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VIRULENCE IN *CERATOCYSTIS ULMI*[1,2]

Virulentie in Ceratocystis ulmi

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Single-ascospore cultures of *Ceratocystis ulmi* from the progeny of a cross between a culture of compatibility type A from Bedford, Massachusetts, U.S.A., and one of type B from Rotterdam, Netherlands, were morphologically heterogeneous and varied widely in virulence towards young elms inoculated in the greenhouse. Some cultures regularly caused typical wilt in callus cuttings of *Ulmus hollandica* 'Belgica' (susceptible) but none in those of *U. carpinifolia* 'Christine Buisman' (resistant). Some cultures caused wilt in trees of both clones; others in neither. All isolates produced abundant yeast-like cells in liquid culture. All isolates induced typical vascular discoloration except in the terminal inch of twigs. Similar progeny from a cross between cultures of types A and B, both from Naarden, Netherlands, however, were morphologically homogeneous and caused a fairly uniform level of symptoms in young trees of *U. hollandica* 'Belgica' (susceptible) and 'Commelin' (moderately resistant), and in seedlings of *U. americana* after inoculation in the nursery.

INTRODUCTION

The use of elms resistant to the "Dutch" elm disease, caused by *Ceratocystis ulmi* (Buisman) C. Moreau, has long been anticipated as a relief from the high costs of annual treatments with insecticides and of the destruction of breeding places of elm bark beetles. It was considered desirable, therefore, to see whether resistant elms were likely to be attacked in their turn by variants of the fungus, and to consider the consequences of any such attack.

REVIEW OF LITERATURE

Resistance

The search for resistance to *C. ulmi* in elm trees was started in 1928 by Professor WESTERDIJK in the Netherlands (WESTERDIJK, LEDEBOER & WENT, 1931) and was continued there by BUISMAN until 1936 (WENT, 1938), by WENT from 1936 to 1953 (WENT, 1954), and by HEYBROEK from 1953 to the present (HEYBROEK, 1964). Studies on resistance to this elm disease have also been pursued with some success in Russia (OZOLIN, 1958, 1959).

Occasional resistance in individual European elms has led to the release of four clones developed in the Netherlands from European species of elm, namely *U. carpinifolia* Gleditsch. 'Christine Buisman' (BUISMAN, 1936, as selection number 24), and *U. hollandica* Mill. 'Bea Schwarz' (WENT, 1948), 'Commelin' (HEYBROEK, 1961) and 'Groeneveld' (HEYBROEK, 1963a). Many other promising clones are still under trial or have been set aside as potential parents.

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Host resistance or low virulence of the pathogen appears to be common in England (PEACE, 1960) but disease severity in *U. americana* in North America has prompted many efforts to obtain resistant trees (OUELLET, 1956; WELCH, 1959; SMALLEY & RIKER, 1962; SWINGLE, 1961; HOLMES & DEMARADZKI, 1960; ARISUMI & HIGGINS, 1961). Although the tetraploid American elm has reacted less encouragingly than the diploid European species, wide variations in susceptibility and cases of recovery including formation of a false annual ring, have been found (BANFIELD, 1964). Resistant seedlings of American elm have been reported also from Canada (POMERLEAU, 1963). The tall, spreading form of the mature American elm, much desired in North America, neither occurs in oriental resistant species nor is sought in the European breeding program.

The highest levels of resistance occur in certain species: *U. sieboldii* Daveau, *U. sieboldii coreana* Nakai, *U. shirasawana* Daveau, *U. marcocarpa* Hance, *U. parvifolia* Jacquin, *U. wilsoniana* Schneider, *U. nikkensis* Rut., *U. propinqua* Koidz., and *U. pumila* L., especially the variety *U. pumila pinnato-ramosa* Henry (BUISMAN, 1935; OZOLIN, 1958).

Pathogenicity

Variation in pathogenicity among strains of *C. ulmi* has been little studied. It is true that the resistances of the species listed above have not suddenly been broken down by the appearance of new fungal strains, but these tree species have not been widely planted. WENT (1938) found little difference among strains of *C. ulmi* but in a few cases she reported less virulence than usual. WALTER & MAY (1937) found a variant of *C. ulmi* that was brown in culture instead of white or cream-colored; its pathogenicity was normal. TYLER & PARKER (1945) found a wide range of pathogenicity among monoconidial progenies of eight sectors in cultures of mass isolates of *C. ulmi*. Their color variant (black) had unusually high pathogenicity. Their mycelial variant (non-sporulating) was avirulent. Culture filtrates of a dark strain of *C. ulmi* isolated in Sweden (MATHIESEN-KÄÄRIK, 1953) were more toxic to tomato plants than those of lighter-colored strains isolated in the same area. FREDERICK & HOWARD (1951) found, among eight culturally distinct isolates of *C. ulmi*, variations in pathogenicity which were not correlated with titer of toxin produced in liquid shake cultures. SWINGLE (1950) observed wilt symptoms on a Buisman elm he had inoculated a year earlier, and isolated a culture virulent towards this clone. HIGGINS (1959) found that this isolate also differed from four others in forming a larger proportion of non-volatile acids from the carbon utilized in liquid cultures, but did not differ in appearence.

MATERIALS AND METHODS

Trees

In greenhouse studies 100 young trees of *U. hollandica* 'Belgica' (susceptible) and 100 of *U. carpinifolia* 'Christine Buisman' (resistant), 2 to 4 ft. tall and growing in 8-inch pots, were used. These had been grown from "callus cuttings" produced 16 months earlier by TCHERNOFF's double-propagation method (1963), and had been forced into leaf after removal from the coldframes on March 7, 1963. Fifty trees of each clone were put into each of two greenhouse compartments, one kept within a temperature range from about 14° to 24°C, and the other from about 18° to 24°C.

