Verticillium wilt of Fraxinus excelsior

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Verticillium wilt of Fraxinus excelsior

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
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in het openbaar te verdedigen
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des namiddags om vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ABSTRACT

Hiemstra, J.A. (1995). Verticillium wilt of Fraxinus excelsior. PhD Thesis, Wageningen Agricultural University, The Netherlands. xvi + 213 pp, 40 figs., 28 tables, 4 plates with colour pictures, 327 refs., English and Dutch summaries. ISBN 90-5485-360-3

Research on ash wilt disease, a common disease of *Fraxinus excelsior* L. in young forest and landscape plantings in several parts of the Netherlands, is described. By means of a survey for pathogenic fungi in affected trees, inoculation and reisolation experiments it is demonstrated that the disease is caused by *Verticillium dahliae* Kleb. Host specificity and virulence of a *V. dahliae* isolate from ash are compared to those of isolates from elm, maple and potato. Disease incidence and progress, and recovery of infected trees are investigated through monitoring experiments in two permanent plots in seriously affected forest stands. Monitoring results are related to the results of an aerial survey for ash wilt disease in the province of Flevoland to assess the impact of the disease on ash forests. Furthermore, pathological xylem anatomy of infected ash trees is described and mechanisms of recovery are discussed. Finally, results are integrated and related to data on ash and on verticillium wilts of other tree species presented in literature reviews in the starting chapters of the book.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Front cover: Severe wilt and defoliation in roadside tree in the province of Flevoland.

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Stellingen

1. Bij door *Verticillium dahliae* aangetaste essen is herstel de regel en sterfte de uitzondering.
   Dit proefschrift.

2. De ringporige anatomie van de es verklaart zowel de zeer snelle verspreiding van *Verticillium dahliae* door de boom in het jaar van infectie[1] als het zeer hoge herstelpercentage in het jaar daarna[2].
   1 Braun, 1982. Lehrbuch der Forstbotanik. Fischer Verlag, Stuttgart. pp. 182-185: Das Massensterben ringporiger Bäume.
   2 Dit proefschrift.

3. Verkleuring van het vaatweefsel is geen betrouwbaar diagnostisch kenmerk voor de aanwezigheid van *Verticillium dahliae* in bomen.
   Dit proefschrift; S. Wilhelm and J.B. Taylor, 1965, Phytopathology 55:311-316; E.B. Himelick, 1968, Ill. Nat. Hist. Survey B1-1968.

4. Het is zeer waarschijnlijk dat er ook onder de boomsoorten zogenaamde 'symptomless carriers' van *Verticillium dahliae* zijn.

5. De al in het begin van deze eeuw geuite waarschuwing dat men de bosbescherming dient te bestuderen en niet moet beschouwen als een receptenboek, dat men even opslaat om binnen vijf minuten de diagnose te stellen en de genezing te bewerkstelligen, is helaas nog steeds actueel.
   M. de Koning, 1922. Boschbescherming, Thieme & Cie, Zutphen.

6. De wetenschapsvelden van de bosontwikkeling en van de pathogenese bij verwelkingsziekten van bomen vertonen principiële overeenkomsten; in beide gevallen is de interactie tussen processen en structuren bepalend voor het verloop van de ontwikkelingen.

7. Boggling of the mind is a part of the process of gaining insight.
   C.H. Beckman, 1984, Phytopath. Medit. 23:109-129.

8. Een goed onderzoeker merkt ook datgene op waarnaar hij niet op zoek was.

9. Een van de meer misleidende aspecten van wetenschappelijke publicaties is dat de daarin beschreven weg van hypothese tot conclusie vrijwel nooit getrouw weergeeft hoe de resultaten werkelijk zijn verkregen.

John L. Casti, 1992. Verloren paradigma's, Contact, Amsterdam.

10. Indien de Nederlandse samenleving werkelijk geïnteresseerd is in het duurzaam gebruik van natuurlijke hulpbronnen, dan dient zij het landbouwkundig onderzoek uit te breiden in plaats van daarop te bezuinigen.

11. Gezien het veelvuldig misbruik ervan moet gevreesd worden voor de duurzaamheid van de term duurzaam.

12. Indien we er niet in slagen om een einde te maken aan zaken als vermesting, verzuring en verdroging dan moet het gezegde "boompje groot, plantertje dood" omgedraaid worden in "plantertje groot, boompje dood".

Stellingen behorende bij het proefschrift van Jelle A. Hiemstra,
getiteld "Verticillium wilt of Fraxinus excelsior",
te verdedigen op 18 april 1995 te Wageningen.

Wanneer ick door 't Haeghsche bos,
Van mijn beroep een weynigh los,
Of in de naeste velden dwael:
Soo vind' ick eycken, boven kael,
Doch elders groen en wel gestelt,
Gelijck de gaefste van het velt.
Dit vind' ick vremt, dies vraegh ick dan,
Ja, vraegh hier gront, en reden van.

Jacob Cats; Hof-Gedachten,
op 't Gesichte van Boomen enz.
op Sorghvliet (1651-1660)

VOORWOORD

De eerste aanzet voor het in dit proefschrift beschreven onderzoek werd al in het najaar van 1984 gegeven tijdens mijn stage bij de toenmalige afdeling Bosontwikkeling van het Staatsbosbeheer. Tijdens terreinbezoeken in verband met onderzoek naar het beheer van jonge gemengde loofboomopstanden werd ik door verschillende beheerders meegenomen niet alleen naar — uiteraard — de percelen waarop ze trots waren, maar ook naar de 'probleemgevallen'. In het laatste geval betrof het meestal percelen waarin het zogenaamde 'essensterven' optrad. De bijbehorende vraag was dan: "Vertel jij, als 'Wageninger' nou eens wat hier aan de hand is." Dat was toen nog te veel gevraagd. Maar de vraag bleef wel hangen.
Een vervolg kreeg dit tijdens mijn laatste afstudeervak, waarin ik zes maanden besteedde aan het in kaart brengen van de omvang van het probleem en het inventariseren van mogelijke oorzaken. Dat leverde opnieuw geen afdoende antwoord op, maar wel voldoende informatie voor het formuleren van een voorstel voor vervolgonderzoek. Dit werd in 1987 door de Landbouw-Universiteit Wageningen gehonoreerd met een AIO-project. Het onderzoek is gedurende de periode 1987-1991 uitgevoerd bij de vakgroepen Bosteelt en Bosoecologie, later Bosbouw, en Fytopathologie. Het verwerken van de gegevens en het schrijfwerk kostte zoals inmiddels duidelijk zal zijn wat meer tijd.

Nu ook het schrijven achter de rug is wordt het tijd om de vele bij het onderzoek betrokken personen te bedanken. In de eerste plaats de heer O. Buwalda (SBB, Groningen) en Ir. A. Arnoldussen (RIJP). Zonder hun enthousiasme was het misschien nooit zo ver gekomen. zij waren de al genoemde beheerders. Daarnaast moet Ir. P.A. van den Tweel (SBB, afd. Bosontwikkeling) genoemd worden omdat hij degene was die me voorstelde het 'essensterven' als onderwerp te nemen voor mijn laatste afstudeervak.
Prof. R.A.A. Oldeman en prof. J. Dekker dank ik voor hun steun en vertrouwen bij het opzetten en de uitvoering van het project, dr. ir. P. Schmidt en dr.ir. T. Limonard voor alle tijd en belangstelling bij de begeleiding van het onderzoek. De leden van de begeleidingscommissie, ir. A. Arnoldussen, ir. B. van Baren, ir. J.J. Borgesius, M. de Kam, ir. B. van der Pas en ir. P.A. van den Tweel hebben door de jaarlijks terugkerende nuttige discussies mede de richting van het onderzoek bepaald.
Het onderzoek werd financieel ondersteund door het Staatsbosbeheer, later directie Bos- en Landschapsbouw en de Rijksdienst voor de IJsselmeerpolders. Hierdoor was tijdelijke assistentie bij enkele tijdrovende experimenten en bij de verwerking van de gegevens van de proefperken mogelijk. NWO droeg de

kosten van het bijwonen van het Verticillium congres in 1990 in Leningrad. Het ERASMUS-programma van de EEG ten slotte maakte een verblijf van drie maanden aan de Universiteit van Hamburg mogelijk.
Aan de uitvoering van het onderzoek hebben velen hun steentje bijgedragen. Ir. F. Burger, ir. H. Schneider en P. Gilmore wil ik van harte bedanken voor hun doorzettingsvermogen bij de vaak saaie en tijdrovende klussen waarbij zij als tijdelijk assistent fungeerden. De mensen van de tuin: J. Post, A. Zweers, L. Goudswaard en wijlen B. Schalk leverden een onmisbare bijdrage aan de het werk op het proefveld aan de Afweg, in de proefperken in het bos en aan de helaas niet geslaagde potproeven. De inzet van enkele studenten maakte een verbreding van het onderzoek mogelijk. Paul van der Vegt, Marten Denekamp, Robert Schroeijers en Toon Rijkers, bedankt dat jullie een deel van het werk op je namen.
Dr. T. Limonard en drs. G. Bollen hebben mij als niet-fytopatholoog ver genoeg bijgespijkerd om dit onderzoek tot een goed einde te laten komen. Ook de andere leden van de vakgroep fytopathologie dank voor de nuttige adviezen en prettige samenwerking. Hetzelfde geldt voor de nog niet met name genoemde vrienden en collega's binnen de vakgroep Bosbouw, met name de medebewoners van de "bovenkamer". Collega onderzoekers buiten de Landbouwuniversiteit waaronder prof. D.M. Elgersma, dr. W. Gams, dr. H. Heybroek, drs. B.C. van Dam, ing. A. de Haas, ir. N.G.M. Dolmans en dr. H.A. van Kesteren; bedankt voor de leerzame discussies en vaak waardevolle praktische tips.
De lezers van de manuscripten: dr. P. Schmidt, dr. T. Limonard, dr. A.J. Termorshuizen, dr. R.P. Leersnijder, prof. J. Dekker en prof. Oldeman hebben door hun gedegen commentaar bijgedragen aan het stroomlijnen van de uiteindelijke tekst. Ir. J.B. van der Pas heeft de engelse tekst gecorrigeerd. De vele discussies bij prof. Oldeman thuis, waarbij het niet altijd alleen maar over essenverwelking ging, waren zeer stimulerend.
Mijn beide paranimfen, dr. R.P. Leersnijder en dr. A.J. Termorshuizen komt een apart woord van dank toe. Peter bedankt voor vele computeradviezen en de ontwikkeling van het in hoofdstuk 8 gebruikte computerprogramma. Aad, dank voor het zeer uitvoerige commentaar en opbouwende kritiek op de concept teksten. Daarnaast beiden dank voor de morele steun.

Tot slot, dank aan mijn ouders, schoonouders, familie en vrienden voor de blijvende belangstelling. En Hilly, zonder jouw steun en vooral ook geduld was dit alles nooit afgekomen.

Jelle Hiemstra, Wageningen, 2 maart 1995.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 GENERAL INTRODUCTION

Een vreemd gerucht gaat door het land.....
Ze sterven.....bij tientallen.....
Zou het waar zijn?

(A strange rumour is spreading.....
They are dying.....by the score.....
Would it be true?)

From: Het raadsel der Iepenziekte
(The mystery of the Elm Disease)
by S. Broekhuizen, 1937; p. 5.

Recently, in some parts of the Netherlands an unidentified disease of common ash (*Fraxinus excelsior* L.) leading to dieback and death of affected trees caused great concern among owners and managers of broadleaved forests. It was feared that yet another serious disease threatened one of our important broadleaves, after Dutch elm disease and watermark disease had become major diseases of elms (*Ulmus* spp.) and water willow (*Salix alba* L.), respectively. This instigated a research project of which the present book is the final report.

In the first section of the present chapter the victim of this 'new' disease, *F. excelsior*, is described. The second section introduces the research project. In that section also the design of the other chapters and of the book as a whole are outlined.

1.1 Fraxinus excelsior

1.1.1 Taxonomy and natural area

The common or European ash, *Fraxinus excelsior* L., belongs to the family of the *Oleaceae*, a family of mostly trees and shrubs. Besides the genus *Fraxinus* this family includes the well-known genus *Olea* (with *O. europaea*,

the olive) and many genera with species known as ornamental shrubs and trees like *Syringa* (lilac), *Jasminum* (jasmine), *Ligustrum* (privet), *Chionanthus* (fringe tree) and *Forsythia* (Boom, 1980).

All ash species are found in the Northern Hemisphere, mostly in the temperate regions. Most species are trees of the mountains, but in the northern part of their natural area they also grow in the plains (Scheller, 1977). In Europe *F. excelsior* L. (common or European ash) and *F. ornus* L. (flowering or manna ash) are the most common species. The best known species on the American continent (Record and Hess, 1943) are *F. americana* L. (white ash), *F. pennsylvanica* Marsh. (green ash) and *F. nigra* Marsh. (black ash).

In Western Europe *F. excelsior* is the only native ash species. Its natural area (Fig. 1.1) stretches from the Mediterranean region to the southern parts of Scandinavia and from the Atlantic Ocean to the Wolga river. It includes the British Isles and Eire. The geographical and climatological limits of this area are the Atlantic Ocean in the west, the short growing period in the north and the dry and hot summer periods in the east and south. Because of the latter the common ash is limited to forests along streams in the southeastern part of its natural area and to the mountains in the southern part of its natural area (Huldén, 1941; Wardle, 1961).

Fig. 1.1 Native area of *Fraxinus excelsior* L. (after Houtzagers, 1954).

Within its natural area the common ash is present on two categories of soil types. Primarily, it is a species of mixed deciduous forests with *Quercus*, *Tilia*, *Ulmus* and *Acer* spp. on moist, humic, loamy, deep soils. Secondly, it is found in combination with *Fagus sylvatica*, *Acer pseudoplatanus* and *Abies alba* on drier, calcareous soils, although with suboptimal growth (Houtzagers, 1954).

Since the report of Münch and Dieterich (1925), especially in German literature, ashes from these two soil types long have been considered to be two different physiological races: 'Wassereschen' ('water ash', i.e. ash originating from the first category of sites) and 'Kalkeschen' ('lime ash', i.e. originating from the second category). However, despite much and thorough research (e.g. Leibundgut, 1956; Weiser, 1964a,b; Von Schönborn, 1967) no clear differences between the two could be demonstrated. Mayer (1984) explains this by stating that a former continuous range of ash sites has been split into two groups of sites rather recently. This would be the result of the appearance, about three thousand to four thousand years ago, of beech (*Fagus sylvatica* L.) that became dominant on part of the former ash sites. Too little time would have passed for the evolution of physiologically differing races to occur, especially because common ash is a genetically stable species with a large genetic variability (Mayer, 1984).

1.1.2 Morphological and growth characteristics

The common ash is characterized by long, pinnate leaves with 9 to 13 leaflets and opposite, black buds (Fig. 1.2). The tree grows according to the model of Rauh (Fig. 1.3), i.e. with an orthotropic monopodial stem, orthotropic monopodial branches and lateral flowering (Hallé and Oldeman, 1975). The rhythmic growth of all axes results in a whorled or subverticillate arrangement of the branches. Under favourable conditions, two (Roloff, 1989) and sometimes even three (personal observations) successive shoots within one growing season are possible. After death or loss of part of the tree, young ash trees usually regenerate rapidly which is illustrated by its frequent use as coppice or pollards. In large trees regeneration processes after loss of major parts of the crown may lead to the formation of young tree-like structures (reiterated complexes conforming to Rauh's model or parts of it) on the major branches (Fig. 1.4).

Fraxinus excelsior

Fig. 1.2 Morphological characteristics of *Fraxinus excelsior*. a. Twig with characteristic black buds. b. Twig with female flowers. c. Twig with male flowers. d. Pinnate leaf. e. Winged fruits (samara). Reprinted with permission from: Boeijink *et al.*, 1992.

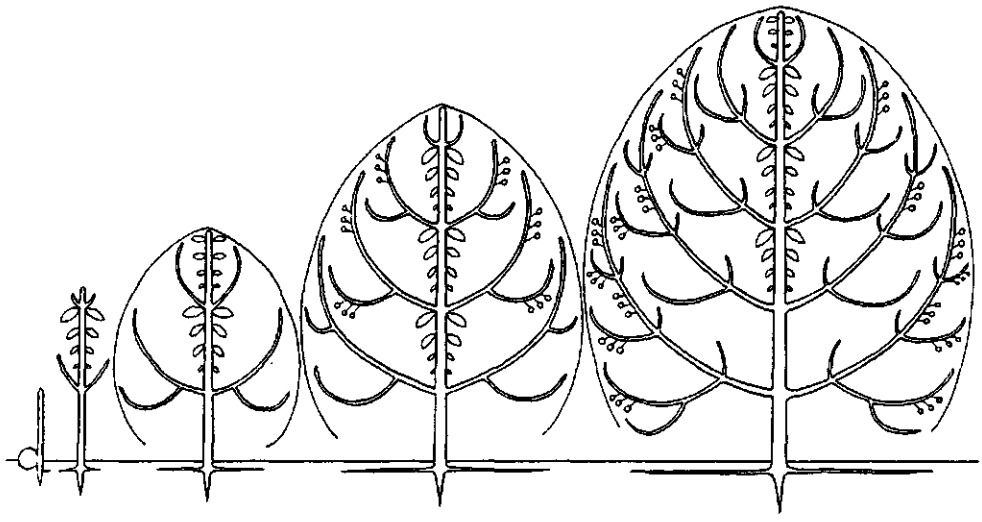

Fig. 1.3 Diagram of the model of Rauh for different developmental stages of a tree. Common ash, of course, has compound leaves instead of the single leaves shown in the diagram. Reprinted with permission from: Hallé, Oldeman and Tomlinson, 1978.

On very suitable sites common ash can reach a maximum height of 40 to 45 meter (Savill, 1991). Heights of 20 to 30 meter, however, are more common. Especially during the first 10 to 20 years height growth may be very fast. Ash trees normally do not grow very old, although they may reach an age of about 300 years. In coppice-form they even may become 500 years old (Wardle, 1961; Thill, 1970; Mayer, 1984).

The root system of common ash is of the heartroot type with far-reaching horizontal roots clustered in the upper soil layer and deeply penetrating sinkers (i.e. vertical roots; Köstler *et al.*, 1968). The roots are often concentrated in certain parts of the soil, resulting in rootless areas within the rooted soil volume. The fine rootlets are strongly concentrated in the upper 10 cm of the soil. They often spring directly from the coarse roots. Most of them live only one year (Wardle, 1957). The tree has mycorrhizae of the vesicular arbuscular type (Jeník and Kubíková, 1961; Linnemann, 1972).

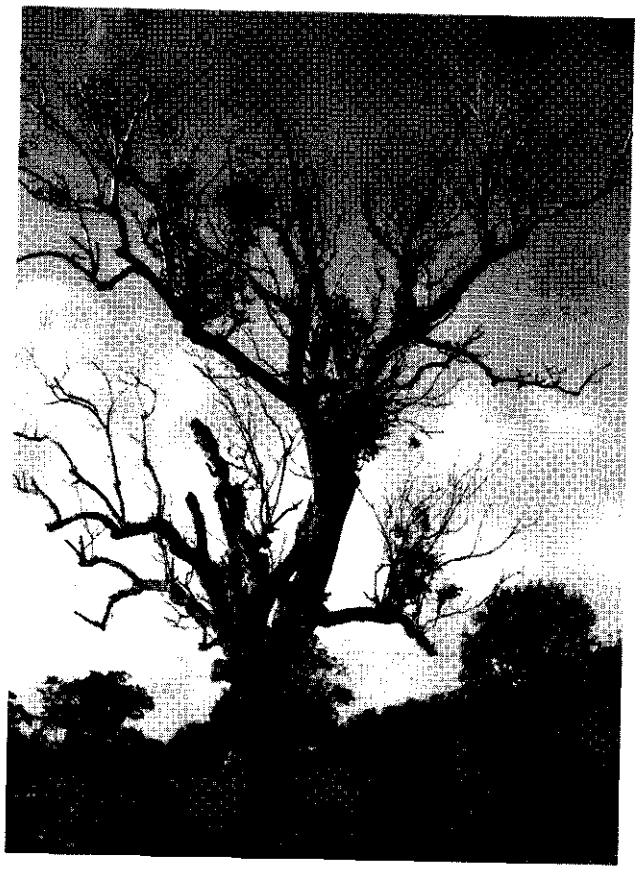

Fig. 1.4 Reiterated complexes conforming to Rauh's model on the major branches of a common ash showing severe crown dieback. Warwick County, Great Britain, October 1987. Photograph by the author.

The ash flowers before the leaves are out. The flowers are wind-pollinated and appear in bunches from lateral buds (Fig. 1.2b,c). They may be male, female or hermaphrodite. Unisexual trees exist but in many cases different flower types are present on one tree (Rohmeder, 1952). The seed (Fig. 1.2e) is a small samara, i.e. a small winged nut which is distributed by wind.

The wood of *Fraxinus* is ring-porous (Grosser, 1977), i.e. with very large spring vessels and quite narrow vessels in the summer wood. Because of their

much smaller diameter the transport capacity of the summer vessels is much lower than that of the spring vessels. Hence, water transport is almost exclusively ensured by spring vessels (Zimmermann, 1983). These vessels are functional for one season only, so diameter growth, i.e. formation of new spring vessels, starts before flushing. The narrow summer vessels may remain functional for several years. Braun (1970) considers this anatomy to be an adaptation to the short period with very fast flushing between the cold winter period and the hot, dry summer period in the native area of most ring-porous trees.

