be carried out in the nursery under field conditions.

The study has led to an increase in the number of methods from which the selectionist may choose, as well as to some refinements of methods used. This may lead to a more reliable and more rapid selection.

At the same time, the study has opened up new problems. The question of the extent and importance of host-specific virulence in the fungus has been raised. A further question is whether the qualitative difference demonstrated in wood discoloration between susceptible and a number of resistant elms after artificial inoculation can be used as a more precise indicator of resistance. Both lines of research seem to merit further study.

SUMMARY

The purpose of this study was to shorten the time needed for selecting elms resistant to *Ceratocystis ulmi* (Buisman) C. Moreau, the causal factor of the Dutch elm disease. By a critical examination an attempt was made to improve the methods in use up to now.

The fungus

The strains of *C. ulmi* were found to vary greatly in pathogenicity. A correlation between virulence and the cultural types of the fungus was not found. Therefore virulence of the *C. ulmi* strains to be used for inoculation purposes in the field, has to be tested on susceptible elm material to be raised beforehand in the glasshouse.

Virulence remains unchanged when strains are kept under mineral oil or in lyophilized condition.

Perithecia are readily formed when an "A" and a "B" type of strain are grown together on a piece of sterilized elm twig. Some ascospore cultures proved to be more virulent than their parent strains.

Probably specialization occurred, since some strains showed a high degree of virulence to one elm clone and a low degree to another one.

Methods of inoculation

Inoculations were performed with a spore suspension consisting of a mixture of five virulent *C. ulmi* strains grown in a nutrient solution of a special composition (p. 412). With a special knife a cut was made into the spring wood and a drop of spore suspension placed on the knife. The inoculum had to be sucked up by the tree.

The spores induced maximal effect when about four days old. The number of spores per ml of inoculum was of less importance. Disease severity was evaluated in such a way that more stress was laid on severely diseased trees than on those that showed only slight external symptoms of disease (p. 413).

The period of sensitivity of the elms

Inoculated trees were able to show disease symptoms, only when they were inoculated during a short period which occurred about 40 to 50 days after bud break. The length of this period depended on weather conditions. Inoculations were most successful under the climatological conditions in the Netherlands when performed about mid-June. In that time the xylem vessels of the spring wood are

fully developed. The physiological condition of the trees is also of the highest importance: unless growth is vigorous, susceptible backward trees do not show disease symptoms and may escape. This may happen after transplanting in the year of inoculation.

Pathological anatomy

The presence of internal symptoms may be a help in screening on resistance. The susceptible *Ulmus hollandica* 'Belgica' elm showed a "spreading" type of discoloration of the wood, whilst the resistant 'Bea Schwarz' elm and clone 296 showed a "spotted" type. Most elms showed, however, a pattern between the two. A strict correlation between the type of pattern and the degree of susceptibility was not obtained.

Conditions which have to be fullfilled for effective testing

These conditions are given on page 436, as the "ten commandments".

Selection of elms in the juvenile stage of development

Contrary to the results from past experiments, it was shown that one-year-old seedlings and callus cuttings are able to show disease symptoms after inoculation. A comparison between the callus cuttings which show juvenile characters and one-year-old grafts, which are in the adult stage, revealed that the disease indices of both groups of trees were similar.

For testing of clones for resistance, callus cuttings are to be preferred to grafts, since clones of the former show greater uniformity. If they are resistant, their roots can not be affected by the fungus, but this is not so with resistant elms grafted on susceptible rootstocks.

Procedure for a rapid selection

When screening seedlings for resistance, damage of their roots must be avoided. The saplings should therefore be planted out directly after emergence in the nursery at distances of 25 × 30 cm.

The following procedure has then proved satisfactory: A first screening was performed when the elms were one year old. Inoculations were repeated in the following years. In the fourth year only the fast growing, resistant trees with a good shape were selected. From the roots of these plants "callus cuttings" were grown, which were subjected to a preliminary screening in the glasshouse, after which the resistant clones were planted in a clonal test garden for further testing and observation (see scheme on page 441).

If the suggestions given in the "ten commandments" and the proposed scheme of selection is followed, selection of a resistant elm can reasonably be accomplished within eleven years.

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