In the "Drakenburg" nursery, 144 trees of *U. americana* (very susceptible), 144 of *U. hollandica* 'Belgica' (very susceptible) and 120 of *U. hollandica* 'Commelin' (moderately resistant) were used. These were, respectively, two-year-old seedlings from Ohio seed, layers produced four years earlier and 6 to 7 ft. tall, and layers produced three years earlier and 2 to 3 in. diameter at breast height. The American elm seedlings were short and bushy because they had been killed back in 1962/63 by frost.

Inocula

Inocula were prepared from single-ascospore cultures of *C. ulmi*, the progeny of crosses between gross isolates from diseased elm trees or between single-ascospore progeny of such crosses. Abundant perithecia formed readily on autoclaved peeled elm twigs inoculated in test tubes with mycelial transfers of the parent cultures and kept at approximately 15 °C in the dark. The use of twigs in test tubes is a modification devised by TCHERNOFF from the techniques of BUISMAN (1932a) and of SHAFER & LIMING (1950). One month was allowed for perithecial development, although perithecia often matured earlier, as ROSINSKI (1961) also found.

Inocula in the greenhouse comprised offspring of a cross between a culture of compatibility type A isolated from *U. americana* in Bedford, Massachusetts, (culture number 56.14671) and one of type B from *U. carpinifolia* 'Wheatleyi' in Rotterdam, Netherlands (culture number NL7).

Inocula in the nursery, where inoculation was carried out when elm bark beetles were flying, were limited to the progeny of cultures of Dutch origin. In order to simulate a cross that would be expected to occur in nature (BUISMAN, 1932b), cultures of both compatibility types were chosen from isoates made from elm trees in the same town, namely Naarden (culture numbers NL1 and NL6). These were crossed; 174 of their single-ascospore progeny (F_1) were test-crossed for compatibility type: two of these, called (NL1 × NL6) 81 and (NL1 × NL6) 84, then were crossed with each other; 103 of their single-ascospore progeny (F_2) were test-crossed for compatibility type; and, finally, 9 of type A and 8 of type B of these F_2 progeny were used to inoculate the trees in the nursery.

Terminology of "A" and "B" was correlated with that of SHAFER & LIMING (1950) by crosses between current isolates and cultures whose compatibility they had designated, and which were kindly made available by DR. WALTER M. BANFIELD. However, attempts to correlate the A and B terminology of SHAFER & LIMING with the plus and minus terminology of either SWINGLE (1936) or BUISMAN (1932a) or to correlate the latter two were unsuccessful.

The cultures were grown on agar slants, then transferred to flasks of a liquid medium and shaken for two days. The inocula injected into trees consisted of the liquid media and the yeast-like cells that grew there. Both agar and liquid media contained the following nutrients per liter, as modified by TCHERNOFF from ZENTMYER (1942): 20 g glucose, 2 g L-asparagine, 1.5 g KH_2PO_4, 1 g $MgSO_4.7H_2O$, 20 mg $ZnSO_4$, 10 mg $FeCl_3$, 1 mg pyridoxine and 1 mg thiamine.

Single-ascospore cultures

Preliminary attempts to obtain single-ascospore cultures by means of a micro-manipulator resulted in no fungal growth, and attempts to dilute the masses of spores extruded at the tips of perithecia were frustrated by an

adhesive substance that held the spores together in a sticky mass. However, spores from within the bases of the perithecia were seen to float freely and separately in water mounts.

Accordingly, five perithecia from a given cross, from the necks of which the extruded ascospores had been removed, were dipped for ten seconds in 50% ethanol to wet them thoroughly, immersed for ten seconds in 2.5% sodium hypochlorite to kill any adhering spores, rinsed twice in sterile distilled water, and finally broken open in a drop of sterile water. This drop, containing a cloud of ascospores, was washed into a sterile test tube with sterile water and diluted to 10 ml. A dilution series by tenths was established in tubes of sterile water and poured into flasks of cooling potato dextrose agar (PDA) at 36°C. The resulting suspension was briefly swirled and quickly decanted into sterile Petri dishes in thin layers, where it hardened. After two to four days' growth, colonies could be seen under the dissecting microscope, and a transfer of one or a small group of adjacent hyphal tips from each colony's margin was made to PDA in a separate Petri plate, by means of a "cookie-cutter"-tipped platinum needle.

Inocula were produced by shaking, for 48 hr at 23–24°C, Erlenmeyer flasks, each containing the liquid synthetic medium into which conidia had been washed from an agar slant bearing growth of the appropriate culture.

Inoculation

In the following description, the inoculation techniques used in the greenhouse are those of Ir. TCHERNOFF; in the nursery, those of Ir. HEYBROEK. The elms in the warmer chamber of the greenhouse passed from dormancy into full leaf more rapidly than those in the cooler chamber, and absorbed water well in a trial inoculation on April 19. The former trees were inoculated on April 26 and 27; the latter on May 2 and 3. The first two days were bright and sunny; the third cloudy, and the last rainy. Yet the inoculum was taken up equally well on each day. Four susceptible and four resistant elms were inoculated with each of 23 single-ascospore cultures.

Each tree was supported in a vertical position while a surgical chisel with a two mm-wide point was held against its trunk about ten cm above the soil. A drop of inoculum was placed upon the chisel point. Surface tension of the medium prevented the inoculum from running down the dry trunk. The chisel was pushed through the bark, then slightly withdrawn and, as the drop of inoculum was gradually drawn in, further drops were added from a fine pipette until at least five drops had entered the trunk. The chisel tip was kept touching the wound to help keep the inoculum there. The process was repeated on the opposite side of the trunk.