The strong concentration of the water transport on a limited part of the outer growth ring makes ring-porous trees vulnerable to disturbance of the water transport by injuries as well as by vascular wilt pathogens (Braun, 1970; Zimmermann, 1983). In such cases, however, the water transport may be maintained by the narrow and much safer summer vessels. For this reason Carlquist (1988, p. 28) sees this type of wood anatomy as 'a strongly pronounced adaptation for conducting large volumes of water in earlywood, combined with conductive safety (evidently with much reduced volume-handling capability) in latewood'.

1.1.3 Silviculture and use

In Dutch forestry only trees from seed are used. In amenity and roadside plantations some clones may also be found, viz. 'Altena', 'Atlas', 'Diversifolia', 'Eureka', 'Geessink' and 'Westhof's Glorie' (Lombarts, 1989; Anon., 1990). In all cases these clones are grafted on *F. excelsior* seedling rootstocks.

Monographic treatments of site requirements, growth and production of ash are given by Volquardts (1958), Thill (1970) and Knorr (1987). More ecologically oriented reviews were published by Huldén (1941) and Wardle (1961). The most important aspects are summarized below.

The large geographic area of its natural distribution indicates that common ash has no very strict climatic requirements. If sufficient water is available it grows well in cool temperate areas as well as in areas with high summer temperatures. Low winter temperatures generally do no harm but common ash is very sensitive to late spring frost (Peace, 1962) that may cause forking by killing the apical buds of stem and branches, after which both subapical buds reiterate Rauh's model (cf. Fig. 3.11 in Oldeman, 1990b).

The soil requirements of common ash are much more pronounced. It shows its best growth on deep, moist, well-drained and aerated, fertile soils of about neutral reaction. On drier or less fertile soils the tree grows much more slowly, especially when older. Ash does not tolerate prolonged flooding, waterlogging or compacted soils. Hence, for wood production to be commercially attractive, common ash should be grown only on the best sites (Evans, 1984). 'An expensive tree on rich soils', it was called by Ackermans, Creemers and ter Harmsel (1979).

It is often stated that ash grows best on limy soils but this certainly is not the whole truth (Thill, 1970). If soils are rich enough, ash will grow well without lime. Indeed it can grow on strongly calcareous soils without the occurrence of lime-induced chlorosis unlike, for example, *Quercus robur*. Van den Burg (1981) reports that ash grows well on soils with a pH (H_2O) ranging from pH 5 to pH 8.

Finally, for fast growth and harmonious crown development ash requires high light conditions. During its seedling stage, however, it is highly shade-tolerant. Even after up to 30 years of suppression with very slow growth (less than one cm per year), seedlings may resume normal growth after opening of the canopy (Van Miegroet and Lust, 1972).

In the past the mechanical qualities of ash wood, being strong, elastic and shock-resistant, made it apt to be used for weapons (spears, arrows, handles of battle-axes), tool handles, parts of all kinds of agricultural equipment, vehicles and boats, and in early airplane industry (Kollmann, 1941). In mountainous regions farmers often used the leaves and young branches as fodder, especially for sheep and goats (Pfeil, 1851).

Nowadays ash wood still is used for sports equipment (hockey sticks, cricket stumps, baseball bats, bars, etc.) and in tool handles. Because the strength is caused mainly by the summer wood, wood for these purposes should contain a relatively high percentage of summer wood. Therefore it should be grown relatively fast (Aldridge and Hudson, 1954; Heilig, 1981). Well-grown ash of good quality is used for veneer, planking, parquet floors or furniture. Rather specific is the use as stirring sticks in aluminium smelters (Schepers and Van Haperen; 1992). Ash is also suitable for pulpwood and much appreciated as firewood. The latter is clearly illustrated by the last lines of an old anonymous rhyme discussing the value of many tree species for use as wood fuel (cited by Wilkinson, 1978; p. 12):

'But ash logs, all smooth and grey,
Burn them green or old;
Buy up all that comes your way;
They're worth their weight in gold.'

1.1.4 Importance in Dutch forestry and landscaping

In the past two centuries, forestry in the Netherlands was developed by reafforestation of marginal lands, agricultural activities continuing to occupy the more productive areas. Therefore, until recently (see next section), ash forests were rather rare in our country. Ash was mainly limited to small remnants of riparian forest types (mainly in the eastern parts of the country), forests on rather steep fertile slopes (Limburg, in the deep south) and small forest plots in parklands situated on estates. Another important ash territory was the large ash-coppice area in the lower parts of the central provinces of Zuid-Holland and Utrecht. Besides these forest areas, ash always has been an important tree in plantings around farms and along roads because of its valuable and much-used wood (De Beaufort, 1853).

In the second half of the present century the importance of ash in Dutch forestry increased considerably. This had the following reasons:
- Better sites became available for afforestation in the newly reclaimed polder areas and in the traditional agricultural areas during re-allotment projects.
- Changing forest policies increasingly favoured large broadleaved forest areas.
- Major diseases (i.e. Dutch elm disease in elms, watermark disease in willows and several rusts in poplar) discouraged the use of some other broadleaved species.

The increased use of ash in the newly established forests in the IJsselmeerpolders (Nuesink, 1980; Bos, 1984) and in roadside, recreational and amenity plantations in other areas, produced approximately 5000 ha of ash forest around 1980 (CBS, 1985). Especially in the coastal provinces and in the province of Flevoland ash stands form a substantial part of the forest area (Appendix I). In 1980 most of the ash forests were less than 40 years old because of the recent plantings. Except for the ash forest area, a further 5000 km roadside and line plantations existed in 1980 (SBB, unpubl.) Finally ash is an important species in many mixed broadleaved stands, including many small woodlots planted for recreational and landscaping purposes.

In the long term planning of the Dutch government (Anon., 1986) a further increase of the ash forest area is intended, leading to a doubling of the area by the year 2050. In the light of the recent developments in European agriculture this even may be a conservative estimate. The need to decrease the overproduction of agricultural products and the pollution by intensive agriculture probably will lead to large areas of agricultural land to be abandoned by agriculture. This may lead to a strong extension of the area with fast-growing trees, including ash (WRR, 1992: fig. 4.10 and 4.11).

1.1.5 Major diseases and pests

In Northern America and in Great Britain two serious diseases occur in ash. North-American ashes, mainly *F. americana*, are affected by a disease called ash dieback or ash decline. Among the symptoms of the disease are slow growth, sparse foliage, small and chlorotic leaves, tufting of leaves at branch ends, cankers on branches and trunks, dying back of twigs and branches, and formation of epicormic (reiterated) shoots. Although many biotic and abiotic agents, including fungi, viruses, drought and frost damage, have been suggested as primary or secondary causes, and a close association of mycoplasmal infection with decline has been reported, the disease is still not completely understood (Sinclair *et al.*, 1987).

In Great Britain a disease, also called ash dieback or ash decline, is affecting *F. excelsior* since the 1950's and appears to have become more widespread in recent years (Evans, 1984). The main symptom is progressive dieback of twigs and branches over several years. The direct cause of the disease is still unknown, but large trees were shown to suffer more from the disease than small trees and single trees more than trees in groups. Also correlations were found with the presence of roads or ditches near trees and with the intensity of arable farming around the trees (Pawsey, 1983; Hull and Gibbs, 1991).

In the Netherlands the above problems are unknown and until recently common ash was known as a species relatively free of serious pest and disease problems. Before the occurrence of ash wilt disease, bacterial canker caused by *Pseudomonas syringae* subsp. *savastanoi* pv. *fraxini* (Janse, 1981b) was the most damaging disease, as the cankers caused by this disease seriously reduce the commercial and aesthetic value of affected trees. In the Netherlands this disease is often, but not always, found on sites unsuitable for

ash (Janse, 1981a). Occasionally stem and branch cankers may be caused by some other fungi like *Nectria galligena* Bres. and *Phomopsis* spp. Honey fungus, *Armillaria* ssp., may also attack ash but is mostly restricted to weakened trees (Smith *et al.*, 1988).

Many insects are known to feed on ash (Wardle, 1961; Schwerdtfeger, 1981; Anon., 1982). However, they hardly ever cause real problems. Ash bark beetle (*Leperisinus varius* F.) prefers weakened trees (or firewood logs during seasoning) for egg deposition. On healthy trees they cause beetle scab ('ash-rose') by boring for maturation and hibernation. Because these bore holes are superficial they do not affect wood quality.

Ash leaves may be eaten by caterpillars of *Operophtera brumata* L. (Winter moth) and *Erannis defoliaria* Cl. (Mottled umber moth). Leaf psyllids (*Psyllopsis* spp.) cause blistering and swelling of leaves, mainly on young plants, and the gall midge *Dasyneura fraxinea* Kieff. raises pustules on leaves. The gall mite *Aceria fraxinivora* Nal. causes cauliflower-like galls on the flowers, and the Ash bud moth (*Prays fraxinella* Bjerkander) causes forking by mining terminal buds. On ash stems the Willow scale *Chionaspis salicis* L. and the Ash scale *Pseudochermes fraxini* Kalt. may occur.

Of much more importance, especially in young plantations, may be the damage caused by mammals like mice, rabbits, deer, etc. which girdle stems by eating the bark away.

1.2 Outline of the project

1.2.1 Background and motives

As was shown above, in the last decades the importance of ash in Dutch forestry and landscaping has increased considerably and further increase is intended. However, in the end of the 1970's a previously unknown disease — commonly hitherto called 'essensterven' (dying of ash) — was observed in ash stands in several parts of the country. The rapid increase of the number of reports of this disease caused great concern among forest owners and managers. This instigated a preliminary investigation that was carried out during the summer of 1986 (Hiemstra, 1987a; Hiemstra *et al.*, 1990).

The investigation showed that the symptoms of the disease resemble those of some well-known and devastating wilt diseases of trees, like Dutch elm disease and watermark disease of willow in our country, and oak wilt in

Northern America. For this reason the name 'ash wilt disease' was coined (Miller and Hiemstra, 1987). It was shown that affected trees were present in most of the important ash areas of the country. Besides, the highest disease incidences (up to 20%) were reported in recently afforested areas where ash is an important tree species (i.e. provinces of Flevoland, Groningen and Zeeland).

Because of these alarming observations, it was feared that the new disease indeed might cause serious problems in existing ash stands and frustrate the intended increase of the ash forest area. Hence, in May 1987 a new research project was started at the department of Silviculture and Forest Ecology (now merged into the department of Forestry) in co-operation with the department of Phytopathology, both from the Wageningen Agricultural University. The project was supported by the former Lake IJssel Polders Development Authority (Rijksdienst voor de IJsselmeerpolders), the State Forest Service (Staatsbosbeheer) and the Research Institute for Forestry and Landscape Planning (RIOBL 'de Dorschkamp', now Institute for Forest and Nature Research IBN-DLO).

1.2.2 Aim and research questions

As stated above, the fear for serious management problems in existing and future forest stands and urban plantations of ash, caused by ash wilt disease, led to the start of this research project. Hence, corresponding with the aim of plant pathology in general as formulated by Cowling and Horsfall (1980), the aim of this project was to understand the ash wilt disease so well that it can be managed effectively.

For effective management of a disease problem, knowledge about the affected species, the causal pathogen and the development of the disease is essential. In the case of ash wilt disease, information on the affected species could easily be gathered by means of a literature survey, summarized in the previous section. In contrast, information on the cause and development of the disease was lacking. Hence it was decided that the study would concentrate on those aspects. This resulted in three main research questions to be answered:

1. What is the cause of the disease?
2. What is the effect of the disease on individual ash trees?
3. What is the effect of the disease on forest stands of ash?

1.2.3 General approach

The cause of the disease

This aspect is covered by Chapters 2-5. The study started with delimiting the problem by coining a proper name (i.e. ash wilt disease; Section 2.1), describing the symptoms in detail (Section 2.2), and describing the distribution of the disease (Section 2.3). This part of the research was largely based on the results of a preliminary investigation in 1986 (Hiemstra, 1987a), supplemented by field observations in the summer of 1987.

The next step (Section 2.4) was pinpointing the probable cause or causes. This was done by critically examining the available information and excluding as many potential causes as possible. Again the data from the preliminary investigation were the prime source of information. Some information gaps (e.g. presence of mycorrhizae and plant-parasitic nematodes in affected stands) were filled in by short-term research projects by others as cited. On the basis of all accumulated information, the hypothesis of ash wilt disease to be caused by a vascular wilt pathogen, probably a *Verticillium* sp., was formulated.

This is followed by a literature survey on *Verticillium* wilts of woody plants in Chapter 3.

In order to test the hypothesis, a nationwide survey for the presence of pathogenic fungi in diseased ash trees and an inoculation experiment investigating the pathogenicity of *Verticillium dahliae* in ash were carried out in the summer of 1988 (Chapter 4). The results proved that the ash wilt disease is indeed caused by *Verticillium dahliae* Kleb.

This raised the question why the disease became only recently so important. Both host and parasite are native to the Netherlands, whereas no previous outbreaks of ash wilt disease have been described. A possible explanation might be the occurrence of a new host-specific or more aggressive strain of *V. dahliae*. This possibility was investigated by means of the inoculation experiment described in Chapter 5 in which three tree hosts of *V. dahliae* where inoculated with four isolates of the fungus obtained from different host plants.

The effect of the disease on individual ash trees

The work on this aspect is discussed in Chapters 6 and 7. In Chapter 6 this is done at organ level describing the anatomical anomalies in the xylem of inoculated young ash tree stems. In Chapter 7 the effect of the disease is

discussed at the whole-tree level. Disease progress in individual trees (i.e. symptom development and occurrence of recovery, dieback or death in the next year) and growth of these trees are analyzed. In both chapters, naturally infected trees as well as inoculated trees are studied.

The effect of the disease on forest stands of ash
This aspect is dealt with in Chapters 8 and 9. Disease incidence, distribution and progress in affected forest stands were studied in two permanent plots established in ash stands severely affected by ash wilt disease. The condition of the trees in these plots was recorded several times a year from 1987 to 1991. The results of the monitoring and their meaning for understanding disease impact are discussed in Chapter 8. Chapter 9 presents another approach of the same subject. In this chapter disease incidence and impact in the province of Flevoland are discussed on the basis of the results of an aerial survey.

Concluding chapter
In the final Chapter 10 all results are reviewed in an integrated manner. Based on the results of the research and supplemented by data from literature, a description of the interactions between the pathogen and the host is presented. By analyzing the impact of the disease, this chapter also provides an answer to the question whether or not the situation is indeed as menacing as suggested by the quotation for the Dutch elm disease at the start of the present chapter. In a second section, the implications for nursery-growing and silviculture of ash are discussed. Finally, the new questions that arise from the results are outlined and the contents of further indispensable research are indicated.

Resuming, the Chapters 1, 2 and 3 set the stage. Chapters 4 and 5 deal with the pathogen. Chapters 6, 7, 8 and 9 cover the diseased trees. In the latter group of chapters, the organization level studied (cf. Oldeman, 1990a) raises from the organ level, to the tree level, to the stand level and finally to the level of a group or mosaic of stands. In the concluding Chapter 10 all results are integrated in a portrait of the interaction between *Verticillium dahliae* and *Fraxinus excelsior*.

2 NAME, SYMPTOMS, DISTRIBUTION, AND POSSIBLE CAUSES OF THE ASH WILT DISEASE

The aim of this chapter is twofold: to delimit the problem and to lay the groundwork for the main hypothesis of the research. Delimiting the problem is achieved by coining a proper name for the disease (Section 2.1) and describing its symptoms (Section 2.2) and distribution (Section 2.3). These three sections summarize the results of a preliminary research project, executed in the summer of 1986 (Hiemstra, 1987a; Hiemstra *et al.*, 1990).

Besides this summary there are also some new aspects. At the end of the section covering the symptoms of the disease, the code system is explained that was used throughout this study for describing symptoms and progress of the disease. Additionally, data collected during the years 1987-1991 are included in the section on distribution of the disease.

Based on the data of the previous sections and on the results of a few additional short-term research projects by others the most probable cause is deduced in the last section of this chapter. This results in the hypothesis that the disease is caused by the vascular wilt pathogen *Verticillium dahliae* Kleb.

2.1 Name of the disease

In the first reports on the disease a large variety of symptoms was listed (De Kam, 1984; Hoogesteger and Arnoldussen, 1985). In all cases dieback and death of trees were mentioned as important issues. Hence, the disease was generally referred to as **'dying of ash'** (Dutch: **'essensterven'**). Field observations during the preliminary research (Hiemstra, 1987) showed that the vast majority of diseased trees displayed the same disease syndrome with defoliation, preceded by wilt symptoms, being the most obvious early symptom (Fig. 2.1). Hence, the name **'ash wilt disease'** has been introduced to replace the vague and unspecific term 'dying of ash' (Miller and Hiemstra, 1987; Hiemstra, 1988). In this book the name 'ash wilt disease' will be used to refer to the disease.

2.2 Symptoms of the disease

2.2.1 Symptoms and symptom development

Symptoms

As stated above, the most striking early symptoms of the disease are wilting and defoliation. Usually wilting of the leaves is accompanied by a subtle change of their colour towards a lighter, greyish green: 'loss of colour' (Fig. 2.2). In extreme cases withering or complete necrosis of the leaves has been observed (Fig. 2.3). All these early symptoms may affect the entire crown, or only part of it.

Fig. 2.1 Main symptoms of the ash wilt disease: wilt and defoliation. Left: healthy ash tree. Note the regularly outspread position of the leaves and leaflets in the upper crown. Middle: A tree with early wilting symptoms. Particularly in the uppermost part of the crown all leaves droop. Right: A tree which lost almost all leaves after wilting. All trees are 12 years old and have been carefully removed from an ash forest stand to enable a picture to be taken. (Horsterwold section Pz5-a1, 1 September 1987).

Secondary symptoms of the disease may include the next phenomena:
- reduced new flushes with very small, pale green to chlorotic leaves and very short internodes (Fig. 2.4);
- marginal necrosis and scorch of the remaining leaves;
- elongated dead areas of bark on the diseased stem or branches;
- dieback of part of the tree;
- epicormic shoots on surviving parts of the tree, sometimes followed by dieback of these shoots.

Discoloration of the wood does not seem to be a consistent symptom. Some affected trees showed a discoloration in the cambial zone, the wood or the pith of stems or branches. However, in many other trees only a very slight discoloration was visible, if any.

Symptom development

The symptoms become conspicuous in the growing season, often after a period of normal growth. Many newly affected trees show the first symptoms in August or September. Death of affected trees may take place within a few weeks after initial symptoms. However, in other trees the death of the lower parts of the crown and finally of the trunk only took place after several years of languishing, formation of epicormic shoots and gradual dieback.

Particularly in young trees, up to 15 or 20 years old, the processes of wilting and defoliation can occur very suddenly. In such trees no signs were found of a decrease in growth before the wilt symptoms appeared. Measurement of the shoots in some affected trees showed normal growth until the moment that symptoms appeared.

It appeared that in some trees of 20 years and older, as well as the above acute process, a more chronic process was occurring. These trees did not show the sudden complete wilting and leaf fall as described above. Instead, the trees declined much more gradually. In 1987 in the city of Rotterdam some 20-year-old trees were showing a gradual decline including early leaf fall in 1986, extremely late flushing in 1987, sparse and pale-coloured foliage, gradual wilting, partial leaf fall and a partial dieback of the topmost twigs during the growing season of 1987. More recently, this kind of dieback has also been reported in other parts of the country.

Fig. 2.2 (top) Early stages of the disease in a roadside plantation of *Fraxinus excelsior* 'Atlas'. From left to right: Tree showing severe wilt, necrosis and loss of leaves; healthy tree; tree with early wilt and discoloration of leaves; healthy tree. (Rotterdam, Boszoomweg, 21 August 1987).

Fig. 2.3 (left) Serious foliar symptoms on a 6-year-old nursery tree: defoliation and necrosis of leaves while still on the tree (Randwijk, 31 July 1989).

Fig. 2.4 (right) Regrowth in year of defoliation: Reduced new shoots with very small pale green or chlorotic leaves and very short internodes (Horsterwold, section Pz5-a1, 16 October 1990).

2.2.2 Symptom code, disease index and disease class

The disease symptoms were visually assessed using a scale developed specially for ash wilt disease. The condition of each tree under investigation was recorded using a two-tier symptom code system. The first element of the code indicating the type of symptoms; the second element representing the estimated percentage of one individual crown showing these symptoms (see Appendix II for more details).

In order to be able to compare individual trees and to indicate disease progress, the coded symptom data were converted into a disease index, ranging from 0 to 10 with increasing severity of symptoms. The disease index scale only gives wide and general classes of increasing severity of symptoms. The differences between the values of the scale are not fixed; e.g. it cannot be said whether or not the difference between disease index 0 and 1 is equal to the difference between 6 and 7 (ordinal scale; cf. Jongman *et al.*, 1987). Details on the method of conversion and conversion tables are given in Appendix II.

In many cases, a more general classification was needed. Therefore a system of disease classes (DC) was used. Several disease indices were merged, resulting in six disease classes (Table 2.1) ranging from healthy (DC 0) to dead (DC 5). Again, the scale is ordinal which means that there is a rank order between the values (classes), but the differences between values are not fixed.

Table 2.1 Disease classes[1] and their meaning.

Disease class	Meaning
0 Healthy	Completely healthy appearence
1 Dubious	Not healthy but no symptoms[1] of ash wilt disease
2 Slight symptoms	Slight symptoms[1] of ash wilt disease
3 Distinct symptoms	Distinct symptoms[1] of ash wilt disease
4 Dieback	Partial death of tree (dieback)
5 Dead	Tree dead

[1] In an early stage of the disease wilting, withering and loss of leaves (DC 2 and 3) are considered to be the main symptoms. Later on, after defoliation, small, chlorotic regrowth (DC 3) and dieback (DC 4) are also possible symptoms of the disease.