The trees in the nursery were inoculated on June 10, 11, and 12. All three days were bright and sunny but to make certain that the results for any given culture would be comparable, all trees scheduled for a given culture were inoculated on the same day. Each of the 17 cultures was inoculated into eight trees of *U. americana* and eight of *U. hollandica* 'Belgica'; each of 15 of the 17 was inoculated also into eight trees of *U. hollandica* 'Commelin'. All trees were inoculated twice, on opposite sides of their trunks about 90 cm above the ground, except that the short American elm seedlings were inoculated about 10 cm above the ground. Inoculation was made by means of a Stanley Utility

knife with a triangular exposed blade. In this case several drops of inoculum could be placed on the surface of the blade when the knife was held horizontally. As soon as a horizontal cut was made into the wood of the trunk and the wound opened slightly by pressure on the blade, the whole inoculum was rapidly pushed into the cut xylem vessels by the greater pressure of the outside atmosphere. The Stanley knife was washed with alcohol and flamed between cultures.

Eight additional elms in the greenhouse (two of each clone in each compartment) and 16 in the nursery (eight American and eight Belgian) were inoculated with sterile nutrient medium as controls.

Recording of symptoms

All trees were observed two weeks and again one month after inoculation. The conditions of those in the greenhouse were recorded according to TCHERNOFF's scale:

1 = Healthy.
2 = A few leaves yellow or flaccid; doubtful whether diseased.
3 = Many leaves wilted; tree clearly ill.
4 = Many leaves fallen and/or brown; more than one shoot or branch tip dead and curved into a crook.
5 = Stalk (trunk) drying.

The data obtained in the greenhouse were analyzed both in their original form (1 through 5) and also after being weighted, according to TCHERNOFF's custom, by multiplying 1 by 0, 2 by 1, 3 by 2, 4 by 3 and 5 by 4, so that the categories in his rating scale had the numerical values, respectively, of 0, 2, 6, 12 and 20.

The conditions of trees in the field were recorded according to the following scale, used by Ir. HEYBROEK:

0 = Healthy.
$\frac{1}{2}$ = Growth checked; vague, non-specific symptoms; tree may be 0 on next reading.
1 = Slightly wilted or yellowish foliage: a few leaves gone; any tentative symptom.
$1\frac{1}{2}$ = Main branch wilted but not yet dry; or one or a few wilted shoot tips with adjoining wilted leaves, but only on lateral branches, not on top.
2 = One or more wilted shoot with adjoining wilted leaves on main side branch at top.
$2\frac{1}{2}$ = Many such wilted shoot tips throughout tree including at top.
3 = Part of the two-year wood (the growth of 1962) dry and dead.
$3\frac{1}{2}$ = Die-back nearly reaching the three-year wood (shoot of 1961); or two-year wood (shoots of 1962) affected at several places in the same plant.
4 = Die-back reaching the three-year wood (the growth of 1961).

For greater convenience in handling the figures obtained in the nursery, they were first multiplied by 2 to eliminate fractions; thus they ran from 0 to 8. This did not change their relative weights.

Besides the two-week and the one-month records, the trees in the nursery were rated again by Ir. HEYBROEK four months after inoculation. Although this rating was carried out on October 16, the trees in the Netherlands still

retained their foliage at that time, and there was no confusion with the onset of autumn coloration, as they could be compared to the controls. The seedling American elms in the nursery, because of their prior frost injury, the invasion of several by *Nectria cinnabarina* Tode ex. Fr., and the difficulty in rating symptoms on such bushes, were omitted from the analysis of variance.

RESULTS

Single-ascospore cultures

Comparison of haemocytometer counts with the growth in the dilution series indicated that only about 0.1 % to 1.0 % of the visible spores grew.

Greenhouse inoculations

The first symptoms in the greenhouse appeared nine days after inoculation. Ratings of these symptoms, made one month after inoculations with *C. ulmi*, are summarized in Table 1.

Since the scale used for recording disease severity applied only to outward symptoms, each of these trees was also cut or peeled at several points to determine the extent of the discoloration in the sapwood of the above-ground portions. In every tree but one, whether or not there were outward symptoms, the vascular streaking typical of this disease extended the length of the trunk and of all side branches, except the terminal inch. The only tree without vascular discoloration was one of *U. hollandica* 'Belgica'. It also was the only one of the eight trees inoculated with culture number 16 that showed no outward symptoms. It had received the note "took inoculum poorly" and the streak went only about 5 cm from the inoculation point. This tree was considered to have escaped the disease through a failure in inoculation, and it was omitted from rating averages and statistical analyses. One tree from each group of four was chosen for reisolation test, and *C. ulmi* was successfully reisolated in all cases.

Analysis of variance of ratings of outward symptoms one month after inoculations in the greenhouse (Table 3a) showed, in both raw and converted data, highly significant differences (1 % level) between warmer and cooler compartments, between the virulences of the several cultures in each compartment, and between reactions of Buisman and Belgian elms in each compartment. There was no significant difference between virulences of the two compatibility types of the fungus in either location, nor in either elm clone. In both raw and converted data, the interaction between fungus cultures within elm clones and fungus types within each location was significant (5 % level).

In the warmer compartment the symptoms developed especially severely on trees near the two ends of the bench.

Nursery inoculations

The ratings of symptom severity on the Commelin and Belgian elms in the nursery after two weeks, one month and four months are presented in Table 2. Occasional trees in this nursery were severely affected by *Nectria cinnabarina*. During the course of the experiment *Nectria* killed two trees of *U. americana* inoculated with culture number 50 and another inoculated with sterile nutrient medium. These trees were destroyed. A fourth, inoculated with culture number