2.3 Distribution of the disease

The first reports of a new disease problem in common ash date from the late seventies (Hiemstra, 1987a). In 1976 inexplicable death of ash trees was reported on several locations in the province of Zeeland. In the same year it was recorded on one location in the province of Drenthe. In the next year it occurred in several forest stands in the eastern part of the province of Flevoland. From 1979 on it was also observed in many stands in the western part of this province and in the area around Rotterdam. After 1980 the number of reports of dying and dead ashes increased dramatically.

Only in some of these reports, especially in some roadside and landscape plantings, a possible 'cause' could be indicated, including strong changes of the water table, salt spray damage, mechanical damage, and sites not suitable for ash. The vast majority of the ashes reported as dying, however, seemed to suffer from ash wilt disease.

In 1986 a questionnaire was sent to all district officers of the State Forest Service in order to gather information on site and management characteristics of affected stands. No relation was detected between occurrence of the disease and site conditions or management practices (Hiemstra, 1987a; Hiemstra *et al.*, 1990). Affected trees were reported on clay, loam, sand and even peat soils, in all kinds of plantations: roadside, amenity and recreational plantations as well as forest stands, both pure and mixed stands. Only coppice seems to escape from the disease. Some of the sites with affected trees are known to be unsuitable for ash, but the vast majority of affected trees grew on sites regarded as suitable.

Nursery trees can also be affected. In 1987 the disorder was found in some nursery beds with saplings between 4 and 6 years old. *F. excelsior* L. and several cultivars ('Westhof's glorie', 'Atlas', 'Diversifolia') are affected. No reports of the disease in *F. ornus* L. and other *Fraxinus* species have been received.

Fig. 2.5 shows the locations where trees with symptoms of ash wilt disease have been reported up to September 1992. The map is based on an inquiry sent to all district officers of the State Forest Service (Hiemstra, 1987a), reports received from several forest owners in the period 1986-1992, and observations by the author. Although the map certainly is not complete, the scarcity of reports of trees with symptoms of ash wilt disease in the province of Limburg is conspicuous because of the considerable ash forest area in that province (557 ha closed ash forest or 11 % of the total area in the

country; Appendix 1). However, almost all reports on dying ash trees concern young plantings in rather recently afforested areas. Hence, almost invariably the age of affected trees is less than 30 years. There are hardly any reports of ash wilt disease in older trees or of diseased trees in forest areas planted long ago (like many ash forests in Limburg).

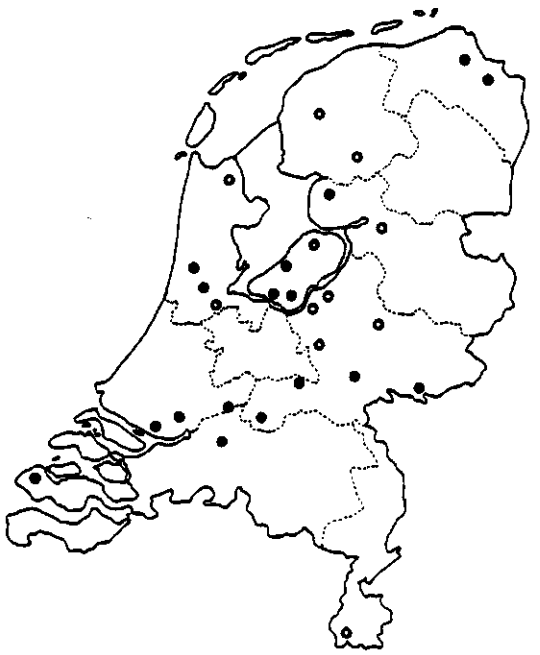

Fig. 2.5 Geographic distribution of ash wilt disease in the Netherlands as reported up to September 1992. Open signs represent individual plantations, dark signs represent several affected plantations.

2.4 Possible causes

At the start of the research in 1987 it was realized that the symptoms of ash wilt disease are very similar to those of some well-known wilt diseases, like Dutch elm disease, watermark disease, oak wilt and *Verticillium*-wilt. All

these diseases are caused by pathogenic micro-organisms. Hence, the involvement in ash wilt disease of a pathogenic organism appeared probable. However, routine checks of diseased ash trees by the Dutch Plant Health Service and the Research Station for Forestry in the years 1980-1986 had never consistently yielded a suspect pathogen (H.A. van Kesteren, pers. comm.; M. de Kam, pers. comm.). So it was decided to start the search for the cause of the disease with an evaluation of all factors that may cause diseases and disorders of trees (Table 2.2). The evaluation was largely based on the results of a preliminary study carried out in 1986. It was completed after some additional, short-term studies carried out in 1987.

Table 2.2 Man-made, abiotic and biotic agents which may promote[1] or cause disorders[2] and diseases of trees (Smith, 1970; Anon., 1982; Manion, 1991).

MAN-MADE AGENTS	
Management methods	- type of planting stock - soil treatment before planting - timing and method of planting, pruning, thinning or felling - use of fertilizers and biocides
Mechanical damage	- traffic - management activities - digging activities - vandalism
Air pollution	- acidification - eutrophication
Miscellaneous	- excess salt - leaking gas and oil pipes
ABIOTIC AGENTS	
Climatic factors	- precipitation extremes - temperature extremes - other climatic factors like lightning, wind, snow and ice
Site factors	- mineral deficiencies and excesses - water shortage and excesses

Table 2.2 (continued)

Fire	-	direct burning
	-	scorching
	-	scar effects
	-	ash and other chemical by-products of conflagrations
BIOTIC AGENTS		
Pathogenic micro-organisms	-	fungi
	-	bacteria
	-	viruses
	-	mycoplasmas, spiroplasmas and ricketsias
Animal feeding	-	nematodes
	-	insects and mites
	-	mammals and birds
Deleterious higher plants	-	parasitic plants
	-	lianas

[1] Including predisposing (cf. Schoeneweiss, 1975) or inciting (cf. Manion, 1991) factors.

[2] In using the terms 'disease' and 'disorder' the recommendation of the Federation of British Plant Pathologists (FBPP, 1973) is followed that the term 'disease' should apply only to malfunctions caused by pathogenic organisms or viruses, and that those caused by other factors should be termed 'disorders'.

2.4.1 Reduction of the number of possible causes

The preliminary study showed that the first two categories of possible causes as listed in Table 2.2 could be rejected. A number of the biotic factors could also be excluded such as injury by higher plants or feeding damage caused by larger animals. What is left is a disease caused by pathogenic micro-organisms or nematodes.

Analysis of the type of symptoms leads to the same conclusion. The early symptoms of ash wilt disease (i.e. sudden wilt, defoliation, necrosis, withering) indicate water stress in the tree. Water stress in plants may be caused by pathogens or by adverse environmental conditions. However, the preliminary study showed the latter to be an improbable cause of ash wilt disease. In ash wilt disease wilting therefore is likely to be caused by a

pathogen.

Two kinds of wilt diseases can be distinguished; one results from vascular infection and the other from root destruction (e.g. Krupa and Dommergues, 1979). The resemblance of the symptoms of ash wilt to those of vascular wilt diseases of trees, like Dutch elm disease and oak wilt, point to vascular infection as the cause of ash wilt disease. The anomalies observed during a wood-anatomical investigation (obstruction of vessels by gums and tyloses, occurrence of fungal hyphae and reduced xylem formation) also indicated a vascular infection (Miller and Hiemstra, 1987). In order to confirm this hypothesis, some short-term research projects were carried out, investigating the condition of the root system and the presence of fungal pathogens in the vascular system of diseased ash trees.

2.4.2 Condition of the root system

Three aspects of the root system were investigated; the condition of the fine roots, the presence of plant-pathogenic nematodes, and the presence of vesicular-arbuscular mycorrhizae (VA-mycorrhizae). For this purpose soil and root samples were collected in two permanent plots, one located in the province of Groningen in an ash stand on a sandy soil planted in 1973, the other located in the province of Flevoland in an ash stand on a loamy soil planted in 1977 (see Appendix III). Both stands were seriously affected by ash wilt disease in 1986, i.e. the year in which the plots were selected.

In the field no signs of rot in major roots were found. The condition of fine roots was assessed using samples from six diseased and five healthy ash trees in the plot in Flevoland. This revealed no major difference in the percentage of dead small roots between both groups (Hiemstra, Mihàlyi and Tuomela, unpubl. data). Hence, it was concluded that root rot is no prime factor in the ash wilt disease.

Samples from eight healthy and eight diseased trees from both plots were used in a survey on the presence of VA-mycorrhizae (Van der Vegt, 1988). In both plots, all trees examined possessed VA-mycorrhizae. The average mycorrhizal frequency was 39 % and 49 % for the samples from the plots in Groningen and Flevoland, respectively. No significant differences in the mycorrhizal frequencies of diseased and healthy trees or between the two plots were found.

Nematode populations were surveyed in combined soil and root samples taken near 36 diseased and 46 healthy trees (Denekamp, 1989), most trees being located in the two permanent plots. Because the soil type strongly affects the nematode population, some complementary samples were collected in two ash stands on different soil types. These stands were located on a clay soil in the province of Noord-Holland and a sandy soil in the province of Groningen. Finally ten trees were sampled in an ash stand on a loamy soil in the province of Flevoland which was completely free of ash wilt disease. Analysis of the soil samples showed the average numbers of plant-parasitic nematodes to be below the damage threshold for acute wilt symptoms. Still the average number of nematodes from some genera was higher in samples from diseased trees than in samples from healthy trees.

Especially interesting is the genus *Pratylenchus*, an endoparasite on many woody plant species. These nematodes are known for their synergistic action with some vascular wilt fungi (*Fusarium* spp., *Verticillium* spp.) which infect plants through their roots. On the average, in soil samples from diseased trees twice as much nematodes belonging to this genus were present as in samples from healthy trees in the same forest plot (averages of 41 versus 19 per 100 grams of fresh soil, respectively). Within each plot, however, the numbers varied strongly and the differences between healthy and diseased trees were statistically not significant.

The average number of saprophagous nematodes in soil samples from diseased trees was slightly higher than in samples from healthy trees (1000 versus 800 per 100 grams of fresh soil). This is attributed to the increased amount of organic material under diseased trees resulting from early defoliation. Again, there was a large variation in numbers and differences were statistically not significant.

Because of the above results it was concluded that the symptoms of ash wilt disease cannot be attributed to dysfunction of the root system resulting from lack of mycorrhizae or destruction of the roots by root rot or nematode attack.

2.4.3 Presence of vascular pathogens

In 1986, samples were taken from six trees affected by ash wilt disease (Hiemstra, 1987a). Three trees showed severe dieback of major branches (trees probably affected in 1985 or even earlier) and the three other trees

showed severe wilting (first affected in 1986). From a discoloured sector in the stem of the first three trees two *Fusarium* spp. and two *Phoma* spp. were isolated. All isolations from the wood outside this sector were negative. From the twigs and small branches of the trees with severe wilting almost every attempt led to the isolation of one and the same fungus, which at that time (Hiemstra, 1987a) was erroneously identified as a *Cephalosporium* sp.

In 1987 samples were collected from four recently wilted trees from three different locations (Hiemstra *et al.*, 1990). Isolations from wood samples taken from the stems of these trees in almost all cases proved to be negative, even when the stem contained a discoloured sector. From one- and two-year-old twigs of these trees, however, one fungus was isolated frequently. This fungus showed a great resemblance to the fungus that had been isolated from the twigs of affected trees in 1986. After proper identification, confirmed by CBS Baarn (Centraalbureau voor Schimmelcultures) it appeared that in 1986 as well as in 1987 *Verticillium dahliae* Kleb. had been isolated, which is a well-known wilt pathogen of many plant species, including several tree and shrub species (see Chapter 3).

2.4.4 Hypothesis

The above evaluation of possible causes led to the hypothesis that the ash wilt disease is caused by the vascular wilt pathogen *Verticillium dahliae* Kleb.

This hypothesis was based on the following four considerations:
1. *V. dahliae* was isolated from the twigs of several diseased trees.
2. This fungus is known to cause vascular wilts in several woody hosts.
3. The symptoms of ash wilt disease resemble those of a vascular wilt disease.
4. All other disease-causing agents (Table 2.2) can be rejected because the symptoms they cause do not resemble those of ash wilt disease, or because the results of the preliminary studies do not show a consistent association between the presence of these agents and the occurrence of ash wilt disease.

3 VERTICILLIUM WILT OF WOODY SPECIES; A LITERATURE REVIEW

In the previous chapter the hypothesis was proposed that the ash wilt disease is caused by *Verticillium dahliae* Kleb. This chapter provides information on the genus *Verticillium* and on wilt diseases of tree hosts caused by *Verticillium* species. Where appropriate, information concerning other hosts (i.e. herbaceous species, often agricultural crop plants) is included.

3.1 The genus Verticillium

The genus *Verticillium* is distributed worldwide with over a hundred species. Among them are saprophytic, plant-pathogenic, entomo- and hyperparasitic species. The genus was erected by Nees von Esenbeck in 1816 (*ex* Isaac, 1967). This genus of Hyphomycetes with predominantly hyaline vegetative hyphae is named after the morphology of its conidiophores. These are erect, septate and verticillately branched, bearing whorls of slender phialides (Fig. 3.1). The conidia, mostly cylindrical, one-celled and hyaline, are borne at the apices of the phialides in slimy heads or, exceptionally, in chains (Isaac, 1967; Domsch *et al.*, 1980).

The genus is strongly heterogeneous. The section Nigrescentia comprises all species with dark resting structures (Gams and Van Zaayen, 1982). Among them are the well-known plant-pathogenic species *V. dahliae* Kleb. and *V. albo-atrum* Reinke & Berth., some mainly saprophytic species like *V. nubilum* Pethybr., *V. nigrescens* Pethybr. and *V. tricorpus* Isaac, and *V. theobromae* (Turc.) Mason & Hughes that attacks banana causing 'cigar end' and internal rot of the fruit.

V. albo-atrum and *V. dahliae* are major plant pathogens. Diseases caused by these two species are widespread in both temperate and tropical regions. Some 300 cultivated crop plants in 43 families, including various of the world's major crops, are susceptible to them (Pegg, 1984). Since there are no effective therapeutic measures to control the disease, they constitute a real menace to world agriculture (Smith *et al.*, 1988). They are differentiated by the form of their resting structures. In *V. dahliae* these are dark brown or black microsclerotia. *V. albo-atrum* produces dark brown to blackish resting mycelium.

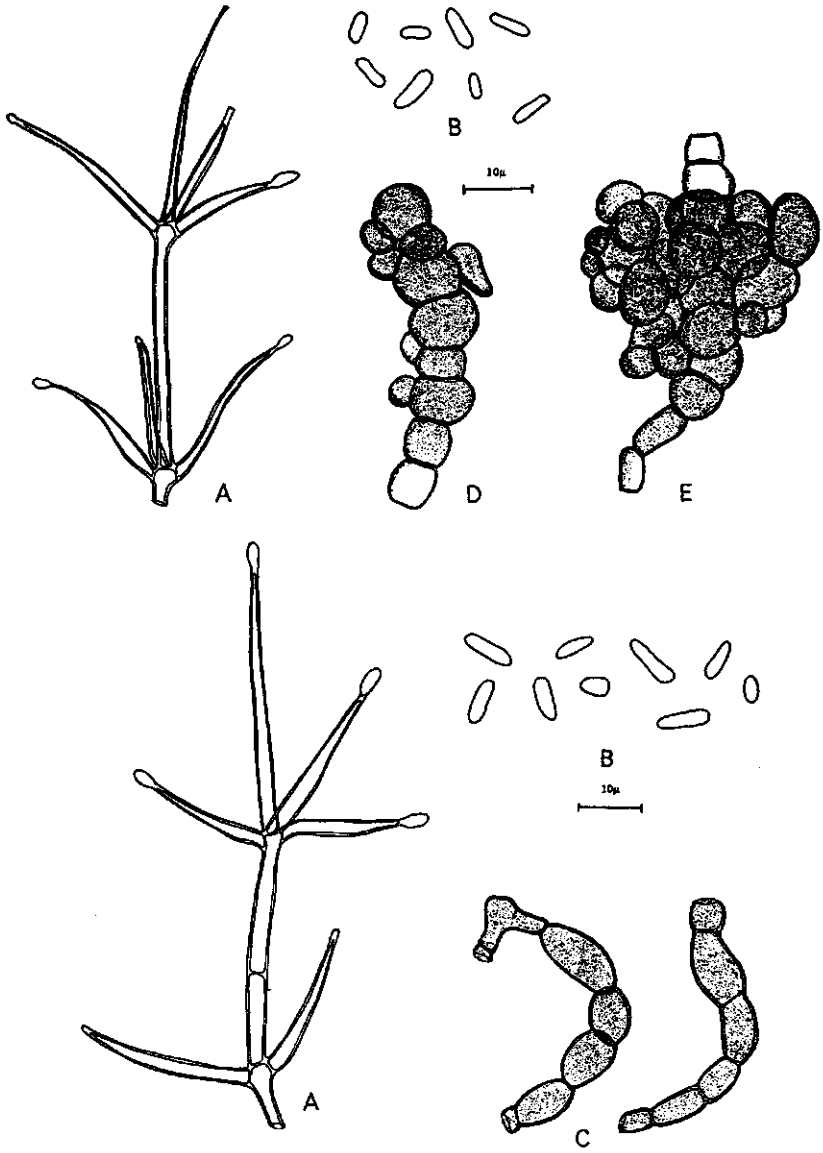

Fig. 3.1 Conidiophore (A), conidia (B) and resting structures (C, D, E) of *Verticillium dahliae* (top) and *Verticillium albo-atrum* (bottom). The horizontal bar represents 10 μ. Reprinted, with permission, from IMI (1970a,b).

Verticillium albo-atrum was first described in 1879 by Reinke and Berthold, who identified it as the pathogen causing potato wilt. *Verticillium dahliae* was first described by Klebahn after isolating it from wilting dahlias in 1913 (Isaac, 1967). Since 1913 the relationship between these two species has been the subject of much controversy as many authors considered them as one large, variable species, called *V. albo-atrum*. For this reason many reports attributing disease syndromes to *V. albo-atrum* certainly refer to *V. dahliae* (IMI, 1970a). In some cases this can easily be seen as microsclerotia are mentioned in the description of the fungus or because authors refer to the 'ms-type' (microsclerotial type) of *V. albo-atrum*.

Today, *V. albo-atrum* and *V. dahliae* are widely recognized as different species, not only because of their morphological differences, but also because of ecophysiological differences; i.e. differences in temperature reactions and pathogenicity (Isaac, 1967; IMI, 1970a,b; Domsch *et al.*, 1980). The optimum temperature for growth of *V. albo-atrum* is 21 °C, no growth occurs at 30 °C. For growth of *V. dahliae* the optimum temperature is 23.5 °C, and growth is still possible at 30 °C (Domsch *et al.*, 1980). The differences in optimum temperature are reflected in the geographical distribution of both fungi. *V. albo-atrum* is widespread in the temperate zone, while *V. dahliae* is present in the temperate as well as subtropical regions (Pegg, 1984; Smith *et al.*, 1988).

V. albo-atrum causes economically important diseases in lucerne, cucumber, hop, tomato and potato. It is, however, far less common than *V. dahliae* which has an extremely wide range of annual and perennial dicotyledonous hosts (Domsch *et al.*, 1980; Smith *et al.*, 1988). The woody hosts of *V. dahliae* will be reviewed in the next section. Among its herbaceous hosts are potato, tomato, pepper, aubergine, melon, watermelon, cotton, okra, strawberry, rapeseed, sunflower and artichoke. Monocotyledonous plants (e.g. wheat and barley) and many weeds have been reported as symptomless carriers.

Generally, isolates of *V. dahliae* or *V. albo-atrum* are not host-specific. Isolates from one host may usually cause disease in a wide range of other species and genera, and can infect still more without causing obvious symptoms (Smith *et al.*, 1988). It has frequently been shown that isolates from herbaceous hosts may also attack woody hosts (Van den Ende, 1958; Adams and Tattar, 1975; Donohue and Morehart, 1978). However, some host-specificity has been shown for *V. albo-atrum* isolates from lucerne and for *V. dahliae* isolates from Brussels sprouts, pepper and peppermint (IMI,

1970a,b).

Many plant pathologists have reported *V. albo-atrum* as a more virulent pathogen than *V. dahliae* in hosts susceptible to both pathogens, e.g. hop, cotton, lucerne and potato. For the woody host *Liriodendron tulipifera* L. this was confirmed by Morehart *et al.* (1980). However, differences in pathogenicity may occur within both *Verticillium* species (Isaac, 1967). Hence, 'the role of strains within an individual species assumes a greater importance than infection by one or other species in the same host' (Pegg, 1984; p. 178).

3.2 Woody hosts and symptoms

A large number of woody plants from very different genera are susceptible to Verticillium wilt. They all belong to the class of Dicotyledons. Woody Monocotyledons (e.g. palms) and Gymnophytes (= Gymnosperms) are not affected (Sinclair *et al.*, 1987). Older reports rarely differentiated between *V. dahliae* and *V. albo-atrum* (Section 3.1). In such cases, *V. albo-atrum* is mentioned as the pathogen causing Verticillium wilt in trees. However, from descriptions of the fungus, that are occasionally provided in such reports, it can be concluded that the pathogen in woody hosts usually is *V. dahliae* (Phillips and Burdekin, 1982). Moreover, Smith and Neely (1979) identified all 51 *Verticillium* isolates from 22 different tree and shrub species as *V. dahliae*.