TABLE 1. Outward reactions of Belgian elm (susceptible) and Buisman elm (resistant) 16-month callus-cuttings, one month after inoculation in two greenhouse compartments on April 26 – May 2 with F_1 single ascospore cultures of *Ceratocystis ulmi* from the cross "Bedford" × "Rotterdam". Each figure is the average of four trees' ratings of severity of outward symptoms. Raw scale = 1, 2, 3, 4, 5; weighted scale = 0, 2, 6, 12, 20; both range from healthy to trunk dying.
Uitwending zichtbare reacties van de vatbare 'Belgica'-iep en de resistente 'Christine Buisman'-iep na inoculatie in twee afdelingen van de kas van 26 april tot 2 mei met een éénsporecultuur van Ceratocystis ulmi, *afkomstig van de kruising 'Bedford' × 'Rotterdam'. Elke index is een gemiddelde van de beoordeling van vier bomen. Index = 1, 2, 3, 4, 5; omgerekend = 0, 2, 6, 12, 20; beide reeksen lopen van gezond tot afsterven.*

Location 1: Warmer greenhouse
Kasafdeling 1: Hogere temperatuur

Location 2: Cooler greenhouse
Kasafdeling 2: Lagere temperatuur

Culture number *Nummer van de cultuur*	Symptom severity rating *Hevigheid van de symptomen*				Culture number *Nummer van de cultuur*	Symptom severity rating *Hevigheid van de symptomen*			
	Raw scale *Index*		Weighted scale *Index omgerekend*			Raw scale *Index*		Weighted scale *Index omgerekend*	
	'Belgica'	'Cristine Buisman'	'Belgica'	Christine 'Buisman'		'Belgica'	'Christine Buisman'	'Belgica'	'Christine Buisman'
			Cultures of compatibility type "A" *Cultures van compatibiliteitstype „A"*						
5	3.5	2.8	9.0	5.0	69	2.5	1.0	5.0	0
36	1.0	2.0	0	2.0	96	3.8	2.3	10.5	3.5
40	1.0	1.0	0	0	109	3.8	2.8	10.5	5.0
45	1.0	1.0	0	0	122	3.5	1.5	9.0	1.0
50	1.0	1.0	0	0	160	3.3	2.0	8.0	3.5
Subav. *Gemid.*	1.5	1.6	1.8	1.4		3.4	1.9	8.6	2.6
			Cultures of compatibility type "B" *Cultures van compatibiliteitstype „B"*						
9	3.3	3.0	8.0	7.5	78	2.8	1.5	5.0	1.0
16[2]	3.2[1]	2.3	6.7[1]	3.0	87	3.8	1.5	10.5	1.5
23	1.0	1.3	0	0.5	115	3.3	2.0	7.5	3.0
27	1.0	1.8	0	1.5	144	3.0	2.8	6.5	6.5
33	1.5	1.5	1.0	1.0	172	3.0	3.8	6.5	10.5
56	1.8	1.3	2.0	0.5					
58[2]	1.3	1.0	0.5	0					
Subav. *Gemid.*	1.9	1.7	2.6	1.9		3.2	2.3	7.2	4.5
			Culture of uncertain compatibility *Cultuur van onzekere compatibiliteit*						
					133[2]	3.3	3.3	8.0	7.5
Avera. *Gemid.*	1.7	1.7	2.3	1.7		3.3	2.2	7.9	3.9

[1] Based on only three trees / *Gemiddelde van slechts drie bomen*
[2] Omitted from analysis of variance / *Buiten beschouwing gelaten bij berekening van significantie*

80, was mowed off during routine care of the nursery. The figures in Table 2 are averages of the remaining trees.

Analysis of variance of these data (Table 3b) showed no significant difference in virulence of cultures except among the type-A cultures in the four-month

TABLE 2. Outward reactions of Belgian elm (susceptible) four-year layers, Commelin elm (moderately resistant) three-year layers and American elm (susceptible) two-year seedlings at two weeks, one month and four months after inoculations on June 10–13 in the nursery with F_2 single-ascospore cultures of *Ceratocystis ulmi* from the cross "Naarden" × "Naarden". Each figure is the average of eight trees' ratings on a scale 0, 1, 2, 3, 4, 5, 6, 7, 8, ranging from healthy to part of 1961 wood dry and dead.

Uitwendig zichtbare reacties van vier jaar oude en drie jaar oude afleggers respectievelijk van de vatbare 'Belgica' en de resistente 'Commelin'-iep en van twee jaar oude zaailingen van de vatbare Amerikaanse iep, twee weken, één maand en vier maanden na inoculatie op 10–13 juni in de kwekerij met éénsporecultures van Ceratocystis ulmi, *afkomstig van de kruising „Naarden" × „Naarden". Elke index is het gemiddelde van de beoordeling van acht bomen. Index: 0, 1, 2, 3, 4, 5, 6, 7, 8, lopend van gezond tot afsterven van het in 1961 gevormde hout.*

Culture number *Nummer van de cultuur*	*U. americana*[4]			*U. hollandica* 'Commelin'			*U. hollandica* 'Belgica'		
	6/27	7/10	10/16	6/27	7/10	10/16	6/27	7/10	10/16
				Compatibility type "*A*" *Compatibiliteitstype „A"*					
5	2.0	2.5	3.0	2.4	1.9	1.9	5.0	5.0	5.9
20	3.0	4.1	4.0	2.3	2.4	2.6	4.9	5.0	5.6
35	3.0	4.4	4.3	1.5	1.1	2.6	4.7[1]	4.9[1]	4.7[1]
40	4.5	5.1	4.4	2.3	1.4	2.5	4.7[1]	4.7[1]	4.3[2]
50	3.6[1]	4.9[1]	4.5[2]	0.1	0.0	2.3	5.0	5.3	5.4
55	2.6	4.6	4.5	1.4	1.4	2.0	4.5	4.7	4.0
60	3.0	3.9	3.9	2.3	1.8	2.3	4.5	5.0	4.1
65	1.9	3.1	2.8	1.8	1.6	2.3	5.0	5.1	5.1
70	2.5	4.0	4.4	1.4	1.0	1.5	5.0	5.1	6.0
Subav. *Gemid.*	2.9	4.1	4.0	1.7	1.4	2.2	4.8	5.0	5.0
				Compatibility type "B" *Compatibiliteitstype „B"*					
10	2.8	4.1	4.0	2.9	2.3	2.6	5.1	5.1	6.0
15	3.5	4.8	4.0	1.9	1.5	2.9	5.0	5.0	6.3
25	3.9	4.3	4.0	2.0	1.9	2.8	4.9	5.1	5.0
30	4.4	5.0	4.8[2]	1.8	1.3	2.8	4.1	4.8	5.1
45	3.3	4.9	4.3	2.6	2.4	2.4	4.3	4.8	5.0
75	2.4	4.3	4.9	2.5	2.1	2.5	5.0	5.1	5.8
80[4]	2.4	4.4[1]	3.8[2]	– –[3]	– –[3]	– –[3]	5.0	5.1	5.9
85[4]	1.4	4.3	4.0	– –[3]	– –[3]	– –[3]	5.0	5.4	6.0
Subav. *Gemid.*	3.0	4.5	4.2	2.3	1.9	2.7	4.8	5.1	5.6
Avera. *Gemid.*	3.0	4.3	4.1	1.9	1.6	2.4	4.8	5.0	5.3