In Europe (Phillips and Burdekin, 1982; Butin, 1983) as well as in Northern America (Sinclair *et al.*, 1987) the reports of Verticillium wilt in tree species almost invariably concern shade, fruit and ornamental trees or young trees in nurseries. In European forests, the disease has never been reported. The present literature review revealed only two recordings of Verticillium wilt in Northern-American forests, i.e. wilt of *Ceanothus interregimus* Hook. & Arn. (Harrington and Cobb, 1984) in a coastal forest in California caused by *V. albo-atrum* and wilt of *Liriodendron tulipifera* L. in urban and forest settings in Delaware caused by both *V. dahliae* and *V. albo-atrum* (Donohue and Morehart, 1978).

Indexes of woody hosts for *Verticillium* have been prepared for Northern America by Engelhard (1957), Himelick (1969), Carter (1975) and Sinclair and Hudler (1984). For Great Britain the woody hosts are reviewed by Piearce and Gibbs (1981). Verticillium wilts of fruit-tree species are listed by

Parker (1959). Besides these reviews numerous reports on isolation of *V. dahliae* or *V. albo-atrum* (often including *V. dahliae*) from one or more woody species exist. Among the most frequently affected trees are maples, especially *Acer platanoides*, *A. saccharum* and several Japanese maples, *Catalpa* species, and many fruit-tree species including apricot, avocado, cacao, cherry, olive, persimmon, pistachio and plum (Table 3.1).
Genera known not to be affected by Verticillium wilt include *Alnus*, *Betula*, *Fagus* (although isolation of *V. dahliae* from a *F. sylvatica* tree without foliar symptoms has been reported by Van Dam, 1991), *Platanus*, *Populus*, *Quercus* and *Salix*. Among the fruit trees *Malus* (apple) and *Pyrus* (pear) spp. usually are not affected.

The symptoms of Verticillium wilt in trees are rather unspecific. Hence positive diagnosis of Verticillium wilt can only be made by isolating the fungus from the diseased trees. Generally, the symptoms include three aspects: leaf symptoms (wilt, discoloration, defoliation), vascular symptoms (discoloration of the xylem and plugging of vessels) and decline (stunting and dieback). Detailed descriptions of Verticillium symptoms on woody hosts are presented by Himelick (1968 and 1969), Smith (1979), Piearce and Gibbs (1981), and Sinclair and Hudler (1984).

Although the symptoms in many trees have several traits in common, the specific symptoms often depend on the host species involved. In most tree species rapid wilting and defoliation are the typical symptoms of Verticillium wilt. Abscission of leaves may take place when still green, as in *Fraxinus americana* and *F. pennsylvanica* (Himelick, 1968), or after yellowing and scorching as in elm, Catalpa and maple species. The presence of other external symptoms like stunting, dieback of twigs and branches or sparse foliage depends on the host species too. Occasionally, sensitive trees, like maple and Catalpa, develop elongated dead areas of bark on stem and branches. These areas are contiguous with a sector of discoloured wood in the stem.

Within infected plants *Verticillium* often causes discolorations that appear as streaks or bands in the sapwood in radial and tangential cuts and as spots or partial to complete rings in one or more annual layers of the wood in cross cuts. The colour of the streaking varies with the species affected. In maples it is greyish green to olive-green, in many other species like Catalpa, elms and cherry trees it is brown, and tulip tree may have black streaks. In some other host species, including ash (Himelick, 1968), lilac (Van der Meer,

1925) and olive (Vigouroux, 1975), no discoloration at all or only a faint tan streaking may be visible.

Table 3.1 Woody plants reported to be susceptible to *V. dahliae*. Only the most common and well-known species are included, especially many ornamental shrubs have been omitted. Compiled from Engelhard (1957), Himelick (1969), Piearce and Gibbs (1981) and Sinclair and Hudler (1984).

Genus	Species mentioned
Forest and landscape trees	
Acer	*campestre, ginnala, macrophyllum, mono, negundo, nigrum, palmatum, pennsylvanicum, platanoides, pseudoplatanus, rubrum, saccharinum, saccharum, tataricum*
Aesculus	*hippocastanum* (occasionally)
Ailanthus	*altissima*
Castanea	*sativa* (occasionally)
Catalpa	*bignonioides, speciosa*
Cercis	*canadensis, siliquastrum*
Fraxinus	*americana, excelsior, nigra, pennsylvanica, quadrangulata*
Liriodendron	*tulipifera*
Magnolia	*grandiflora, soulangeana, stellata*
Robinia	*pseudoacacia* (occasionally)
Tilia	*americana, cordata, euchlora, glabra, parvifolia*
Ulmus	*americana, campestris, carpinifolia, fulva, glabra, montana, parvifolia, procera*
Fruit trees and shrubs	
Coffea	*arabica*
Olea	*europaea* (olive)
Persea	*americana* (avocado)
Pistacia	*vera* (pistachio)
Prunus	*amygdalus* (almond), *armeniaca* (apricot), *avium* (sweet cherry), *cerasus, cerasifera, communis* (almond), *domestica* (plum), *laurocerasus, lusitanica, mahaleb, mume, pennsylvanica, persica* (peach)
Rubus	*allegheniensis, idaeus, occidentalis, rosaefolius, ursinus*
Theobroma	*cacao*

Table 3.1 (continued)

Ornamental shrubs	
Amelanchier	*canadensis*
Berberis	*thunbergii, vulgaris*
Buxus	*koreana, microphyllum, sempervirens*
Cornus	*alba, florida*
Cotinus	*coggygria*
Daphne	*cneorum, mezereum*
Elaeagnus	*angustifolia*
Erica	*australis, persoluta*
Koelreuteria	*paniculata*
Ligustrum	*amurense, vulgare*
Lonicera	*morrowii*
Rhus	*aromatica, glabra, typhina*
Ribes	*sanguineum*
Rosa	*multiflora* and others
Sambucus	*racemosa* and others
Spiraea	*spp.*
Syringa	*vulgaris*
Viburnum	*burkwoodii, lantana, lentago, tinus, tomentosum*

Many tree species show extensive dieback after defoliation. This is often followed by the formation of adventitious shoots, especially from the base of the stem (traumatic reiteration; Oldeman, 1990b). With regard to the rate of symptom development and the extent of dieback in maple species, Piearce and Gibbs (1981) distinguish between three types of decline: acute wilt, chronic wilt and overwinter attack: 'In "acute wilt", all the foliage suddenly wilts and turns brown. The withered leaves remain attached to the tree which quickly dies. In "chronic wilt", leaf symptoms develop more slowly and do not always affect the whole tree. Defoliation is followed by dieback of certain branches. The tree may recover or may die over several seasons. In 'overwinter attack' trees which had appeared healthy in the autumn fail to produce leaves in spring.'

Although many affected trees die, recovery in the year after infection is not uncommon. Some trees even produce a new flush of leaves some weeks after defoliation (Himelick, 1968; Vigouroux and Castelain, 1969). Especially

in olive recovery has been reported frequently (Wilhelm and Taylor, 1965; Thanassoulopoulos *et al.*, 1979; Blanco-López *et al.*, 1984; Tjamos *et al.*, 1991). But also in other fruit-tree species, viz. cherry (Van der Meer, 1925), apricot (Taylor and Flentje, 1968; Vigouroux and Castelain, 1969), peach (Ciccarese *et al.*, 1990), and cacao (Emechebe *et al.*, 1974); and in ornamental trees like Catalpa (Carter, 1975) recovery of infected trees is well-known. Even in very sensitive maple species infected trees may recover (Sinclair *et al.*, 1981; Regulski and Peterson, 1983).

3.3 Disease cycle

The literature on the disease cycle of *V. dahliae* in agricultural crops is extensive, but in tree species it is limited. Therefore, this section has to draw heavily upon the results of research into Verticillium wilts of agricultural crops. Nevertheless, where available, data concerning tree hosts are included. Because Verticillium wilts of tree species almost invariably are caused by *V. dahliae*, this section concerns only *V. dahliae*, unless stated otherwise.

Generally, *V. dahliae* invades its hosts from the soil. Hyphae from germinating microsclerotia infect the young roots and, in plants not immune to vascular infection, grow into the xylem where they produce conidia. These are distributed through the plant by passive transport with the ascending sap stream. In the aboveground parts of living hosts *V. dahliae* is mainly restricted to the xylem vessels but when the host dies it invades other tissues where large quantities of new microsclerotia are produced that act as new inoculum.

In the disease cycle of *V. dahliae* (Fig. 3.2) several phases can be distinguished. Here, the disease cycle is divided in five phases; (1) survival and spread of the fungus, (2) germination of microsclerotia, (3) invasion of the root, (4) colonization of the vascular system, and (5) symptom development.

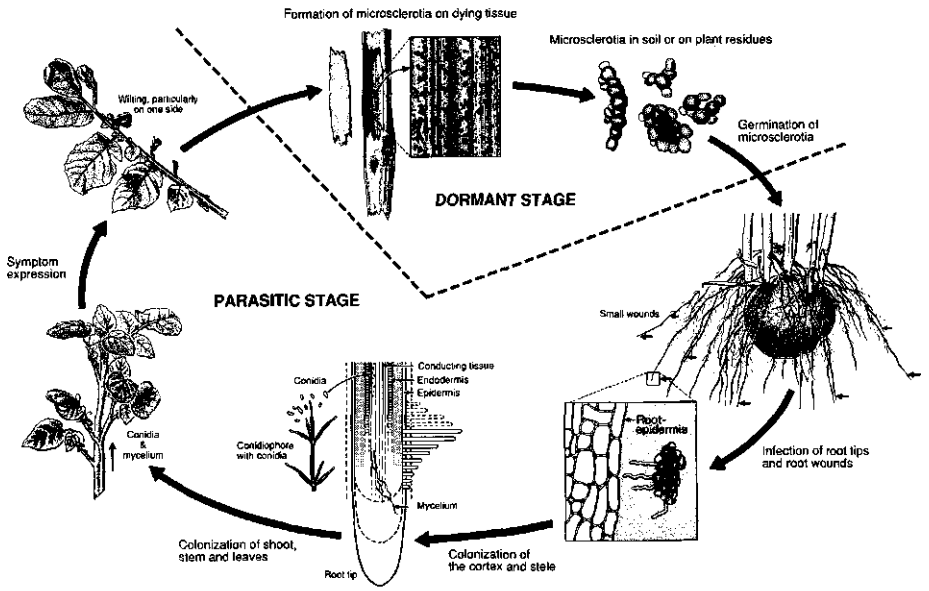

Fig. 3.2 Disease cycle of *Verticillium dahliae* in potato. Courtesy of M.P.M. Nagtzaam, Department of Phytopathology, Wageningen Agricultural University.

3.3.1 Survival and spread of the fungus

The abundance of *V. dahliae* is due to two major factors: (1) its very persistent resting structures, and (2) the very broad range of plants on which it can grow. In contrast to conidia and hyphae, which in soil lose their viability within a period of weeks (Schreiber and Green, 1962; Green, 1969), microsclerotia can withstand adverse and antagonistic soil conditions for a long time. Green (1980) demonstrated that microsclerotia in moist soil at 4 °C may survive for at least 5 years. Wilhelm (1955) reported that, in fields free of susceptible plants, populations of microsclerotia, although declining, remained viable for at least 14 years.

The persistance of soil populations of *V. dahliae* is strongly enhanced by the very broad host range of the fungus. The host range not only contains many agricultural crops (see previous sections), but also many weed species some

of which may act as symptomless carriers (Wooliams, 1966; Evans, 1971; Johnson *et al.*, 1980; Vargas-Machuca *et al.*, 1987). Besides, *V. dahliae* also has the capacity of superficial growth and production of new microsclerotia in the roots of many non-hosts, including some Gramineae (Martinson and Horner, 1962; Lacy and Horner, 1966; Krikun and Bernier, 1987) and Liliaceae (Malik and Milton, 1980).

V. dahliae forms microsclerotia in the organs of its hosts, especially leaves and herbaceous stems, when these become moribund. After incorporation into the soil the microsclerotia survive, first concentrated in host tissues and later free, after decomposition of these tissues. Debris of *Verticillium*-infected plants in this way can increase the soil inoculum of *V. dahliae*. This phenomenon is well-known in cotton (Benken and Khakimov, 1964; Evans *et al.*, 1966) and herbaceous agricultural crops like potato and tomato (Pegg, 1974). In tree hosts, microsclerotia may be formed in infected petioles, as has been shown for olive (Tjamos and Tsougriani, 1990). Development and longevity of microsclerotia vary strongly, depending upon soil temperature, soil and plant tissue moisture, and soil microbial activity (Nadavukaren and Horner, 1961; Brinkerhoff, 1969; Green, 1980; DeVay and Pullman, 1984).

Within cultivated fields, the fungus appears to spread primarily by the movement of infected plant tissue and infested soil during cultivation. Most microsclerotia are found in the upper layers of the soil (Wilhelm, 1950; Green, 1957). Because of their formation on dead plant material, many of them may also be found on the soil surface. Hence they can be spread by dust storms and excess irrigation or rain water causing surface run off (Easton *et al.*, 1969). They may also be dispersed from one field to another with wind-blown leaves of infected host plants like cotton. Blank and Leyendecker (1951) showed experimentally that incorporation of petioles from infected cotton plants into a field soil free of *V. dahliae* can cause serious infection in the next cotton crop. And Wilhelm and Taylor (1965) argued this mechanism to be the cause of serious Verticillium wilt in olive orchards that were not infected by *V. dahliae* before the presence of cotton on neighbouring fields.

V. dahliae may also be distributed from one area to another by means of infected planting stock or plant parts like potato tubers (Pegg, 1974) or fruits and seeds (Richardson, 1979) and weed plants (Evans, 1971). In tree nurseries, finally, the fungus may be transmitted by using scions, buds or rootstocks of infected plants (Sinclair *et al.*, 1987).

3.3.2 Germination of microsclerotia

Microsclerotia are composed of cells that vary in size, cell-wall thickness and degree of pigmentation. Schreiber and Green (1962, 1963) report that during germination the germ tubes arise from the thinner-walled, less-pigmented cells. They suggest that the cellular heterogeneity reflects physiological differences which may contribute to non-simultaneous germination of all viable cells when environmental conditions seem to be suitable. In combination with the ability to germinate repeatedly (Menzies and Griebel, 1967; Farley *et al.*, 1971), this strongly enhances the persistence of *V. dahliae* in soils.

In natural soils microsclerotia remain dormant until they are induced to germinate by root exudates. Only microsclerotia near a growing root respond to the exudates. Huisman and Gerik (1989) indicated that the effective sphere of influence of cotton roots is limited and at best is less than a millimetre wide. Schreiber and Green (1963) showed by fractioning of exudates of tomato roots that the stimulatory effect of the exudate is in the basic fraction, suggesting that amino-acids or other nitrogen-containing compounds are responsible for overcoming fungistasis. They also showed the effect of the exudates of some hosts to be stronger than that of other hosts. Nevertheless, the induction of germination is not specific for host plants, i.e. plants in which vascular infection may occur. It can also be caused by the exudates of the roots of plants immune to vascular infection, including monocotyledonous plant species (Lacy and Horner, 1966; Levy and Isaac, 1976; Fitzell *et al.*, 1980).

This may explain the often-reported beneficial effect of using monocotyledonous crops in rotations for Verticillium wilt control. Although the fungus may grow and even produce new microsclerotia on the roots of some wilt-immune species (Martinson and Horner, 1962), it was reported to die on the roots of most of them without producing microsclerotia (Evans, 1971). Hence, induction of germination of microsclerotia without abundant proliferation of microsclerotia on roots or aerial parts of wilt-immune plant species may be a major factor in decreasing the inoculum density of the pathogen (Schnathorst, 1981).

3.3.3 *Invasion of the root*

Because of the methodological difficulties inherent to investigations in a soil medium, fungal invasion of roots under natural conditions has been studied only to a limited extent. Most researchers studied colonization of plant roots under laboratory conditions. Others sidestepped the problem by directly introducing the pathogen into the xylem of the host. Nevertheless, a number of features concerning the invasion of roots have been established.

A detailed study by Garber and Houston (1966) showed that *V. dahliae* can colonize all parts of unwounded young cotton roots, from the root tip up to the hypocotyl. The cells of the root cap as well as the epidermal cells were readily penetrated either between or directly through the cells. The cortex was also colonized, again by inter- and intracellular growth. In the case of intracellular growth, appressorium-like structures and hyphae narrowed at the point of cell-wall penetration were observed. After penetration of the endodermis, some hyphae grew into xylem vessels. This was accomplished within three days. Penetration of vessels was invariably through a pit. Up to this point in the process of invasion no differences were detected between susceptible and resistant cotton varieties.

In tree hosts, the same pattern of invasion was observed under laboratory conditions in young roots of cacao (*Theobroma cacao*) by Emechebe *et al.* (1972) and in *Fraxinus pennsylvanica*, *Cercis canadensis* (both susceptible), *Gleditsia triacanthos* and *Platanus occidentalis* (both resistant) by Born (1974). In all these species penetration into the xylem vessels was observed within a week. Again, until that point of infection no differences were observed between susceptible and resistant species.

Observation of cotton roots showed that under natural conditions infection by *V. dahliae* occurs primarily near the root tip. No colonization was observed in the first millimetre behind the root tip but colony densities gradually increased over the next few millimetres, reaching maximum values about one centimetre from the apex, and remaining at this level further away (Gerik and Huisman, 1985). This may be explained by the high amounts of exudates in the zone of elongation and the combination of lower exudation rates and higher microbial metabolism on older roots (Gerik and Huisman, 1988; Huisman and Gerik, 1989).

The cortical infections remain localized, measuring longitudinally usually not more than two millimetres (Evans and Gleeson, 1973; Huisman and

Gerik, 1989). The colonies are randomly scattered with colony frequency per centimetre root being directly related to the inoculum density in the soil. Different plant species may yield different colony frequencies although this is not related to susceptibility (Evans and Gleeson, 1973; Lacy and Horner, 1966). Huisman and Gerik (1989) stated that the colonization frequency is probably a unique property of each combination of plant and fungal species. Especially on species immune to vascular infection most colonies apparently are superficial since they are very vulnerable to surface disinfectants (Evans and Gleeson, 1973). On host plants, however, Gerik and Huisman (1988) report preferential growth in the inner cortex.

In cotton roots, penetrating hyphae may be impeded by the inner tangential wall of epidermal cells, by gum-like deposits in the cell walls of cortical cells and by the endodermis (Garber and Houston, 1966). Generally, the endodermis is reported to have a major role in preventing invasion of the xylem (Talboys, 1958a; Garber and Houston, 1966; Born, 1974; Emechebe *et al.*, 1972). This results in large discrepancies between the frequencies of root colonization and those of systemic infection. In cotton, for example, a ratio of around 5000 cortical colonies to each systemic infection was consistently observed (Huisman and Gerik, 1989).

Thus, the extravascular tissues of the root contribute to the overall resistance of the plant by reducing the occurrence of vascular infections (Talboys, 1964; Beckman and Talboys, 1981). In wilt-immune plants (i.e. plants immune to vascular infection) this mechanism apparently is highly effective. However, in many other plants vascular infections do occur. From then on differences between resistant and susceptible host plants become apparent (Garber and Houston, 1966; Harrison and Beckman, 1982).

3.3.4 Colonization of the vascular system

Many workers investigating the distribution of *V. dahliae* and *V. albo-atrum* in infected plants observed sparse hyphal growth and large quantities of free-floating conidia inside the xylem vessels. Because the stems of infected plants also were often colonized much faster than could have been accomplished by hyphal growth, it was concluded that within the xylem the pathogen spreads primarily by means of conidia transported upward with the sap stream (Talboys, 1962; Garber and Houston, 1966; Presley *et al.*, 1966). Especially in woody hosts this mechanism is of great importance as it enables the fungus

to spread throughout the above-ground parts of its host well within one growing season, even in large trees. This would be impossible if the fungus had to rely on hyphal growth only.

In elm trees this mechanism has been clearly demonstrated by Banfield (1941). By adding coloured suspensoids to conidial suspensions he showed that in inoculated elm trees conidia of *V. dahliae* and *Ophiostoma ulmi* may ascend five feet in eight to fifteen seconds, and reach an upward distribution of 20 feet in less than ten minutes. The importance of the sap stream in the spread of *V. dahliae* within tree hosts was also demonstated by Dochinger (1956a) and Caroselli (1957) who showed that the distribution of the fungus was curtailed in trees that were defoliated immediately prior to inoculation.

The changed behaviour of *V. dahliae* after penetration of a vessel, i.e. production of conidia instead of profuse hyphal growth, is stimulated by the chemical and physical characteristics inside the vessel. It has experimentally been shown that low oxygen levels (Ioannou *et al.*, 1977) and a negative pressure potential (Duncan and Himelick, 1988) both stimulate conidial production. Within the vessels conidia are produced by microcycle-conidiation (a form of budding) or by simple conidiophores (Emechebe *et al.*, 1972; Schnathorst, 1981). Free-floating conidia eventually lodge at vessel ends or against protruding parts of the vessel wall like the rims of bordered pits, helical thickenings and perforation rims, where they may germinate. The hyphae then penetrate through bordered pits into adjacent vessels and produce secondary conidia (Beckman *et al.*, 1976).

The spread of the fungus may be limited by several resistance mechanisms of the host (Dimond, 1970; Beckman and Talboys, 1981; Bell and Mace, 1981; Beckman, 1987). The essential first step in resistance is trapping of the free-floating conidia as this provides time for the defensive mechanisms of the host to develop in front of the advancing pathogen (Beckman and Talboys, 1981). Trapping of conidia is achieved by the grid of lateral and end-walls of the vessels.

This might suggest a direct relation between vessel length and degree of articulation of the vessels at the one hand and susceptibility to vascular pathogens at the other hand. In elm trees (*Ulmus* spp.) this mechanism indeed contributes to resistance to *Ophiostoma ulmi*, the cause of Dutch elm disease (Elgersma, 1970 and 1982). However, Beckman *et al.* (1976) concluded that in different cotton varieties, although trapping of conidia is essential, resistance versus susceptibility to Verticillium wilt is not directly related to

vessel length or degree of articulation but is determined by different potential of the cultivars to respond actively to vascular infections.

The defensive response of infected plants has chemical (e.g. phytoalexins) and physical (e.g. vascular occlusion) aspects (Harrison and Beckman, 1982). Timing and spatial arrangement are the essential parameters that determine the outcome of the complex interactions between the offensive mechanisms of the vascular pathogen and the defensive mechanisms of its host (Beckman, 1984). In resistant-type interactions, gel plugs are formed rapidly above the trapping sites. These plugs cut off the water flow and embed the secondary conidia, thus immobilizing the parasite. By two to three days after initial infection of the xylem, the parenchymatous cells that ensheath the vessels produce tyloses which completely wall off the infected vessel above the trapping site (Beckman, 1987 and 1989). Finally, gel plugs, tyloses and surrounding cell walls become infused with several secondary metabolites like oxidized polyphenols (tannins) and phytoalexins that are exuded from paratracheal parenchyma cells (Mace, 1989). This causes vascular browning, a classic symptom of vascular infection. Because of their antibiotic nature, both compounds contribute chemically to the resistance of the occlusive structures against fungal action. Somers and Harrison (1967) showed that the main tannin fraction extracted from severely discoloured wood of apricot trees (*Prunus armeniaca*) greatly inhibited spore germination and hyphal growth of *V. dahliae in vitro* at a concentration well below that actually present in the infected wood.

The defensive reactions in susceptible hosts are essentially the same as in resistant ones, but in susceptible hosts the rates, intensities or spatial arrangement of these reactions are inadequate (Beckman, 1989; Mace, 1989). Because of the resulting incomplete immobilization of the pathogen many secondary infection sites develop where the contest starts over again. By this leap-frog movement the pathogen eventually may become systemic in the vascular xylem of susceptible hosts. This results in widespread local occlusions (Beckman, 1964) although not fast enough to contain the fungus completely. These occlusions are a major factor in symptom development (Subsection 3.3.5).

In perennial dicotyledons the confinement processes as described above are supplemented by a defensive response of the cambium. As long as infected trees are not killed, the cambium may respond to the presence of the pathogen by forming a specific tissue largely consisting of parenchymatous cells, the so-

called barrier zone, a tangential layer that serves to isolate or separate the infected wood on the inside from the healthy wood that continues to be formed on the outside. This mechanism is a general defense mechanism against injury and decay in tree species (Shigo, 1984) but has also been reported to take place after infection by vascular pathogens, including *V. dahliae* (Tippett and Shigo, 1981).

But even without formation of a barrier zone the ability to produce secondary xylem greatly contributes to resistance of woody hosts to vascular pathogens (Talboys, 1958b). If reinfection does not take place, infected trees may simply outgrow the disease because of the limited capacity of lateral distribution of *V. dahliae* (Talboys, 1968; Sinclair *et al.*, 1981). These two aspects explain the frequent recovery of trees infected by *Verticillium*, even after serious symptom development (Section 3.2).

Although the resistance of a host to vascular infection is largely due to its capacity of active response, its anatomical and physiological characteristics are of importance too. Hence, changes in these factors during the course of the life of a plant or within a growing season may affect the relative susceptibility of a plant. In the secondary xylem of woody species the vessel length and diameter depend upon the time of formation. In juvenile plants the vessels are smaller than in mature plants and in many tree species vessels formed early in a growing season (earlywood) are larger (longer as well as wider) than those formed later in the same growing season (latewood). Especially in ring-porous species like ash, elm and oak the differences are extremely large (Zimmermann, 1983). These changes in vessel sizes may affect the susceptibility to vascular pathogens by influencing the spread of conidia after initial penetration of the xylem (Banfield, 1941).

In elms (*Ulmus* spp.) resistance to Dutch elm disease is related to small dimensions of the vessels (Elgersma, 1970). Decreasing susceptibility of elm species to Dutch elm disease during the growing season also has been related to decreasing vessel dimensions (McNabb *et al.*, 1970). Verticillium wilt of tree species seems to be influenced by vessel dimensions in the same way. Holmes (1967) suggested a common resistance mechanism for both diseases since he found that elm clones resistant to Dutch elm disease were also the ones that were most resistant to Verticillium wilt. Rauscher *et al.* (1974) do not completely agree on this point. In their experiments with seedlings from three elm species and two clones from each of five different elm species or hybrid families the response to the two diseases corresponded only in one

highly resistant and two highly susceptible elm clones. Finally a seasonal decline in susceptibility to *V. dahliae* was reported for *Acer platanoides* and *A. saccharum* but not for *A. saccharinum* (Schreiber and Mayer; 1992).

Another important factor that may affect resistance to vascular infection is the composition of the xylem sap. Duncan and Himelick (1987) showed that the type of amino acids present affects conidial production and growth of *V. dahliae* on Czapek-Dox medium as well as on xylem sap of *Acer saccharum* amended with individual amino acids. Regulski and Peterson (1983) showed that xylem sap collected from five different maple species varies in ability to support growth and conidial production of *V. dahliae* and that seasonal variations exist in this ability.

Resistance, however, is not a direct result of the composition of the xylem sap. Xylem sap of boxelder (*Acer negundo*), a species known to be rather resistant to *Verticillium* in the field, also supported growth of *V. dahliae*. Moreover, Kessler (1966) showed that *V. dahliae* can grow very well on the xylem sap of non-hosts like *Betula lenta* and *Prunus serotina*. Sap of a very sensitive host (i.e. *Acer rubrum*) even was not the most favourable for growth of the fungus. Nevertheless, seasonal variations in the composition of xylem sap may contribute to the seasonal variations in susceptibility and in internal inoculum densities in maple (*Acer* spp.) as reported by Schreiber and Mayer (1992). Changing quantities of nutrients probably also explain why in annual hosts like cotton (Presley and Taylor, 1969), tobacco, chrysanthemum and potato (Pegg, 1974) symptoms usually do not develop until the onset of flowering or tuber formation.

3.3.5 Development of the disease syndrome

During the last four decades many researchers were involved with pathogenesis of vascular wilt diseases. This has resulted in many reviews on factors and processes that contribute to development of the disease syndrome, e.g. Ayres (1978), Beckman (1964, 1984 and 1987), Pegg (1974), Schnathorst (1981), Talboys (1968 and 1972), Van Alfen and MacHardy (1978) and several contributions to two recent volumes on vascular wilt diseases edited by Mace, Bell and Beckman (1981) and Tjamos and Beckman (1989).

It is generally agreed that a disturbance in the water balance in infected plants is at least partially responsible for the disease syndrome caused by *V. dahliae* and other wilt pathogens. The cause of this internal water stress,

however, has been subject to much debate. Two conflicting theories have been advanced; limited water flow caused by vascular occlusion and increased loss of water resulting from toxicosis.

The theory of limited water flow in infected plants is supported by the observation that in the early stage of the disease the symptoms are reversible. They often disappear during the night or after infected plants have been provided additional water (Caroselli, 1959; Duniway, 1973). Besides, vascular discoloration and plugging of vessels are generally recognized features of wilt diseases. Thus in many reports on vascular wilts it has been concluded that the wilt symptoms result from water stress caused by vascular occlusion which leads to disruption of the internal water transport.

Other authors argued that the symptoms of wilt diseases result from increased loss of water caused by a toxic effect of fungal metabolites on membrane permeabilities and stomatal function in leaves. Several fungal toxins have been identified and it was shown that culture filtrates of pathogenic vascular fungi may cause symptoms of wilt and desiccation in many plant species (Talboys, 1968; Pegg, 1974, 1981 and 1989). For *V. dahliae* isolated from woody hosts this was shown, amongst others, by Van der Veen (1930), Caroselli (1955) and Van den Ende (1958). Hence many authors accepted the view that 'wilt, desiccation and dieback are primarily attributable to fungal toxins, while the internal discoloration is largely an expression of host response' (Piearce and Gibbs, 1981; p.4).

Nowadays, however, most authors support the theory of vascular occlusion. In a literature review on water relations in wilt diseases caused by *Ceratocystis*, *Fusarium* and *Verticillium* spp. Hall and MacHardy (1981) conclude that desiccation of leaf tissue results from reduced flow of water to leaves caused by an increased resistance to liquid flow in the xylem of infected plants. Beckman (1987, p. 101), after extensively reviewing the literature, concludes that 'the concept of toxins "acting in advance" to cause the final wilt syndrome not only has turned out to be invalid but has distracted us from the main events that occur early in the interaction' (i.e. between the pathogen and its host). This view now seems to be generally accepted (Ayres, 1978; Van Alfen, 1989; DeVay, 1989).

For explaining symptom development in trees infected by *Verticillium* it is necessary to go back to the phase of colonization of the xylem (Subsection 3.3.4) and to analyze the differences between susceptible and resistant hosts.

As Beckman (1984) has pointed out, especially the timing and location of the defensive reactions of the host are of crucial importance in the interaction between pathogen and host. Another important aspect is that in vascular wilts the pathogen remains within the non-living conducting elements of the xylem until the disease has reached an advanced stage. Hence, the distribution of the pathogen within its host strongly depends on the number of entries into the vascular system and on the anatomical architecture of the host xylem (Talboys, 1968).

The vessels in the stem of a dicotyledonous tree form a complex branching and anastomosing network. This design contributes significantly to the safety of water conduction by providing a network of alternative pathways. Because of the network structure with its many interconnections, local obstructions of the sap stream are easily overcome by lateral transfer of water and increased flow in intact vessels (Zimmermann, 1983). Studies of water movement around sawcuts provide clear evidence of this. If two cuts are made, halfway across a tree trunk and from two sides, it is often found that water transport is not interrupted and that the tree survives. Emechebe *et al.* (1974) showed that most of the 15-week-old cacao seedlings (*Theobroma cacao* L.) treated this way remained turgid even if the two cuts were only 6 centimetre apart.

Hence limited loss of transport capacity after local occlusion as in resistant plants does not necessarily cause wilt symptoms. In susceptible hosts, however, the many widespread local occlusions eventually clog the vessel network and symptoms of water stress develop. If the pathogen reaches the petioles of these plants, the effect is even stronger as the vessels here are much smaller, i.e. more easily plugged, and bypasses are barely available (Talboys, 1968). The water stress leads to wilt, desiccation and defoliation. If the plugged vessels are not replaced by new uninfected xylem, dieback symptoms even may result.

In the above process fungal metabolites may be of influence too. But generally their role is limited to the direct environment of the fungus (Beckman, 1987; Bishop, 1988). Here, on the one hand, they are involved in inciting the defensive reactions of the host. On the other hand, they may counteract them as well. Especially fungal enzymes may interfere with the formation of gel plugs in infected vessels. Two types of toxic fungal metabolites should be distinguished: metabolites of low molecular weight ('real' toxins) and high molecular weight substances, particularly polysaccharides and glycopeptides. The latter group of fungal metabolites may be considered as physiologically

clogging the vessels by plugging the pores in the pit membranes rather than being toxic (Bishop, 1988; Sutic and Sinclair, 1991). This may explain why culture filtrates of vascular pathogens can induce symptoms of wilt diseases (Van Alfen, 1989).

Callose layers and other deposits on cell walls are also important aspects of the host-pathogen interaction (Bishop and Cooper, 1983 and 1984; Street *et al.*, 1986; Newcombe and Robb, 1989). On the one hand, these deposits are part of the defensive mechanisms of the host. By sealing off the vessel walls (including the pit membranes) they assist in localizing the pathogen. On the other hand, because of the same mechanism, they contribute to the development of water stress inside the plant.

Several more host and pathogen factors have been claimed to play a role in the complex interaction between the pathogen and its host, including plant growth hormones (especially IAA, ABA and ethylene), phenolic substances and phytoalexins. These factors are extensively reviewed in several contributions to two recent volumes on vascular wilt diseases edited by Mace, Bell and Beckman (1981) and Tjamos and Beckman (1989).

3.4 Control of the disease

Development of disease symptoms is the final outcome of the complex interactions between the pathogen and its host. Environmental and management factors strongly affect these interactions either by their influence on the pathogen or by predisposing the host toward greater susceptibility (Schoeneweiss, 1975). In the first part of this section the most important factors will be discussed. In the second part some remarks will be made on how disease can be controlled by manipulating environmental or host factors.

3.4.1 The effect of environmental factors

Extensive information on factors that influence disease incidence or symptom development in Verticillium wilts can be found in reviews prepared by Bell (1973), Brinkerhoff (1973), Pegg (1974), Schnathorst (1981) and Katan (1989). The most important factors that emerge from these reviews are previous land-use, other soil organisms, soil characteristics and weather conditions.

Previous land-use is of great importance because it determines to a great extent the soil inoculum level of *V. dahliae* for the subsequent years. In annual crops like cotton (Huisman and Gerik, 1989) and potato (Nicot and Rouse, 1987), disease incidence is primarily related to the soil inoculum level of *V. dahliae*. This also holds for cotton (Blanco-López *et al.*, 1989) and pistachio (Ashworth and Zimmerman, 1976). Many authors related severe outbreaks of Verticillium wilt in orchards of olive, pistachio and several *Prunus* spp. to increase of the soil inoculum of *V. dahliae* by cropping of susceptible annual crops like potato, tomato and cotton in the years before planting of the trees, or by intercropping of orchard trees with these crops (Snyder *et al.*, 1950; Wilhelm and Thomas, 1950; Wilhelm and Taylor, 1965; Ashworth and Zimmerman, 1976; Ndubizu, 1977; Thanassoulopoulos *et al.*, 1979; Saribay and Demir, 1984).

Cultivation practices are of importance too, especially by aiding the distribution of the fungus or by wounding of the roots of the host. As *V. dahliae* usually enters its host through the root, the additional infection courts provided by wounding of roots (e.g. by tillage), may increase disease incidence. Under experimental conditions this was confirmed for maple (Caroselli, 1957) and cacao trees (Emechebe *et al.*, 1975). Vigouroux and Castelain (1969) reported that in apricot orchards with increasing frequency of soil cultivation disease incidences also increased.

V. dahliae may be carried from one tree to the next by contaminated tools like axes, saws or increment borers (Dochinger, 1956b). Sinclair *et al.* (1981), however, considered contamination resulting from pruning activities of no practical importance as the downward movement of the fungus is only a small fraction of its upward movement, if any. The use of infected planting stock is another way in which management practices may aid distribution of the fungus. It has been reported that young trees may be infected but remain symptomless until after transplantation from the nursery grounds (Sinclair *et al.*, 1981; Smith, 1983).

Among soil organisms, especially fungi and nematodes are known to influence the incidence of Verticillium wilts (Bell, 1973; Brinkerhoff, 1973; Pegg, 1974). Antagonistic action of fungi and synergistic action of plant parasitic nematodes to *V. dahliae* in agricultural crops have been reported by many researchers. For tree hosts such interactions were reported by Dwinell (1967), Ndubizu (1977), Smith (1979) and Sinclair and Hudler (1984). Migratory nematodes in particular, e.g. *Pratylenchus* spp., and root knot nematodes

(*Meloidogyne* spp.) are often reported to act synergistically with *Verticillium* spp.

Another group of important soil organisms are the earthworms. They do not directly influence the host plant or the pathogen. However, because of their feeding habits they strongly mix and aerate the soil. Ndubizu (1977) showed that microsclerotia of *V. dahliae* are capable of passing through earthworms without losing infectivity. Hence distribution of *V. dahliae* within the soil is influenced by the action of earthworms. Of even more importance is their habit to incorporate dead plant parts, that may contain microsclerotia of *V. dahliae*, into the soil.

Of the soil characteristics especially soil moisture and nitrogen level are important. Low soil moisture has repeatedly been reported to increase symptoms in infected trees (Caroselli, 1954 and 1957; Phillips and Burdekin, 1982; Morehart and Melchior, 1982). High nitrogen levels are reported to increase disease incidence as well as intensity of symptoms (Van der Meer, 1930; Dochinger, 1956b; Dwinell, 1967; Phillips and Burdekin, 1982; Sinclair and Hudler, 1984).

Some other authors, however, disagree by stating that the type and amount of fertilizer applied does not seem to be related to disease incidence or intensity (Caroselli, 1957; Ndubizu, 1977; Smith, 1983). An explanation for this apparent contradiction has been forwarded by Phillips and Burdekin (1982, p. 72) who stated that 'with increasing nitrogen additional growth of the tree may mask the external symptoms'.

Disease incidence has been reported to be reduced by high potassium levels (Dwinell, 1967; Bell, 1973; Smith, 1983). Soil texture and pH seem to be relatively unimportant. In a review of the environmental effects on Verticillium wilt Schnathorst (1981) mentions that in cotton the disease is not limited to alkaline clay soils as stated by many early reports, but that it in more recent years also has been reported on sandy soils and on neutral to acid clay soils.

Temperature and rainfall are important climatic factors in Verticillium diseases. Rainfall influences soil moisture and temperature and running off surplus water may distribute microsclerotia (Subsection 3.3.1). The effect of temperature is related to the temperature requirements of *V. dahliae*. Low soil temperatures retard germination of microsclerotia and growth of hyphae, hence disease development is inhibited. High soil temperatures may kill the

fungus and lead to reduced inoculum densities in soil. This principle is used in solarization of soil (Subsection 3.4.2).

Air temperatures over 28 °C have been related to increased resistance of host plants and holding off or reduction of symptoms in many agricultural crops (Brinkerhoff, 1973). In tree species recovery of infected trees during hot summer periods has been reported for apricot (Taylor, 1963; Taylor and Flentje, 1968), avocado (Zentmeyer, 1949) and olive (Wilhelm and Taylor, 1965; Vigouroux and Castelain, 1969). Finally, Smith and Neely (1979) reported decreased susceptibility of several tree species during the hot and dry period in July.

3.4.2 Strategies for control

There are no therapeutic measures to control the diseases caused by *V. dahliae* and *V. albo-atrum* (Smith *et al.*, 1988). Hence the measures practised in management of these diseases aim at reducing the amount of soil inoculum and preventing infection of susceptible plants. In agricultural crops this is achieved by cultural practices including the use of resistant crop cultivars, rotations with grass crops, avoiding excess irrigation and excess nitrogen fertilization, soil solarization and fumigation (DeVay and Pullman, 1984). Unfortunately, most of these measures generally are not applicable for management of Verticillium wilt in tree species, except on nursery grounds.

For control of Verticillium wilts in tree species prevention, i.e. use of disease-free plants in disease-free soil, is the most important measure. Hence, in growing planting stock of susceptible species, fields where previously susceptible crops like potato, tomato, cotton and eggplant have been grown should be avoided (Smith, 1979). Occasionally infested fields may be grassed down or left fallow for several years in order to decrease the soil inoculum (Phillips and Burdekin, 1982; DeVay and Pullman, 1984). Additional control measures on slightly infested fields include chemical control of weeds, balanced fertilization and prevention of water excess and shortage. In order to prevent the build-up of soil inoculum, infected plants and plant debris should be removed and destroyed immediately. Soil fumigation can be effective but, because of the costs, only in high-value crops.

The only option for management of Verticillium wilt in established trees in orchards and landscape plantings is to promote natural recovery. For this purpose, generous watering and fertilization with balanced fertilizers or a

formulation containing abundant potassium are recommended (Himelick, 1968; Smith, 1979; Piearce and Gibbs, 1981; Smith, 1983; Sinclair and Hudler, 1984). In one case soil amelioration with wood shavings has been reported to be effective (Wilhelm and Taylor, 1965). Chemical control of disease in infected trees is not a practical approach (Smith, 1979). Surface-acting fungicides do not reach the fungus because of its internal location, and application of systemic fungicides to the soil or direct injection into symptomatic trees also have not been successful (Talboys, 1984), probably because by the time of symptom development the widespread vascular occlusions also prevent distribution of the chemical (Born, 1974).

Dead trees should be removed and burnt. Several authors also advise pruning and burning of all dead branches from infected trees (Himelick, 1968; Smith, 1979; Phillips and Burdekin, 1982; Sinclair and Hudler, 1984). This measure, however, does not remove the fungus from an infected tree (Tattar, 1978). At the best, pruning of dead and symptomatic branches may support recovery because the wound-associated compartmentalization processes supplement the tree's ability to localize xylem infections (Sinclair *et al.*, 1981).

In areas with high summer temperatures, soil solarization (by covering the soil with transparent polythene sheets during summer) has been shown to be very effective in reducing the soil inoculum of *V. dahliae* (Katan, 1981). In orchards of several fruit tree species, including olive (*Olea europaea*), pistachio (*Pistacia vera*), almond (*Prunus dulcis*) and avocado (*Persea americana*) this treatment has proved to be beneficial without causing serious damage to the trees (Ashworth and Gaona, 1982; Tjamos, 1991; Tjamos *et al.*, 1991). Because of the strongly decreased soil inoculum level little reinfection takes place in the first years after solarization and recovered trees stay healthy. However, the treatment has to be repeated every three to five years.

In forests none of the above measures are practicable. Here the only one method for controlling Verticillium disease is prevention, which means use of disease-free plants in disease-free soil. If this is not possible, the only alternative is use of resistant species or varieties.