1 = Based on seven trees / *Gemiddelde van zeven bomen.* 2 = Based on six trees / *Gemiddelde van zes bomen.* 3 = No 'Commelin' elms inoculated with this culture / *Geen 'Commelin'-iepen met deze cultuur geïnoculeerd.* 4 = Omitted from analysis of variance / *Niet betrokken in de significantieberekening.*

TABLE 3. Analyses of variance of the data summarized in Tables 1 and 2, for outward symptoms of elms inoculated with single-ascospore cultures of *C. ulmi*.
Significantie van de gegevens, samengevat in de tabellen 1 en 2 betreffende uitwendig zichtbare symptomen van iepen, geïnoculeerd met éénsporecultures van C. ulmi.

Sources of variation *Bronnen van variatie*	Degrees of freedom *Vrijheidsgraden*	Mean squares *Gemiddelde kwadraten*

a. 'Belgica' and 'Christine Buisman' elms rated one month after inoculation in the greenhouse /*'Belgica' en 'Christine Buisman'-iepen, beoordeeld een maand na inoculatie in de kas.*

		Raw data *Ruwe gegevens*	Converted data *Herleide gegevens*
Locations (L)	1	54.0562 ss	714.0250 ss
Types (T)	1	.3062 ns	.6250 ns
LT	1	1.0563 ns	13.2250 ns
Clones (C)	1	15.0062 ss	255.0250 ss
LC	1	15.0063 ss	198.0250 ss
TC	1	1.8063 ns	55.2250 ns
LTC	1	1.0562 ns	30.6250 ns
Cultures (R): LT	16	3.3875 ss	45.8375 ss
CR: LT	16	1.0938 s	17.6000 s
Eror/*Fout*	120	.5163	8.6167

b. 'Belgica' and 'Commelin' elms rated a half, one and four months after inoculation in the nursery /*'Belgica' en 'Commelin'-iepen, beoordeeld een halve maand, één en vier maanden na inoculatie op de kwekerij.*

		½-*month*	*1 month*	*4 months*
Compatibility type of culture (T)	1	5.2750 s	5.3777 ns	17.3361 ss
Clones within type A (C: T_A)	1	326.8680 ss	437.5070 ss	269.5069 ss
Clones within type B (C: T_B)	1	145.0417 ss	288.1667 ss	187.0417 ss
Cultures within type A (Cu: T_A)	8	1.8542 ns	2.0070 ns	2.3559 ss
Cultures within types B (Cu: T_B)	5	2.0500 ns	1.1000 ns	1.1500 ns
CCu: T_A	8	3.1493 ss	2.4132 ns	4.4913 ss
CCu: T_B	5	1.0917 ns	.7417 ns	1.4417 ns
Error/*Fout*	210	1.1780	1.5042	.7685

ns = Not significant / *Niet significant*
s = Significant, to 5% level / *Significantie 5%*
ss = Highly significant, to 1% level / *Significantie 1%*

readings but, as expected, a highly significant difference between elm clones. The interaction between elm clones and fungus cultures was not significant, again except among type-A cultures in the two-week and four-month readings.

Inoculations with sterile nutrient medium, both in greenhouse and in nursery, resulted in no evidences of injury or disease and wounds healed promptly. The trees so inoculated yielded no fungus in culture.

DISCUSSION

Single-ascospore cultures

Spores counted by haemocytometer were seen almost always occurring singly, and very seldom in pairs. In any case, because germination percentage

was only 1% or less, in only a few of such rare pairs could both spores be expected to germinate. Moreover, each culture in the present experiments mated with cultures either of type A or of type B but not with both. If any of the cultures used had originated from pairs or from clumps of spores, at least half of the instances where two spores jointly led to colony formation should have included both compatibility types (since A and B appear to be inherited approximately equally). Such a colony should then have been able to mate with test cultures of both types A and B, or even to produce perithecia alone on a suitable substrate. This did not occur even once in the many hundreds of cultures tested. These three lines of reasoning are considered sufficient bases for the conclusion that the cultures obtained by modified dilution method used were indeed from single ascospores.

If micro-endospores perform a sexual role, as suggested by OUELLETTE & GAGNON (1960), at least they did not lead to the development of both A and B compatibility types in any of the hundreds of single-ascospore colonies derived by the method described here, even though some of these colonies later developed many visually-distinguishable sectors.

The sticky substance, which caused extruded ascospores to adhere to one another, probably was produced in the neck of the perithecium. However, it is possible that the spores are surrounded, even before they enter the neck, by a gelatinous sheath which becomes mucilaginous through drying or when exposed to air. If so, this sheath did not prevent the spore walls from appearing to touch in the rare cases where two spores were seen adjacent in the haemocytometer.