4 ASH WILT DISEASE; ONE MORE VERTICILLIUM WILT

4.1 Introduction

Preliminary trials (see Chapter 2) suggested that the ash wilt disease was associated with *Verticillium dahliae* Kleb., a widespread soil-borne pathogen prevalent in many agricultural crops in the Netherlands. The extremely wide host range of this fungus includes annual and perennial dicotyledonous plants (Smith *et al.*, 1988) including *Fraxinus* spp. (Sinclair *et al.*, 1987).

However, Verticillium wilt in European ash trees in landscape plantings and forest stands has never been a problem so far. Also most ash trees affected by ash wilt disease did not show discoloration of the wood which is a common symptom in Verticillium wilts of other tree species (Piearce and Gibbs, 1981). Besides, during routine checks of diseased ash trees by the Plant Protection Service and the 'Dorschkamp' Research Institute for Forestry and Landscape Planning in the early eighties, *V. dahliae* was isolated only once (H.A. van Kesteren, M. de Kam; 1987, pers. comm.).

Therefore new experiments were necessary to determine whether or not the disease is caused by *Verticillium dahliae* Kleb. The objectives of the experiments were:

1. To investigate if *V. dahliae* is consistently associated with ash wilt disease.
2. To examine the pathogenicity of *V. dahliae* in healthy ash trees.
3. Re-isolation of *V. dahliae* from inoculated trees after development of symptoms of ash wilt disease.

4.2 Isolation of *Verticillium dahliae*

First an isolation experiment was carried out on two groups of trees in order to develop and test the method of isolation. Thereafter a survey of 150 ash trees including healthy trees and trees affected by ash wilt disease was carried out.

4.2.1 Material and methods

Group 1
In August 1987, five trees from different locations showing severe symptoms of ash wilt disease were examined. The trees were six to thirteen years old and belonged to different genetic types (Table 4.1, group 1). Samples of 20 cm in length were taken from both stem and branches of each tree. Also some roots and leaves were sampled. In the laboratory a subsample was cut from the centre of each sample. The stem subsamples consisted of 2 cm thick disks. Branch and root subsamples were about 10 cm long. From all subsamples the bark was peeled off in order to prevent contamination of the wood by fungi and bacteria living in and on the bark of trees. The superficial part of the transverse surface of stem samples that might have been contaminated during cutting of the subsample was also removed using a sterile scalpel. Small chips (< 5 mm) were cut under sterile conditions from the exposed transverse surface (stem samples) or tangential surface (branch and root samples). The chips were placed on a malt agar or potato dextrose agar medium in plastic Petri dishes and incubated at 20 °C in the dark. Plates were inspected regularly. The leaf stalks were surface-sterilized by flaming after dipping them in 70 % alcohol. Then small parts were cut from the leaf stalks and treated like the other samples.

Group 2
In August 1988, sixteen 5-to-20-year-old ash trees (Table 4.1, group 2), varying in disease intensity, were examined. One branch was sampled of each tree. In most cases only the current year's shoot was sampled but from some trees both the current and last year's shoot were sampled. Of each sample four wood chips were placed on malt agar as described for group 1.

Survey
In September 1988, 150 ash trees in forest stands and landscape plantations were sampled in a nationwide survey. Fig. 4.1 shows the locations. In each area both diseased and healthy trees were sampled. Only vigorous dominant or co-dominant trees were sampled. The age of the trees ranged between 10 and 25 years. Samples of about 30 cm length were taken from the last year's shoot of three branches per tree in the upper part of the crown. Samples were stored in plastic bags at 5 °C until processing (usually the next day, at the latest within one week).

Table 4.1 Data on 21 ash trees sampled during development (group 1) and testing (group 2) of the method used for isolating *V. dahliae* from diseased trees.

Number of trees	Location	Age (years)	Type
GROUP 1			
1	Horsterwold-1	12	seed-grown *F. excelsior*
1	Horsterwold-2	13	seed-grown *F. excelsior*
1	Rotterdam-1	2 + 6	'Diversifolia'[1]
1	Rotterdam-1	2 + 6	'Westhof's Glorie'[1]
1	Rotterdam-2	6	seed-grown *F. excelsior*
GROUP 2			
3	Rotterdam-3	20	'Atlas'[1]
5	Rotterdam-4	8	seed-grown *F. excelsior*
8	Walcheren	5	'Eureka' and 'Westhof's Glorie'[1]

[1] All cultivars grown on *F. excelsior* seedling rootstock.

In the laboratory 10 cm long subsamples taken from the centre of each sample were washed under running tap water after which the bark was peeled off in a laminar air flow cabinet. Small chips from the tangential wood surface were placed onto malt agar (4 chips per plate, 1 plate per branch) and incubated at 23 °C in the dark. Plates were inspected after 10 and 21 days.

The state of health of the sampled trees was recorded using a code indicating the presence and intensity of disease symptoms. Based on this code the trees were classified into six disease classes, ranging from healthy to dead (cf. Table 4.3). The details about the symptom code and the conversion into disease classes are given in Appendix 1.

4.2.2 Results

Group 1

V. dahliae was isolated from four of the five trees. It was most frequently isolated from the last year's part of a branch but also frequently from the current year's part and once from a leaf stalk. It was not isolated from root

and stem samples, not even from discoloured wood, if present (Table 4.2).

Group 2

V. dahliae was isolated from all trees with distinct symptoms of ash wilt as well as from the trees that had been qualified as dubious (Table 4.3). It was also isolated from three out of five trees with slight symptoms of ash wilt, but not from the one healthy tree that was examined. If the current and the last years's part of a branch were compared, *V. dahliae* was more frequently isolated from the last year's part of the branch.

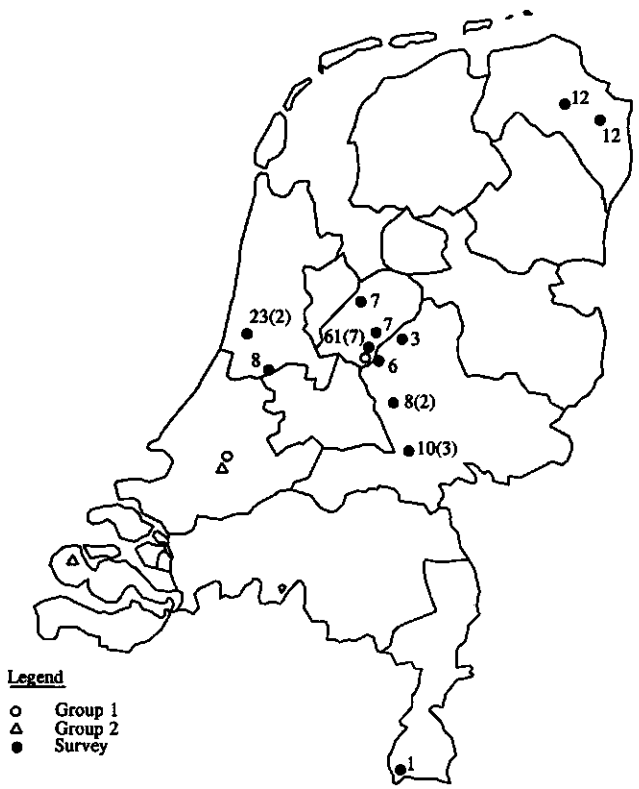

Fig. 4.1 Location of the trees examined for the presence of *Verticillium dahliae* during the trials (group 1 and 2, see text) and during the survey. For the survey the number of sampled trees per location is indicated and (if more than 1) also the number of sampled stands (in brackets).

Table 4.2 Results of isolation of *Verticillium dahliae* (Vd) from several parts of 5 young ash trees (group 1) with severe symptoms of ash wilt disease (Disease class 3).

Sampled part of tree		Number of sampled trees	Number of chips placed on agar	Number of Vd-positive chips[1]
Root		2	16	0
Stem and branches	discoloured sector	1	22	0
	discoloured spots	5	33	0
	wood without discolouration last growth ring	4	53	0
		3	46	0
Twigs	two year old	2	22	11 (2)
	one year old	4	55	37 (4)
Leaf stalks		1	8	1

[1] Between brackets the number of trees from which the Vd-positive chips originated.

Table 4.3 Results of isolation of *Verticillium dahliae* (Vd) from twigs (1 per tree) of 16 young ash trees (group 2) with symptoms of ash wilt disease of varying intensity.

Disease Class	Number of trees	
	Sampled	Vd-positive
0 (healthy)	1	0
1 (dubious)	2	2
2 (slight symptoms of ash wilt)	5	3
3 (distinct symptoms of ash wilt)	8	8
4 (dieback)	0	-
5 (dead)	0	-

Survey

From 150 trees that were sampled during the survey 37 were classified healthy (Disease Class 0), 18 were classified dubious (DC 1), 2 showed only slight symptoms (DC 2), 89 showed distinct symptoms (DC 3) and 4 trees showed partial dieback (DC 4). *V. dahliae* was isolated from 63 % of the trees with distinct symptoms of ash wilt (DC 3) and 10 % of the trees in disease classes 1 and 2. It was never isolated from healthy trees nor from trees with partial dieback. The trees from which *V. dahliae* was isolated included trees from all sampling areas.

Three other fungi (*Phoma macrostomum* Mont., *Phoma pomorum* Thüm. and *Aureobasidium pullulans* (de Bary) Arnaud were frequently isolated from trees of each disease class (Table 4.4). Several other fungi were much less frequently isolated, including *Coniothyrium sporulosum*, *Microsphaeropsis olivacea*, a *Papulospora* sp., *Phoma enteroleuca* var. *enteroleuca*, *P. exigua*, *P. viburnicula*, a *Phomopsis* sp., a *Pleurophoma* sp. (*'coral luteous'*), and a *Septoria* sp.

Table 4.4 Fungi isolated most frequently from 150 survey trees; number and, between brackets, frequency per disease class.

	Disease class[1] (DC)				
	0	1&2	3	4	1-4
Total number of trees per DC	37	20	89	4	113
Trees positive for					
Verticillium dahliae Kleb.	0 (0)	2 (10)	56 (63)	0	58 (51)
Phoma macrostomum Mont.[2]	12 (32)	5 (25)	24 (27)	2	31 (27)
Phoma pomorum Thüm.	7 (19)	2 (10)	18 (20)	2	22 (19)
Aureobasidium pullulans (de Bary) Arnaud	2 (5)	4 (20)	14 (16)	0	18 (16)

[1] See table 4.3 for the meaning of the disease class codes.

[2] Var. *macrostomum* and var. *incolorata* (Boerema and Dorenbosch, 1970).

4.2.3 Discussion

Sampling method

The aim of the survey was to investigate if *V. dahliae* is consistently associated with ash wilt disease. Therefore healthy as well as diseased trees had to be sampled. Since monitoring of further development of these trees was envisaged for a subsequent experiment, non-destructive sampling was needed.

Preliminary investigations (see Chapter 2) suggested that *V. dahliae* was most easily isolated from twigs and young branches. Examination of several parts of a first group of trees (Table 4.2) confirmed this finding. Again *V. dahliae* was only isolated from twigs and young branches. This corroborates the findings of Himelick (1968) who stated that in cases of Verticillium wilt of trees only samples from living branches with actively wilting leaves are suitable material for laboratory diagnosis. Thus, after another test with similar results (Table 4.3), it was decided to sample branches.

Only the last year's part of the branches was sampled because this part gave best results during the trial experiments. In order to obtain a higher degree of certainty, three branches per tree were sampled. No samples were taken from dead branches (DC 4) or completely dead trees (DC 5) because dead wood is usually invaded by many secondary (saprophytic) fungi other than the pathogen that killed the tree (cf. Butin and Kowalski, 1986; Griffith and Boddy, 1988). The small numbers of sampled trees in disease classes 1 and 4 result from the very few trees in these classes that were available for sampling.

Method of isolation

The method of isolation used during the survey is similar to that used by Blanco-López *et al.* (1984) except for the growth medium. During preliminary trials isolation was also attempted on potato dextrose agar (Blanco-López *et al.*, ibid.) and using a moist-chamber method (McKeen and Thorpe, 1971), but malt agar gave better results (Hiemstra, unpubl.).

Since bacteria never caused any problems during the preliminary investigations, plain malt agar was used. However, during the incubation of plates from the survey, bacteria developed rather frequently on wood chips from the first series of samples from both healthy and diseased trees. Therefore, during the second part of the survey, malt agar containing 100

ppm tetracycline was used to prevent growth of bacteria. With regard to *V. dahliae*, the results before and after addition of tetracycline were essentially the same. Bacterial growth therefore is considered not to have affected the isolation results.

Presence of *Verticillium dahliae*

The only fungal species isolated that is known to be pathogenic was *Verticillium dahliae*. It was isolated from over 60% of the trees with distinct symptoms of ash wilt and from some of the trees in disease classes 1 (dubious) and 2 (slight symptoms) but never from healthy trees. Although incidental isolation has been reported before (Buisman, 1933; Anon., 1951; Van den Ende, 1958), these results represent the first large scale isolation of *V. dahliae* from *F. excelsior*.

V. dahliae was often isolated out of only one of the three branches sampled per tree (Table 4.5) and even in many cases out of only one of four wood chips per branch (Table 4.6). This means that it might have been isolated more frequently if more branches per tree and/or more wood chips per sampled branch had been examined. Hence, the percentage of 63 % of the diseased trees from which *V. dahliae* was isolated probably underestimates the number of infected trees.

This hypothesis is supported by the results of a repeated isolation using the samples of a group of ten trees from DC 3 from which *V. dahliae* was isolated remarkably infrequently during the first examination. Samples of seven healthy trees from the same location were also re-examined. After repeated isolation two more diseased trees but still none of the healthy trees yielded *V. dahliae*. Thus the percentage of 63 indeed should be considered an underestimation.

Table 4.5 Number of branches per tree from which *Verticillium dahliae* (Vd) was isolated for 58 Vd-positive trees (3 branches sampled per tree).

	Number of Vd-positive branches per tree			
	1	2	3	1, 2 or 3
Number of trees	24	12	22	58
Percentage of all Vd-positive trees	41	21	38	100

Table 4.6 Number of wood chips per branch from which *Verticillium dahliae* (Vd) was isolated for 114 Vd-positive branches (4 wood chips examined per branch).

	Number of Vd-positive chips per branch				
	1	2	3	4	1, 2, 3 or 4
Number of branches	45	28	20	21	114
Percentage of all Vd-positive branches	39	25	18	18	100

Presence of other fungi

All other frequently isolated species were present in both healthy and diseased trees and should therefore be considered as endophytes. *Phoma macrostomum* and *P. pomorum* were isolated from trees in each disease class with equal frequencies. Both fungi are known as inhabitants of dead and diseased material from many tree species (Boerema and Dorenbosch, 1970; Dorenbosch, 1970; Boerema *et al.*, 1970) and have been isolated from *Fraxinus* species before (unpubl. data, Plant Protection Service Wageningen). *Aureobasidium pullulans* was frequently isolated from trees in disease classes 1, 2 and 3 and also, but less frequently, from healthy trees. This fungus is a common saprophyte on leaf surfaces and is also known as a common endophyte of bark and wood of many tree species (Domsch *et al.*, 1970). Most of the less frequently isolated fungi are known as weak parasites, wound parasites or common saprophytic species and some of them have also been isolated from *Fraxinus spp.* before. *Phoma enteroleuca* var. *enteroleuca* is known as a facultative parasite (Dorenbosch, 1970).

4.3 Inoculation of healthy trees

4.3.1 Material and methods

Design of the experiment

Two methods for inoculation (treatment 1 and treatment 2) were tested. The treatments were repeated on a new set of trees several times during the growing season of 1988. On each date one or more rows of trees were treated. Symptoms were assessed regularly during the growing season.

Trees

The trees used in this experiment were three-year-old seedlings of common ash (*Fraxinus excelsior L.*, origin Echteld-01) from the nursery of 'De Dorschkamp' Research Institute which had been replanted two times at the nursery (1+1+1 stock). In April 1988 these seedlings were planted in an experimental plot in rows of ten trees with a nominal spacing of 1 × 1 m. At that time the plants had a diameter of about 12 mm (at 10 cm above soil level) and a height of about 70 cm. The plot was situated near Wageningen on a clay soil which was supposed to be free of *V. dahliae*. As far as known, on this site susceptible crops like potato were never grown and for a period of at least ten years prior to planting of the ash trees a grass crop was grown on this site.

Inoculum

The inoculum for both treatments was prepared in a culture on liquid Czapek-Dox medium in Erlenmeyer flasks. The flasks were put in a shaking machine at room temperature in the dark for about seven days. The isolate that was used originated from a diseased ash tree located in the province of Flevoland (isolation experiments, group 1). After filtration over double cheese-cloth, the liquid of the cultures, containing about 10^7 conidia/ml, was used as conidial suspension in treatment 1. The clumps of fungal material (hyphae and microsclerotia), that usually develop in these cultures, were used for treatment 2.

Treatment 1

Trees were inoculated by making a horizontal incision a few millimeters deep through the bark into the xylem of the stem. At the same time a drop of the conidial suspension was brought into contact with the wound. The suspension then was sucked into the xylem by the low xylem pressure potential (cf. Elgersma, 1969). This method was applied to ten trees on each of the following dates: 3/6, 16/6, 12/7, 2/8, 17/8 and on 5 trees on 15/9. In each application an equal number of trees was used as control. Control trees and inoculated trees were alternating within the rows (chessboard pattern). The control trees were inoculated with sterile Czapek-Dox liquid medium on 3/6 or sterile water on the other dates. After treatment, the wounds were covered with moist cottonwool and wrapped with parafilm.

Treatment 2
A T-cut was made through the bark of the stem and a clump of microsclerotia and hyphae then was inserted between the bark and the xylem. This method was applied to ten trees on 12/7 and 2/8 and to five trees on 16/6 and 17/8. On each date an equal number of trees was used as control which received a few drops of sterile water. Control trees and inoculated trees were alternating within the rows. Again wounds were covered with moist cottonwool and wrapped with parafilm.

4.3.2 Results

With both treatments **control trees** never developed symptoms of ash wilt.

Treatment 1
Most of the trees developed symptoms (Fig. 4.2) within a month after inoculation. First the colour of the leaves changed from bright-green to a dull, yellowish or greyish green ('loss of colour'). At the same time or somewhat later the leaves started wilting. Many leaves also developed marginal necrosis. In most cases the trees gradually deteriorated and after some time the leaves were shed completely. After about six weeks all inoculated trees could be clearly distinguished from the control trees by these symptoms. Many of the trees that had been treated early in the growing season developed new shoots and leaves a few weeks after leaf fall. The new shoots and leaves initially were very small and chlorotic. Later the colour became more normal but the leaves remained small.

Treatment 2
These trees also developed disease symptoms. However, the symptoms were less conspicuous. Generally, development of the symptoms started later and its progress was much slower. The intensity of symptoms in this group also varied more strongly. The first wilt symptoms usually became visible after 5 to 6 weeks. These early symptoms were often confined to one side of the plant.

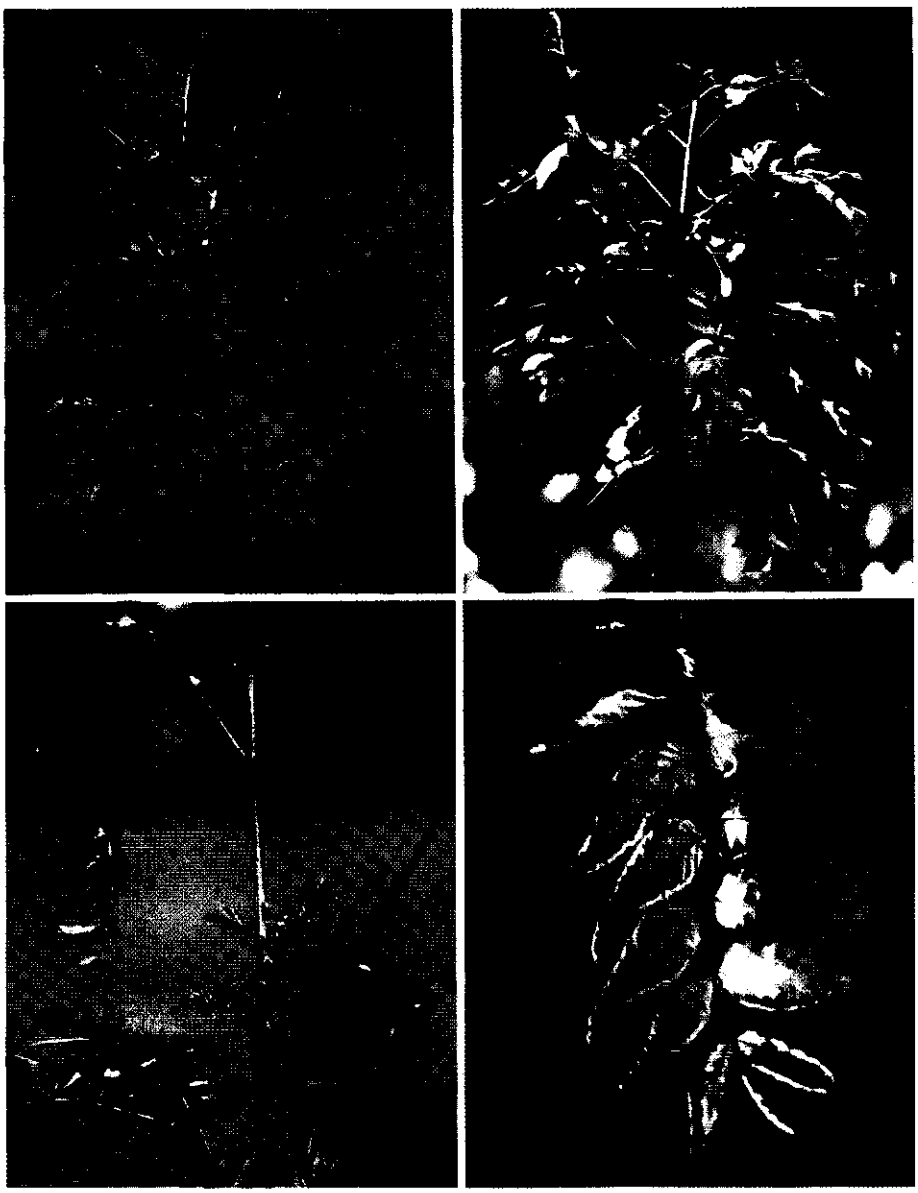

Fig. 4.2 Symptom development in young ash trees stem-inoculated with *V. dahliae*. Top left: Healthy control tree. Top right: Discoloration, wilt and necrosis of leaves in inoculated tree. Bottom left: Defoliation and new shoots on inoculated tree. Bottom right: Discoloration and marginal necrosis of leaves in inoculated tree.