Greenhouse inoculations

TCHERNOFF's customary conversion of the data emphasizes severe symptoms, as well as having the advantage of removing the assignment in raw data of a positive number (1) to healthy trees. Yet, where symptoms were rated according to his scale and then converted, the difference between the raw and weighted data did not affect statistical significances.

The observation of most interest is that *many* single-ascospore cultures (from a cross between isolates of *C. ulmi* from widely separated locations) induced severe disease in the Buisman elm. This clone is widely planted in the United States and other countries, although less so in the Netherlands where it is severely affected by *Nectria cinnabarina. Nectria* has not thus far been a problem on Buisman elms in the United States. The Buisman elm has generally been considered as a possible solution to the problems resulting from the death of vast numbers of American elms, even though the shape of the mature tree is different.

It is unfortunate that the parent cultures were not also tested in the same experiment. Because it was anticipated that the ability to overcome the resistance of this clone would be rare, efforts were made to extend the number of trees available in the greenhouse to the greatest possible number of sibling cultures. The type-A parent cultures, however, had been inoculated the previous summer into two five-foot Buisman elms in a nursery in Amherst, Massachusetts, and no disease symptoms had been observed although typical symptoms had resulted from parallel inoculation of American elm. One type-B culture from Massachusetts, and another from Ohio, kindly made available by Dr. ROGER U.

SWINGLE, caused wilt then in both Buisman and American elms (Fig. 1).

The development of more severe symptoms near the two ends of the warmer greenhouse, and throughout the cooler one, suggests that cooler temperatures may have encouraged fuller expression of symptoms. However, temperature differences should have been slight, because of the forced circulation of air. TYLER (1945) found that temperatures from 15° to 29°C favored disease development and he reported inhibition of wilt only above 32°C or below 12°C, extremes not encountered in the present experiments.

TYLER also reported that suppression of invasiveness was concomitant with inhibition of wilt. Others (BANFIELD, 1964; FREDERICK & HOWARD, 1951; WESTERDIJK, LEDEBOER & WENT, 1931) found that the intensity of vascular discoloration caused by *C. ulmi* was proportional to that of foliar wilting. However, BANFIELD (1964) found no such correlation with elms inoculated later than August 20. All the present trees (except one), whether wilted or not, had equally thorough xylem discoloration and were equally invaded, regardless of their location within the greenhouse.

Resistance to *C. ulmi* is often encountered in young seedlings (CAROSELLI & FELDMAN, 1951; HEYBROEK, 1957) although it has seemed not directly tied to the juvenile phase of the plant's growth (TCHERNOFF, 1964). The resistant reaction of many trees of the susceptible clone in the greenhouse might be ascribed by some to their youth. However, the present trees were not seedlings, but cuttings. Even if this objection were applicable to callus cuttings, it could militate against the validity of only the non-pathogenic cultures, and could not explain the fact that many young elms of both clones ('Belgica' and 'Christine Buisman') in the greenhouse showed severe wilt after inoculation with certain cultures of *C. ulmi*. Moreover, there is high statistical significance to differences between cultures in the greenhouse experiment. Thus the variability of reaction to inoculation in the greenhouse may be taken to reflect real differences in virulence between sibling single-ascospore cultures of *C. ulmi*.

The abundant evidence from other sources that there is a difference in susceptibility between the Buisman and the Belgian elm is confirmed here in the statistical evidence from the greenhouse inoculations.

The temperature of the greenhouse and/or the timing of the inoculation after the elms were forced into leaf appear to have been highly critical. In the future greenhouse experiments, these factors must be very carefully controlled. If these factors are indeed critical, it is conceivable that the virulence of some cultures was masked at the given temperature and/or time of inoculation, although other cultures caused symptoms under the same conditions.

Nursery inoculations

It is less surprising, perhaps, that the offspring of a cross between cultures from trees in the same town should possess little variability in virulence. The macroscopic appearances of these cultures were strikingly similar, lacking the usual variations in color, texture, rate of growth, frequency of sectoring, and day-night concentric zonation that had been recorded for the offspring of each of several earlier crosses involving parents from more distant origins.

Symptom ratings made two weeks, one month and four months after nursery inoculations were compared in the statistical studies. The slightness of the difference between one month and four month ratings for inoculations with

C. ulmi supports the observations of TYLER & PARKER (1945) who experienced little change in wilt index after 20 days. However, their ratings at 11 days, and the present ones at 14 to 17 days were clearly not yet at maximum severity.

Differences between statistical significances of certain of the ratings obtained at three time intervals after inoculation raise the question of what validity may be accorded to ratings based on subjective observations of tree health. Of the seven analyses in Table 3b, however, all three lines varying in significance with time appear to be attributable to a variability in the virulence of cultures fo compatibility type A as read at different intervals after inoculation. The uniformity of significances in four, and the explicability of the variations in significance in three, of the seven rows of analyses of variance in Table 3b indicate that symptom ratings made in the manner described are indeed reproducible and are a valid basis for drawing conclusions.

Fungal compatibility type appeared to affect the ratings of virulence at two weeks and at four months but not at one month. However, there is no clear evidence here that virulence is tied to compatibility type, especially since compatibility type was not significant in the greenhouse experiment. A final decision must be deferred for experiments designed particularly to test this point. This uncertainty (first line of Table 3b) is a warning against too-rigid adherence to statistical findings based on rating of symptoms on a single date, even one taken at what is considered an ideal time interval after inoculation.