4.3.3 Discussion

Method

The faster development of symptoms in treatment 1 is most probably the result of the much higher number of vessels infected immediately after application. In this treatment large quantities of conidia are directly introduced into a large number of vessels and distribution through the plant by the transpiration stream starts immediately. In treatment 2 immediately after application most of the fungal material is still outside the vessels. The fungus needs time to invade the vessels and produce conidia before extensive spread can take place. Thus, effective colonization of these trees takes place at a later point in time and the plant has more time for activating its defence mechanisms.

Dressing the wound in treatment 2 was necessary to prevent rapid drying out of both the wound and the inoculum. Dressing of the wound in treatment 1 probably would not have been necessary. However, because of the relatively small number of treated trees in this experiment, it was thought prudent to minimize damage which may result from the wounds and to make treatments 1 and 2 comparable.

Because of the much faster development of symptoms, inoculation with a conidial suspension (treatment 1) is considered to be the best test for the pathogenicity of *V. dahliae* in *F. excelsior*. Other advantages are its simplicity and the fact that it requires little time to perform, especially when the wounds are not dressed.

Date of inoculation

The date of treatment (during the growing season) had no effect on the success of the infection. For a complete and quick development of the symptoms, however, the first part of the growing season is most effective since inoculum uptake is fastest. During periods with high air humidity there was hardly any visible uptake of the suspension. However, even trees with very slight uptake of the inoculum developed disease symptoms.

Symptom development

Inoculated trees developed symptoms of discoloration, marginal necrosis, wilt and early leaf fall. All these symptoms are common in Verticillium wilts of several woody species other than ash (Piearce and Gibbs, 1981; Sinclair *et al.*, 1987). Thus it may be concluded that *Fraxinus excelsior* is susceptible to

V. dahliae. This finding is corroborated by the results of two earlier small-scale inoculation experiments with *F. excelsior* and *V. dahliae* (Van den Ende, 1958; Himelick, 1969).

The disease symptoms that developed after inoculation greatly resembled those of ash wilt disease previously described by Hiemstra (1987ab, 1988) and Hiemstra *et al.* (1990). However, contrary to some trees which were naturally affected by ash wilt disease, trees inoculated with *V. dahliae* never showed partial or complete dieback. This may be related to the different places of infection in combination with the way the pathogen spreads within the plant. Spread within tree hosts is mainly by means of conidia transported upward by the sap stream (Banfield, 1941; Caroselli, 1954; Born, 1974; see also Chapter 3). In case of a natural infection, *V. dahliae* enters the xylem in the root. Distribution of conidia in this case leads to infection of both the aboveground parts of the plant and (at least part of) the root system. In inoculated trees the pathogen enters the xylem somewhere on the stem. In this case upward spread of conidia by the sap stream will leave the roots free of *V. dahliae* resulting in infected above-ground parts on a healthy root system. This may explain the absence of dieback in inoculated trees. Another point of difference is that re-infection is possible continuously in case of natural infection because of the presence of the fungus in the soil. In case of inoculation, however, infection is a one-time event, provided that the soil is free of *V. dahliae*.

Discoloration of the wood

In the wood of both inoculated and naturally infected trees no consistent discoloration was visible. At most some infected trees showed a faint tan but some healthy trees also showed this colour. A dark brown to black discoloration as in maple (*Acer* sp.) and many other tree hosts (Piearce and Gibbs, 1981) has never been observed. These findings concur with the reports of Smith (1979) for *Fraxinus americana* and *F. pennsylvanica* and Wilhelm and Taylor (1965) and Sinclair *et al.* (1987) for olive that discoloration of the wood usually is absent, even in severely infected trees. Thus, discoloration of the wood can not be considered of diagnostic value in Verticillium wilt of *F. excelsior*.

4.4 Re-isolation

4.4.1 Material and methods

At the end of the growing season (17 October 1988) nine inoculated and eight control trees were examined for the presence of *V. dahliae*. Samples were taken at several heights of the trees, starting just above soil level. The samples were examined using the same method as during the survey (Section 4.2). Table 4.7 contains more details on the trees and the samples taken.

4.4.2 Results

V. dahliae was isolated from each inoculated tree that was examined, but never from the control trees. It was isolated most often from the upper parts of inoculated stems. All current year's shoots and most last year's shoots of inoculated trees yielded *V. dahliae* but from the stem base (well below the level of inoculation) it was isolated only once (Table 4.7).

A number of other fungi were isolated from both the inoculated and the control trees. Most of them (*Aureobasidium pullulans*, *Microsphaeropsis olivacea)*, a *Papulospora* sp., *Phoma macrostomum*, *Phoma pomorum* and a *Septoria* sp.) were the same as isolated during the survey. Also another fungus (*Phoma medicaginis*) was isolated, but with very low frequencies.

4.4.3 Discussion

Isolated fungi

The re-isolation experiments confirm that the inoculations have been successful as *V. dahliae* was isolated from all inoculated trees that were examined and never from control trees. The other fungal species which again were isolated from control trees as well as from inoculated trees should be considered as endophytes although in literature most of them are known as facultative pathogens or saprophytes (cf. Subsection 2.4.3).

Table 4.7 Inoculated trees used for reisolation, and reisolation results.

	Tree number								
	1	2	3	4	5	6	7	8	9
THE EXAMINED TREES[1]									
Date of inoculation	3/6	3/6	3/6	16/6	16/6	2/8	2/8	2/8	2/8
Method of inoculation[2]	s	s	s	s	s	s	s	m	m
Height of inoculation	67	95	38	18	20	27	23	42	9
Total height at end of growing season	130	195	90	119	168	122	96	146	147
EXAMINED TREE PARTS AND RESULT OF ISOLATION[3]									
Current year's shoot	+		+	+	+	+	+	+	+
Last year's shoot	+	+	+	+	+	-	+	-	-
Remaining part[4] of stem	-	+	-	+	+	+		-	-
Stem just above soil level	-	-	-	+	-	-	-	-	-
Large root just below soil level	-	-							
Small root deeper in soil	-	-							

[1] Only results for inoculated trees are shown. From the 9 control trees that were examined *V. dahliae* was never isolated.

[2] s = trees inoculated with a conidial suspension; m = trees inoculated with microsclerotia.

[3] + = *Verticillium dahliae* Kleb. (Vd) isolated; - = Vd not isolated; no sign means not examined.

[4] remaining part = stem without current and last year's shoot.

Spread of *V. dahliae* in inoculated trees

The results indicate that *V. dahliae* after inoculation low on the stem was spread mainly upward. Four months after inoculation the fungus was re-isolated from the top of all inoculated trees, i.e. between 0.5 and 1.5 metre upward from the place of inoculation. The downward spread was much less as can be seen in Table 4.7. These results seem to confirm that the root system in stem-inoculated trees stays free of *V. dahliae*, as suggested in the previous section of this chapter. However, at the end of the year of inoculation the fungus is still present in the upper parts of all inoculated trees.

4.5 Conclusions

The experiments described in this chapter lead to the following **conclusions**:

1. ***V. dahliae* is consistently associated with the ash wilt disease (Section 4.2).**
2. **Trees artificially infected with *V. dahliae* develop symptoms similar to those of ash wilt disease (Section 4.3).**
3. **After development of these symptoms by inoculated ash trees *V. dahliae* can be re-isolated from these trees (Section 4.4).**

Hence, fulfilling Koch's postulates (Agrios, 1988), it has been demonstrated that *Verticillium dahliae* Kleb. is the cause of ash wilt disease.

This conclusion may not be surprising since *Verticillium dahliae* is a common wilt pathogen affecting hundreds of plant species from many families all over the world, herbs as well as woody plants (Smith *et al.*, 1988).

A well-known European host in which *V. dahliae* can cause serious damage is the olive (*Olea europaea*; Vigouroux, 1975). *Fraxinus* and many other genera of the family Oleaceae (*Forsythia*, *Jasminum*, *Ligustrum*, *Osmanthus*, and *Syringa*) are also reported to be susceptible to infection by *V. dahliae* (Menzinger & Sanftleben, 1980; Sinclair *et al.*, 1987). Some American species of *Fraxinus* (especially *F. americana* and *F. pennsylvanica*) are known to be often affected by *Verticillium dahliae* (Himelick, 1969; Forer & Longenecker, 1973) but reports of *V. dahliae* isolated from naturally infected *F. excelsior* are sporadic and severe attack of common ash (including dieback and complete death of trees) has not been reported before.

The symptoms of ash wilt disease are very similar to those of Verticillium wilts in other tree species. However, in contrast to many other tree species, in ash discoloration of the wood is not a reliable symptom. This explains why the fungus was not found during routine checks in the early eighties (Section 4.1). During such checks wood samples, usually from stems or large branches, were examined for discolorations. Isolations were only made from discoloured wood, if present.

Most authors report that Verticillium wilt of trees is a disease of young trees in nurseries and sometimes of shade and ornamental trees but not (Boyce,

1948; Butin, 1983) or only seldom (Himelick, 1968; Sinclair *et al.*, 1987) of forest trees. **This study is the first report of widespread Verticillium wilt in forest trees.** It coincides with a series of publications reporting Verticillium problems in other tree species getting more important over the last decades (Himelick, 1968; Born, 1974; Kopinga & de Kam, 1987; Worf *et al.*, 1989). The reasons for such developments are not quite understood.

In agricultural crops, changes in agricultural practices, use of infected seed or plant material, presence of symptomless carriers (many weed hosts) and previous cropping with susceptible crops have been advanced as factors contributing to the increase of Verticillium wilt during the last 100 years (Pegg, 1984). Such factors also could be of importance in ash wilt disease in the Netherlands as most of the affected plantations were recently planted on former agricultural land.

4.6 Summary

In this chapter it is demonstrated that the ash wilt disease of common ash (*Fraxinus excelsior L.*) is caused by the fungal pathogen *Verticillium dahliae* Kleb. Initial experiments showed that this fungus can readily be isolated from young branches with wilt symptoms. It was also shown that discoloration of the wood is not a reliable symptom. In 1988 during a nationwide survey in the Netherlands 150 ash trees, both diseased and healthy ones, were examined. *V. dahliae* was isolated from 63 percent of the diseased trees but never from a healthy tree. *Phoma macrostomum*, *Phoma pomorum* and *Aureobasidium pullulans* were isolated from both healthy and diseased trees and should be considered as endophytes. Inoculation of healthy young ash trees by inserting a conidial suspension of *V. dahliae* into the stem led to development of symptoms of ash wilt disease within six weeks after treatment. Inoculation with microsclerotia placed in a T-shaped wound on the stem also caused these symptoms but symptom development needed more time. At the end of the same year *V. dahliae* was easily re-isolated from these trees. After inoculation *V. dahliae* spreads mainly upward, and absence of dieback symptoms after inoculation is attributed to the root system staying free of *Verticillium*.

Acknowledgements
The author wants to express his gratitude to Ir. F.W. Burger who did most of the isolations during the survey, to Ir. H. Schneider who identified the isolated fungi other than *V. dahliae* and assisted during the re-isolation experiments and to Ing. P. Schalk of IBN-DLO (former Institute for Forest and Landscape Research 'De Dorschkamp') who provided the planting stock for the inoculation experiments.

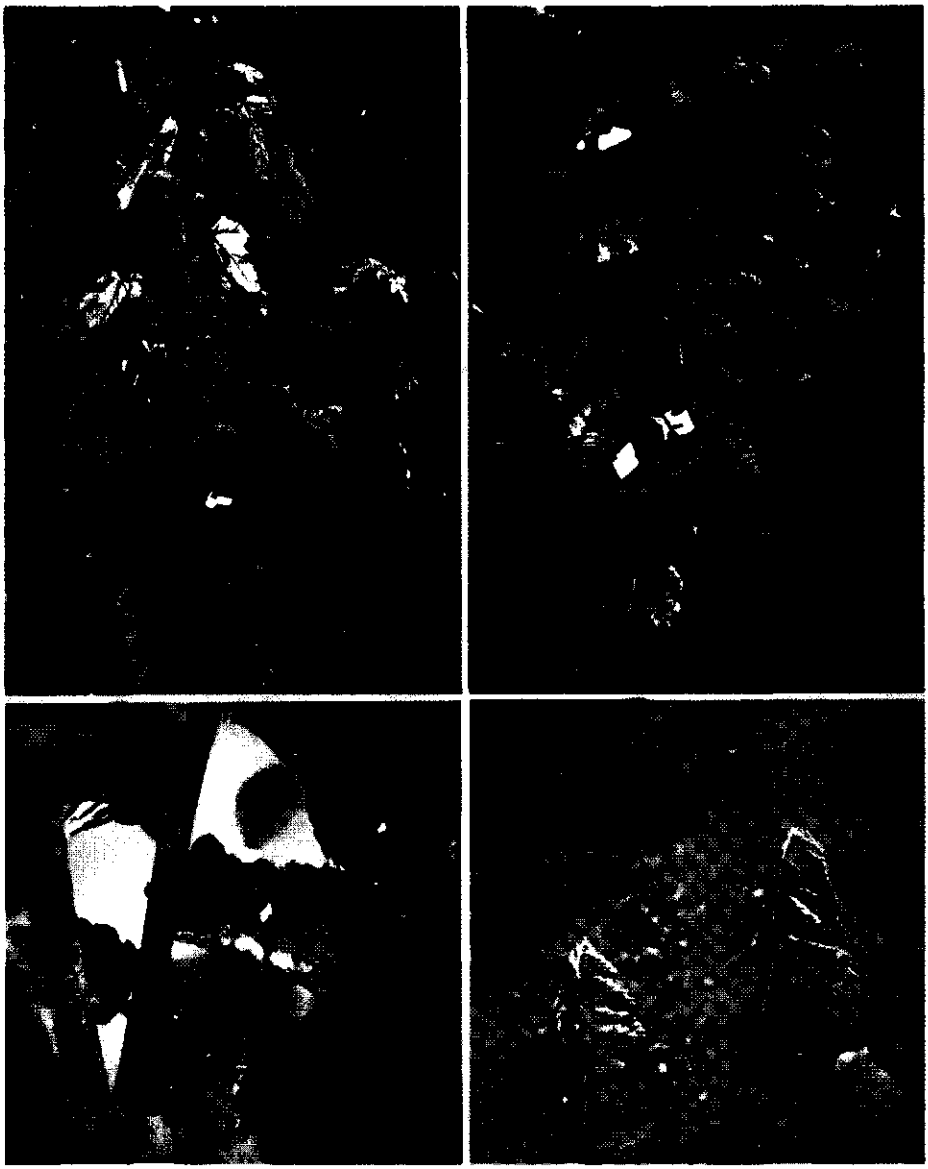

Fig. 5.1 Symptoms in young maple and elm trees stem-inoculated with *V. dahliae*. Top left: Yellowing, marginal necrosis and wilt of leaves in maple. Top right: Yellowing and marginal necrosis of elm leaves. Bottom left: Detail of elm leaves with marginal necrosis. Bottom right: detail of wilting twig ends in elm.

5 HOST SPECIFICITY AND VIRULENCE OF *VERTICILLIUM DAHLIAE* ISOLATES FROM ASH, ELM, MAPLE AND POTATO

5.1 Introduction

In the previous chapter it was shown that the ash wilt disease, reported from several parts of the Netherlands, is caused by *Verticillium dahliae* Kleb. Both the pathogen and its host, *Fraxinus excelsior* L., are native to the Netherlands. Serious problems caused by *V. dahliae* in common ash have not been reported before, but it has been known for a long time that occasionally this pathogen may attack common ash (Anon., 1951; Buisman, 1933; Van den Ende, 1958). This raised the question why the disease appears to occur at such a large scale now. One possible explanation might be the occurrence of a more virulent, host-specific strain of *V. dahliae*. The widespread appearance of such a strain might be the result of selection from the natural soil population after introduction of a new host in areas where it was not previously grown (Vigouroux, 1971, cited by Pegg, 1974; Tjamos, 1981). It might also be a result from spread of a more virulent strain imported from another area, as has occurred with Dutch elm disease (Gibbs, 1978).

V. dahliae has a very large host range (Smith *et al.*, 1988; cf. Chapter 3). Generally, pathogenic isolates from one host species are pathogenic to many other host species too. However, isolates may differ in virulence. In tomato, two races of *V. dahliae* are distinguished depending on genes for wilt resistance in tomato cultivars (O'Garro and Clarkson, 1988). In cotton, defoliating as well as non-defoliating strains are known (Pegg, 1984; Bejarano-Alcázar *et al.*, 1990). On mint, Brussels sprouts and pepper some host-specificity has been observed (Domsch *et al.*, 1980).

In the present chapter, the possibility of the occurrence in ash wilt disease of a strongly virulent, host-specific isolate of *V. dahliae* is investigated. For that purpose the virulence and host-specificity of a *V. dahliae* isolate from ash were examined. In an inoculation experiment with three tree hosts of *V. dahliae*, ash, elm and maple, the effect of isolates from these three host species and from potato on symptom development and growth were compared.

5.2 Material and methods

The inoculation experiment included 590 two-years-old plants; 200 plants of *Fraxinus excelsior* L. and *Acer pseudoplatanus* L. each and 190 plants of *Ulmus carpinifolia* Gled. Dormant plants were received from a commercial nursery and planted in rows of ten with 1 × 1 m spacing on an experimental field (Wageningen, Afweg) in the end of April 1989. The rows were arranged in ten blocks with two adjoining rows of each species (block 10 contained only one row of elm). The sequence of the tree species within each block was determined using a table of random numbers (Cox, 1958; Table A.1).

Treatments were attributed to rows of trees in a direction perpendicular to the species rows. Again two adjoining rows received the same treatment and the sequence of the treatments in each block was determined using a table of random numbers. Per block this operation resulted in 15 square sub-plots of four trees of one species that received the same treatment.

The treatments were applied on 8 and 9 July 1989. *V. dahliae* isolates from four different hosts, i.e. *Fraxinus excelsior* L. (common ash), *Acer platanoides* L. (Norway maple), *Ulmus carpinifolia* Gled. (= *Ulmus minor* Mill., smooth-leaved elm or European field elm) and *Solanum tuberosum* L. (potato) were used to produce inoculum for the treatments 1 to 4, respectively. The inoculum consisted of a conidial suspension of the isolates produced on liquid Czapek-Dox medium (see Chapter 4). Trees were inoculated by forcing a knife with a drop of inoculum on it into the xylem of the lower part of the stem. The inoculum then is drawn into the xylem by the negative pressure potential in the xylem. Control trees (treatment 5) received sterile water instead of inoculum.

After inoculation, the condition of each tree was described regularly using the symptom code and disease index system as described in Chapter 2. Height and diameter of all trees were measured at the times of planting and treatment and at the end of the growing seasons of 1989 and 1990. Heights were measured with a measuring stick in centimetres. Diameters were measured at ten centimetres above soil level with a diameter tape in millimetres.

During the two years of the experiment, the number of trees steadily decreased. In the first year of the experiment, 16 trees were removed for various reasons, including feeding damage caused by rabbits and mechanical damage during cultivation. Some trees were excluded from the experiment because of very poor growth before inoculation. At the end of the growing season of 1989, one complete block (60 trees) was used for re-isolation

purposes. In the second year some maple trees were absent because of death resulting from the infection by *V. dahliae* in the previous year. For these reasons the numbers of trees were different for each treatment—species combination and date of observation. The numbers are included in the tables in the results section.

Data were processed with Lotus-1-2-3 and Statgraphics computer programmes. In order to be able to analyze the differences in virulence of the four isolates per host species and to compare the results for the three host species, the codes for tree species and treatment were combined resulting in fifteen treatment codes, one for each species—treatment combination. After this operation, means of disease index per observation date and of height and diameter growth in 1989 and 1990 were calculated, and an analysis of variance (ANOVA) was performed including multiple range analysis of means of height and diameter growth. A slight gradient in soil conditions within the experimental field resulted in a significant effect of the block number on growth for all treatments. Thus the treatment means were analyzed with the block number as covariate.

5.3 Results

5.3.1 Symptom development

Symptoms in the year of inoculation

Symptom development was different in the three species (Fig. 5.2). Symptom development was fastest in ash trees. Two weeks after treatment some of the ash trees already showed initial symptoms of wilt and withering. After two more weeks, the symptoms in these trees were conspicuous and most other inoculated ash trees also showed beginning symptoms. Two months after treatment almost all inoculated ash trees showed distinct symptoms resulting in an average disease index of about 5 for all treatments (Fig. 5.2A). From this moment on, the average disease index did not increase further. This was the result of the increasing incidence and intensity of symptoms in part of the trees on the one hand, and on the other hand recovery of some of the trees that had developed symptoms very soon after inoculation. Differences between the four treatment groups were small with the *V. dahliae* isolates from ash and potato causing slightly higher disease indices than those from maple and elm.