CONCLUSIONS

Resistance

The present experiments emphasize the usefulness of distinguishing between resistance and immunity. Resistance implies only that the individual is not easily or is only partially overcome. A plant may indeed have so many or such strong mechanisms for resistance that it is not affected at all by a pathogen, and is immune. It may even have more resistance than would be necessary for the appearance of immunity (symptomlessness). But it also may have less resistance. It may finally succumb to disease despite its resistance. Indeed, Dr. CHRISTINE J. BUISMAN, who discovered clone number 24 (1935, 1936), and for whom it was named after her death in 1936, herself pointed out that under good conditions disease symptoms occurred occasionally in heavily-inoculated individuals (1936b). Some recent clones are more resistant than the Buisman elm, but even these are not immune (HEYBROEK, 1963a, 1963b). It is a conscious policy of the Dutch investigators not to require immunity in a new clone that is to be released (HEYBROEK, 1957) and in their early selections they retained every clone with even slight resistance, to be used in crossings. At present they require of a new clone to be issued, that it be more resistant than the Commelin elm.

The fact that resistant plants can sometimes succumb to disease, and consequently may serve as "sieves" which select only those fungal strains that can overcome them, is a reason to use mixed plantings along streets and in yards and parks. Not only can elms be mixed with other species of trees: resistant elms of one clone can even be mixed with elms of other clones whose resistance is based on other genes. It is already known that resistance in the Buisman elm is of a recessive nature while that in some other elms is dominant (HEYBROEK, 1962). The polygenic inheritance in *C. ulmi* for virulence towards particular elm

clones, apparent from the present experiments, is matched by a polygenic inheritance in certain elm clones for resistance to *C. ulmi* attack (ARISUMI & HIGGINS, 1961; HEYBROEK, 1962), which is perhaps accompanied by a cytoplasmic inheritance (HEYBROEK, 1963b, 1964).

The ability now to obtain strains of *C. ulmi* that cause different levels of disease in particular elm clones should make it possible henceforth to determine more easily whether several clones are resistant by virtue of the same or of different genes.

Nevertheless, the fact that a tree's resistance is lower than immunity is not a reason not to plant it. It is merely a reason for remaining alert to possible hazards. Resistant elms in practical use, especially those growing in regions where both compatibility types of *C. ulmi* occur, should be watched closely for disease symptoms, so that new pathogenic races may be destroyed before they can spread.

The use of any single clone of resistant elm on a very wide scale should be discouraged in favor of a broad program involving may different resistant clones of elm and other tree species. As soon as it is possible to distinguish between the different factors in elm for resistance to *C. ulmi*, breeding programs should include crosses designed to bring several of these different factors together in new clones. It is anticipated that clones with such multiple resistances will not be readily overcome by random changes in the pathogenicity of the fungus.

Avirulence

TYLER & PARKER (1945) found a practically nonpathogenic culture in America by selection of sector variants in mass isolates. This method, of course, requires waiting for such sectors to arise and it assumes that differences in virulence are associated with differences in cultural appearance. Their avirulent culture also produced no spores and so had to be inoculated into trees as mycelium. Its lack of invasiveness may, therefore, have been a result merely of its inability to sporulate within the xylem vessels. The avirulent cultures in the present experiments produced conidia on agar media and yeast-like cells in the liquid medium used for inoculum, just as did their virulent siblings. The lack of virulence here could not have resulted from inability to sporulate. It also could not have resulted from inability to invade the tree, since the fungus was found throughout the vessels of the above-ground parts of 183 of the 184 inoculated trees; and yet there were no outward symptoms on 78 of these trees!

The availability of cultures of *C. ulmi* that cause no symptoms in very susceptible host clones can help studies of the nature of pathogenesis. Any hypothetical mechanisms henceforth proposed for symptom production in the "Dutch" elm disease must now be shown to be absent or much reduced or blocked in trees inoculated with invasive-but-avirulent cultures and especially active in those inoculated with highly virulent ones. Conversely, any feature of metabolism found to differ between cultures of these two types is immediately suspect as a possible intermediary in the syndrome.

The ready production of avirulent cultures lends support to the idea proposed by LEACH (1935) that *C. ulmi* may have been a saprophyte associated with bark beetles in elm wood, and that about 1917 a pathogenic mutation may have

arisen in northwestern Europe enabling this saprophyte to start to kill elm trees, or to the suggestion by KÄÄRIK (1960: 39) that *C. ulmi* may have arisen through hybridization between different species of *Ceratocystis* (syn. *Ophiostoma*) not pathogenic to elm.

The existence of cultures of extremely different virulence also gives an opportunity to study the inheritance of virulence by crosses between such types. Crosses among virulent cultures and among avirulent ones appear possible also, since a correlation between compatibility and virulence is not yet clearly proved.

Inasmuch as greenhouse trees that showed no outward symptoms after inoculation with *C. ulmi* still possessed thoroughly invaded and discolored sapwood, an experience reported earlier by several investigators (CAROSELLI & FELDMAN, 1951; FREDERICK & HOWARD, 1951; WESTERDIJK, LEDEBOER & WENT, 1931), evidently the degree of discoloration of sapwood is not a safe indication of the degree of resistance of a tree towards production of outward wilt symptoms.

In view of the wide variation in pathogenicity among single-ascospore cultures of *C. ulmi*, caution should be applied both to the development of resistant elms and their increasing use. TYLER & PARKER (1945) warned: "Racial specialization should be considered in a disease control program involving the development of disease resistant elms." Inoculations in breeding programs for resistance to *C. ulmi* should include an assortment of the most virulent cultures that can be obtained by selection among progeny of various crosses between A and B strains of the fungus.

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SAMENVATTING

Eén-ascosporecultures van *Ceratocystis ulmi*, verkregen uit de nakomelingen van een kruising tussen een cultuur van het compatibiliteitstype A uit Bedford, Massachusetts, V.S., en een type B uit Rotterdam, Nederland, waren morfologisch heterogeen en vertoonden grote verschillen in virulentie ten opzichte van jonge iepen, die in de kas waren geïnoculeerd. Enkele cultures veroorzaakten bij herhaling een typische verwelking bij callusstekken van de kloon *Ulmus hollandica* 'Belgica' (vatbaar), maar zij brachten geen verwelking teweeg bij

Ulmus carpinifolia 'Christine Buisman' (resistent). Andere cultures veroorzaakten verwelking bij bomen van beide klonen, weer andere bij geen van beide. In alle gevallen ontstond na inoculatie de typische houtverkleuring, die echter uitbleef in de toppen van de twijgen (ca. 3 cm). Alle cultures produceerden in een voedingsvloeistof een overvloed van gistachtige cellen.

Eén-ascosporecultures, verkregen uit een kruising tussen de twee compatibiliteitstypen, beide afkomstig uit Naarden, Nederland, waren daarentegen morfologisch homogeen. Zij vertoonden bovendien vrijwel geen verschillen in virulentie na inoculatie in de kwekerij in jonge bomen van *U. hollandica* 'Belgica' en 'Commelin' (respectievelijk vatbaar en matig resistent) en in zaailingen van *U. americana* (vatbaar).

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* Photocopy in English translation available from F. W. Holmes, Shade Tree Laboratories, University of Massachusetts, Amherst, Mass. 01003, at current cost.

A

B

FIG. 1. Symptoms of infection by *Ceratocystis ulmi* in the Buisman elm.
A. General view of the tree. B. Close-up of wilted leaves. The fungus was reisolated from this tree.

Ziektesymptomen in de 'Christine Buisman'-iep, veroorzaakt door Ceratocystis ulmi.
A. Algemeen beeld van de boom. B. Detail-foto: verwelkte bladeren. De schimmel kon uit deze boom weer geïsoleerd worden.

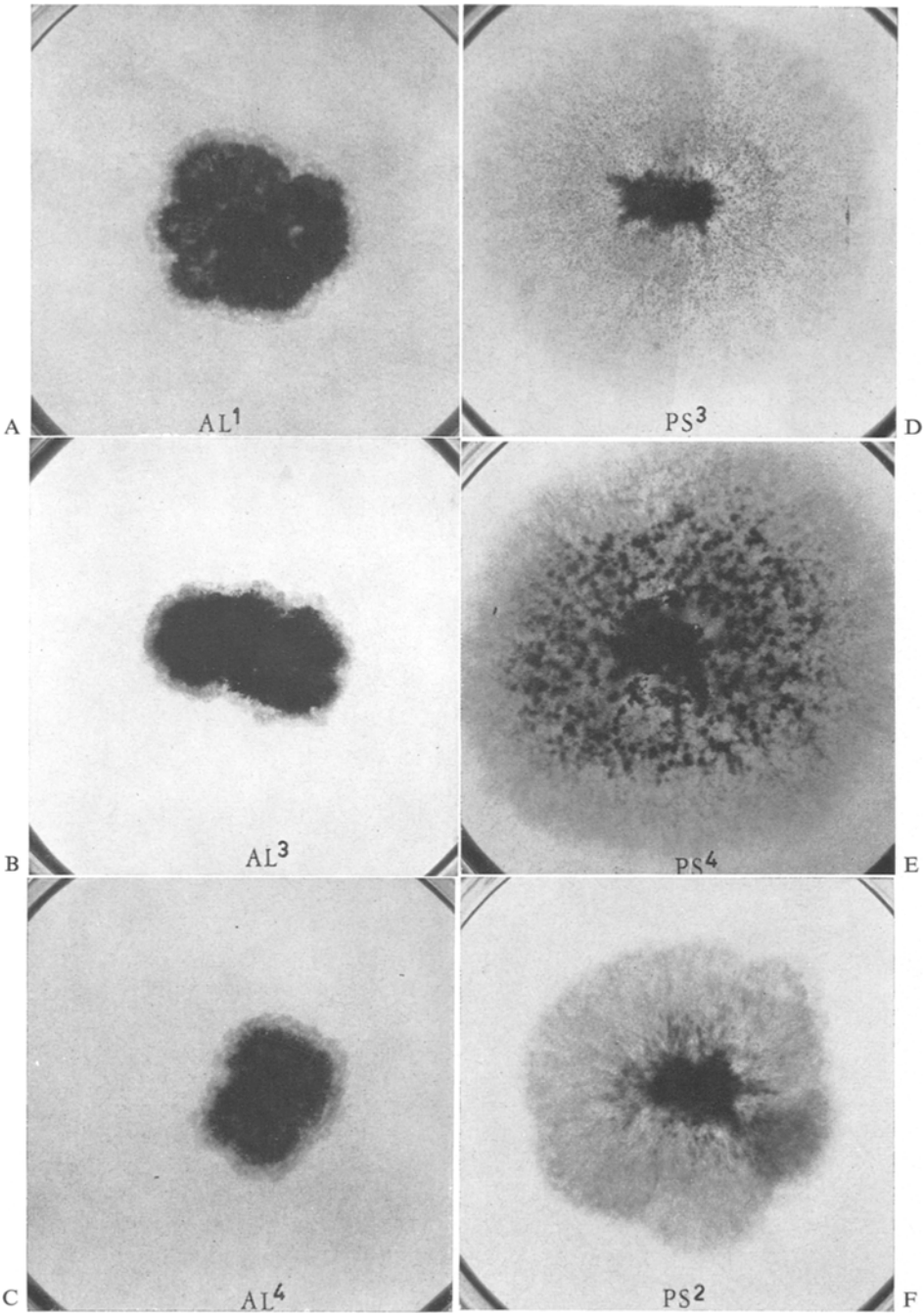

FIG. 1. Three strains of the footrot fungus (A–C) compared with three strains of the ubiquitous soil-borne fungus (D–F) on cherry agar after growing six days in darkness at room temperature. Photographs made with transmitted light.
Drie stammen van de „dode harrel"-schimmel (A–C) *in vergelijking met drie stammen van de algemeen voorkomende grondschimmel* (D–F) *op kersagar na zes dagen groeien in het donker bij kamertemperatuur. De foto's zijn gemaakt bij doorvallend licht.*