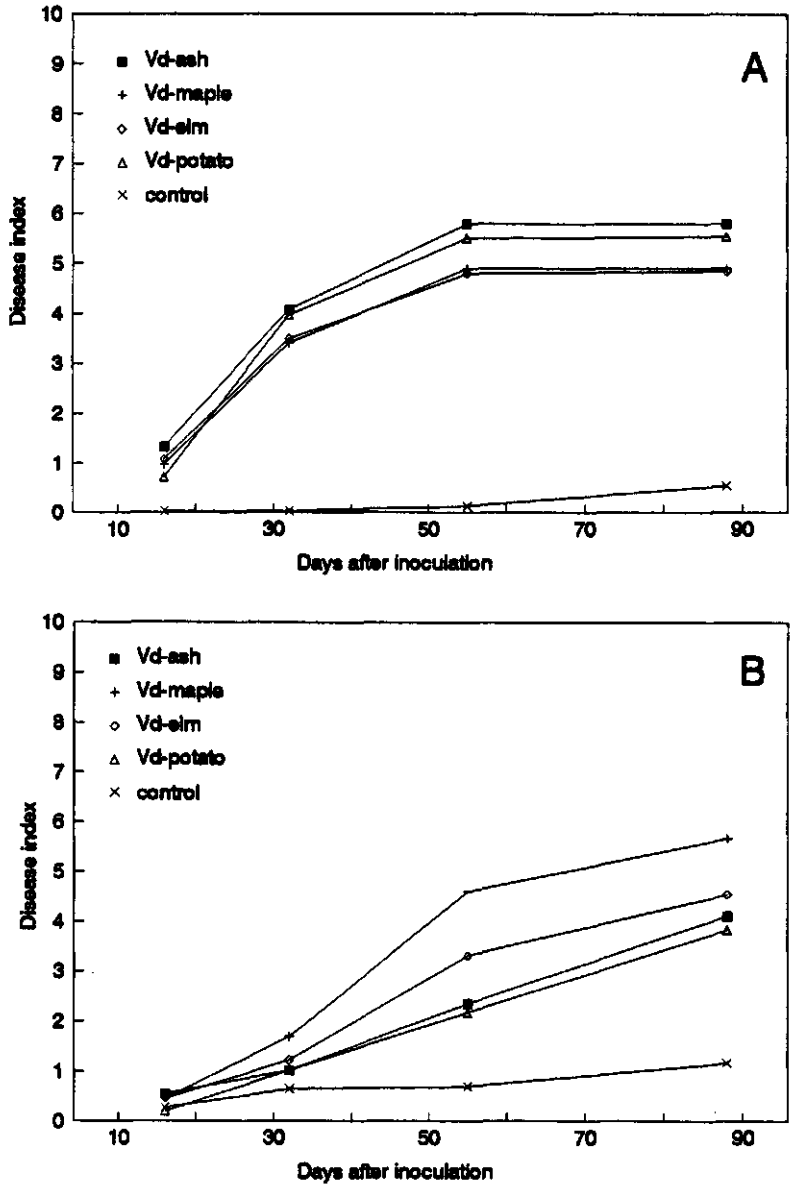

Fig. 5.2 Symptom development in ash (A), maple (B) and elm (C) after inoculation with *V. dahliae* isolates from ash, maple, elm and potato.

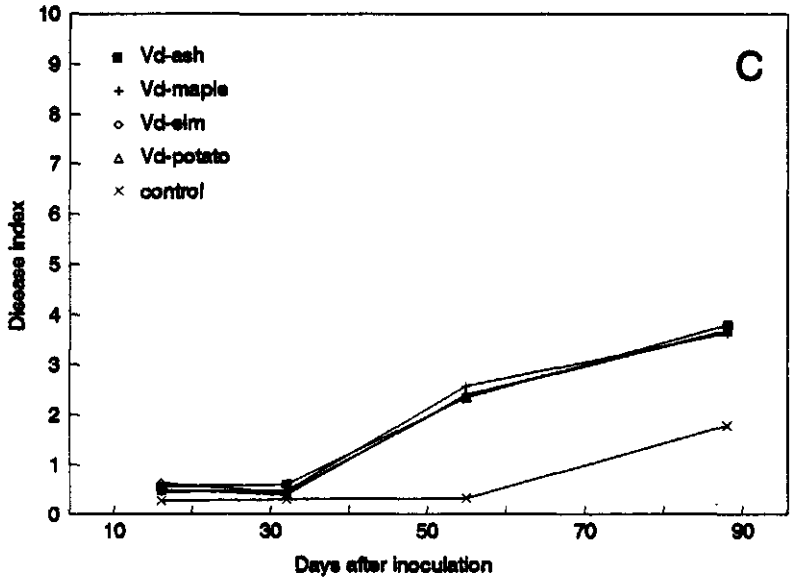

Fig. 5.2 (continued)

The symptoms in inoculated ash trees were the same as described in detail in Chapter 4; i.e. in a very early stage the leaves lost their bright green lustre resulting in a dull greyish appearance, then the leaves drooped, wilted and withered, and finally complete defoliation occurred. Marginal necrosis of leaves was also a common symptom (see Fig. 4.2). In some trees the symptoms were one-sided, and usually the part below the point of inoculation remained healthy. Recovery of trees resulted from reflushing of sleeping buds and from the formation of new shoots on the lower part of the stem.

In maple the first symptoms also were recorded two weeks after treatment. During the season of treatment the number of symptomatic trees as well as the intensity of the symptoms steadily increased for all treatment groups (Fig. 5.2B). In this host there were considerable differences in symptom index between the four isolates inoculated, with the maple isolate causing the most serious symptoms and the ash and potato isolates causing the least symptoms. The symptoms in maple strongly resembled those in ash. Early symptoms in maple were yellowing, marginal necrosis and drooping of leaves (Fig. 5.1) followed by serious wilt and defoliation. In the second part of the growing season some of the maple trees showed recovery through formation of new shoots on the lower part of their stems.

In elm, finally, symptoms developed much more slowly and the final disease index was lower than those of the ash groups and most of the maple groups (Fig. 5.2C). There were no differences in the average disease indices of the four inoculated elm groups. The symptoms in elm (Fig. 5.1) were partly different from those in ash and maple. The first symptom in this species was a change in the colour of the leaves towards a pale yellow-green colour. After some time this discoloration often became very distinct in the young leaves at the end of growing twigs. A second symptom was the development of small necrotic spots along the margins of the leaves and sometimes between the veins. A third common and very conspicuous symptom was the presence of brightly yellow leaves with a broad necrotic brown margin on some twigs or branches. In some inoculated elms only the tops of a few leading branches died.

During sampling for reisolation at the end of the growing season, serious discoloration of the xylem was observed in inoculated elm and maple trees but not in inoculated ash trees. *V dahliae* was easily reisolated from the stemwood of all inoculated trees that were examined.

Symptoms in the year after treatment

In June 1990 there were no differences between the four inoculated treatment groups (Table 5.1) in both ash and elm. In this year all ashes except one and all elm trees resumed growth and were free of symptoms of *Verticillium* wilt. One inoculated ash tree appeared to be dead in June 1990. This tree died in the dormant period between the growing seasons of 1989 and 1990.

In the four inoculated groups of maple trees, however, serious dieback and death of trees were apparent. About 50 % of the inoculated trees showed serious dieback (including death of trees) by June 1990. The number of trees with serious dieback was slightly different in each of the four treatment groups (Table 5.1). Some maples showed recurrent wilt and defoliation symptoms.

Table 5.1 Condition in June 1990 (numbers per disease class) of ash, elm, and maple trees that were stem-inoculated with *V. dahliae* in July 1989.

Disease Class[1]	Inoculated species and Vd source[2]														
	Ash					Maple					Elm				
	a	m	e	p	-	a	m	e	p	-	a	m	e	p	-
0	4	9	4	7	18	5	3	4	11	26	3	4	3	3	5
1	29	27	31	27	16	13	7	7	9	8	30	29	25	30	28
2	0	0	0	0	0	0	1	0	1	0	1	1	0	0	0
3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
4	0	0	0	0	0	18	21	22	15	2	0	0	0	0	0
5	0	0	0	1	0	0	3	1	0	0	0	0	0	0	0
total	33	36	35	35	34	36	35	35	36	36	34	34	28	33	33

[1] 0 = healthy, 1 = dubious, 2 = slight foliar symptoms, 3 = severe foliar symptoms, 4 = dieback, 5 = dead.

[2] Origin of the *V. dahliae* isolate used for inoculation: a = ash, m = maple, e = elm, p = potato, - = not inoculated.

5.3.2 Height and diameter growth

The average height and diameter growth per treatment group are shown in Table 5.2. Because of a known horizontal gradient in the soil moisture content of the experimental plot, the growth data per treatment group were compared by means of a multifactor analysis of variance with the block number as covariate. This showed a highly significant effect of both treatment (i.e. combination of species and *V. dahliae* isolate) and block. Multiple range analysis (95 % confidence intervals) showed statistically significant differences between the control group and all inoculated groups for all three species.

In ash no significant differences were found between the effects of the four isolates. In maple, however, inoculation with the maple isolate reduced growth much stronger than inoculation with one of the other three isolates. The analysis of variance showed the differences in growth to be statistically significant for diameter growth in 1989 and both height and diameter growth in 1990. The effect on maple of the elm isolate (treatment 2-3) was intermediate between that of the maple isolate on the one hand and that of the ash and potato isolates on the other hand. In elm the effect of the elm-isolate was strongest, but the differences with the other isolates were statistically not significant.

5.4 Discussion

The above results illustrate the differences in susceptibility of different tree hosts for *V. dahliae*. However, the relative susceptibility of a specific host depends on the criterion used. When judged by the development of foliar symptoms, elm obviously has the lowest susceptibility of the three host species (Fig. 5.2). This concurs with the report by Dwinell (1967) who found *Ulmus americana* to be much more tolerant to infection by *V. dahliae* than *Acer saccharum*.

In both ash and maple, foliar symptoms developed faster and were more serious than in elm. Since the reactions of these two species differed, it is difficult to determine which one is the most susceptible. In the year of inoculation foliar symptoms in ash trees generally developed faster and were more serious than in maple. However, the ash trees were free of symptoms in the year after inoculation, whereas many inoculated maples showed serious dieback and some died.

Table 5.2 Mean height and diameter growth[1] of all treatment groups.

Inoculated species	Vd-source	1989			1990		
		Number	Height growth (cm)	Diameter growth (mm)	Number	Height growth (cm)	Diameter growth (mm)
Ash	Ash	37	22 ab	3.4 a	32	54 a	9.1 a
Ash	Maple	40	19 a	3.5 a	36	51 a	9.3 a
Ash	Elm	39	20 a	3.2 a	35	48 a	9.2 a
Ash	Potato	39	24 ab	3.3 a	34	46 a	8.1 a
Ash	-	39	34 b	6.2 b	34	113 b	14.5 b
Maple	Ash	40	41 ab	8.0 b	36	60 b	11.7 b
Maple	Maple	39	33 a	5.5 a	34	34 a	8.3 a
Maple	Elm	39	47 b	7.4 b	35	48 ab	9.4 ab
Maple	Potato	40	39 ab	7.8 b	36	68 b	11.7 b
Maple	-	40	60 c	10.7 c	36	107 c	15.4 c
Elm	Ash	38	42 ab	7.0 a	33	106 a	13.4 a
Elm	Maple	38	50 b	8.4 a	34	96 a	13.3 a
Elm	Elm	32	35 a	6.9 a	28	100 a	13.4 a
Elm	Potato	37	44 ab	7.7 a	33	101 a	13.2 a
Elm	-	35	96 c	11.5 b	33	89 a	13.5 a

[1] Different letters indicate statistically significant differences (multiple-range testing with 95% confidence intervals).

The matter becomes even more complicated when reduction of growth of inoculated trees (Table 5.3) is taken into account. As might be expected because of the severe defoliation of most inoculated ash and maple trees, diameter growth in the year of inoculation of these two species is strongly reduced (by 46 % and 33 %, respectively). Despite the rather mild symptoms of most inoculated elms, diameter growth of elm also was reduced rather strongly, i.e. by 35 %, in the year of inoculation. Even more surprising was the reduction of the height growth of inoculated elms by 55 %, which is a much stronger reduction than observed in inoculated ash and maple (38 % and 33 %, respectively). In the year after inoculation the situation was changed drastically. Diameter growth and especially height growth of both ash and maple were still reduced substantially, but no growth reduction at all was observed in inoculated elm trees. Moreover, height growth of inoculated elms was slightly more than that of control trees.

The experiments described show that it is not enough to monitor symptom development when comparing the virulence of *V. dahliae* isolates to woody hosts. The effect on growth of inoculated trees should also be assessed. Besides, in inoculation experiments with perennial hosts, observations should be continued in the dormant period and at least one growth period after the season of infection.

Table 5.3 Growth of inoculated trees (percentage of the growth of the control trees).

Species	Vd-source[1]	1989		1990	
		Height growth	Diameter growth	Height growth	Diameter growth
Ash	a,m,e,p	62	54	44	62
Maple	a,m,e,p	67	67	67	50
	(m)	(56)	(51)	(54)	(33)
	(a,e,p)	(71)	(72)	(71)	(55)
Elm	a,m,e,p	45	65	99	113

[1] a = ash, m = maple, e = elm, p = potato

Many researchers have compared virulence of different *V. dahliae* isolates. In agricultural crops like cotton (Schnathorst, 1981b), potato (Joaquim and Rowe, 1991) and tomato (Tjamos, 1981), generally a continuum from limited to strong virulence is reported. In several woody hosts, including olive (Schnathorst and Sibbett, 1971; Al Ahmad, 1990), American elm (Dwinell, 1967) and several maple species (Dochinger, 1956a; Donohue and Morehart, 1978; Hoitink *et al.*, 1979; Townsend and Hock, 1973), differences in virulence of isolates have also been reported. However, other researchers (Caroselli, 1957; Emechebe, 1974) did not find differences on maple and cacao, respectively.

The results of the present experiment confirm that *V. dahliae* isolates from one host are generally also pathogenic to other hosts of this fungus. This does not only hold true for the three isolates from woody hosts that proved to be pathogenic to at least two other woody hosts but also for the potato isolate that was pathogenic to all three woody hosts. Thus, as reported before by Morehart *et al.* (1980) and Ndubizu (1977), isolates from non-woody hosts may also be pathogenic to woody hosts. For *F. excelsior* this has been reported before by Van den Ende (1958).

The results also show some differences in virulence of the four isolates towards maple. In ash and elm there were no significant differences between the four isolates. Although in ash the isolates from ash and potato (treatments 1-1 and 1-4) caused slightly more serious symptoms (Fig. 5.2), analysis of the growth data (Table 5.2) did not show significant differences between the isolates, neither in the year of inoculation nor in the next year. The same is the case for the four isolates when inoculated into elm. But in maple the isolate originating from maple (treatment 2-2) caused much stronger foliar symptoms than the isolates from ash and potato (treatments 2-1 and 2-4). Also the negative effect of the maple isolate on the growth of inoculated maples was much stronger than that of the ash and potato isolates.

These results concur with the general view that some *V. dahliae* isolates may be more virulent towards the host from which they were isolated, although generally they are pathogenic to several other hosts too (Sinclair *et al.*, 1987). The results clearly demonstrate that the isolate from ash is not specific to the host from which it originates. It can also be concluded that the isolate from ash is not extremely virulent.

Hence, the recent outbreak of ash wilt disease cannot be attributed to the distribution of a host-specific or more virulent strain of *V. dahliae*.

The effects of the isolates from ash and potato were almost identical on all three tree species. This indicates that increase of the soil inoculum by susceptible annual crops like potato may contribute to the occurrence of ash wilt disease in recently planted ash forests. This is especially important as many tree nurseries as well as many of the recently planted ash forest plots where ash wilt disease is present, are situated near or on land formerly planted with potatoes. Such a development, i.e. increase of the soil inoculum on susceptible herbaceous crop plants with subsequent infection of woody species, has been described previously for attack of olives by *V. dahliae* in orchards intercropped by tomato or cotton and for several fruit tree species planted on land previously cropped to potato or cotton (Schnathorst and Sibbett, 1971; Wilhelm and Taylor, 1965; Wilhelm, 1981).

5.5 Summary

In an inoculation experiment with ash, elm and maple the host specificity and virulence of four *V. dahliae* isolates originating from ash, elm, maple and potato was examined. Symptom development and growth of inoculated trees were monitored during the year of inoculation and the year after. All isolates proved to be pathogenic on each of the three tree species. On maple, the maple isolate was more virulent than the other isolates. On ash and elm, no differences in virulence were observed. Therefore, the recent large-scale occurrence of ash wilt disease is not likely to be the result of a host-specific or extremely virulent isolate. For examination of the effect of *V. dahliae* isolates on a tree host, symptom development as well as growth of inoculated trees should be monitored, and inoculation experiments with perennial hosts should be continued for at least two growing periods. An increase of the soil inoculum due to annual agricultural crops may contribute to the occurrence of ash wilt disease in recently planted ash forests.

Acknowledgement

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6 PATHOLOGICAL WOOD ANATOMY OF *VERTICILLIUM*-INFECTED ASH TREES

co-authors: R.J.C. Schroeijers[1] and R.W. den Outer[1]

6.1 Introduction

The external symptoms caused in trees by pathogenic vascular fungi like *Ophiostoma ulmi* (Buisman) Nannf., *Ceratocystis fagacearum* (Bretz) Hunt and *Verticillium dahliae* Kleb. are often dramatic, including severe wilt, defoliation and dieback. Besides these external symptoms, the disease syndrome usually includes several internal anomalies like gel and gum formation, hypertrophy of parenchyma resulting in formation of tyloses and vascular discoloration (Beckman, 1964). These changes are not just signs of the presence of the pathogen, but they are part of the active defensive mechanisms of the host that are incited by the presence of the pathogen (Beckman, 1987; Sutic and Sinclair, 1991).

Cowling and Horsfall (1980) compared defense in plants to the defense of a medieval castle. The outer walls include all plant surfaces and the gates include all the surface openings. In many cases these 'outer walls' and 'gates' of plants manage to keep pathogenic organisms out. In case they do not hold, the struggle between the offensive actions of the pathogen and the defensive actions of the host continues inside the infected plant. Here, again like in a castle, preformed structural defenses as well as improvised new ones form a system of internal 'walls' that may be effective in containing the intruders.

A conceptual framework of the internal walls and the way in which they act in containing invading micro-organisms after wounding of the secondary xylem of trees was provided in 1977 by Shigo and Marx in their essay on the compartmentalization of decay in trees (CODIT-model). They describe four 'walls' of increasing strength that are involved in containing invading micro-organisms. The first three types of walls are located in the tissues that were present at the time of wounding. Type 1 walls, that limit vertical spread, result from vascular plugging and develop in response to injury. Radial distribution is limited by the type 2 walls made up by the margins of each

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growth ring. Type 3 walls, consisting of sheets of ray cells, limit the tangential distribution. Finally wall 4, the barrier zone, separates the tissue present at the time of wounding from new, uninfected tissue formed after wounding. This wall, generally consisting of parenchymatous cells, is the strongest and is formed by the cambium in response to mechanical wounding.

Later the CODIT-model has been extended to encompass infections by vascular pathogens too (Tippett and Shigo, 1981; Shigo, 1984) and barrier zones were defined as zones of protective tissue which form in response to infection as well as to mechanical wounding and serve to isolate the infected wood on the inside from the healthy wood that continues to form on the outside.

Zones of protective tissue between the infected xylem tissues in the centre of trees affected by a vascular pathogen and the new, uninfected tissue at the outside of the stem have been reported for several combinations of tree genera and vascular pathogens (Buisman, 1935; Schoeneweiss, 1959; Shigo and Tippett, 1981; Tippett and Shigo, 1981; Bonsen *et al.*, 1985). In the case of Verticillium wilts, barrier zone formation in the wood of infected trees has been reported in *Ulmus* (Buisman, 1935) and in *Acer* (Tippett and Shigo, 1981).

In ash trees (*Fraxinus excelsior* L.) affected by ash wilt disease (i.e. infected by *Verticillium dahliae*) recovery is common (Chapters 7 and 8). Plugging of vessels by tyloses and granular deposits, and the presence of large groups of parenchymatous cells in the wood of naturally infected trees were described by Miller and Hiemstra (1987). They concluded that these cells might function as a defence barrier, but that further studies were required to substantiate such a claim. Objectives of the work described here were to examine the effect of wound-inoculation with *V. dahliae* on the wood anatomy of *F. excelsior*, and to relate this information to the reported complete recovery of such trees. For this purpose the results are discussed in the light of the CODIT-theory.

6.2 Material and methods

Sampled plants

During the period 1988-1990 samples from 19 young (age 3 to 5 years) ash trees (*Fraxinus excelsior* L.) were examined. These trees were part of inoculation experiments carried out in 1988 and 1989 (cf. Chapters 4 and 5).

Seventeen of the trees were sampled in the dormant period after treatment in early July 1988 or 1989. Ten of them (inoculated trees) had been wound-inoculated by making an incision into the secondary xylem of the lower stem and inserting a drop of a conidial suspension of *Verticillium dahliae*. The other seven (control trees) were treated in the same way but received sterile water only (see Chapter 4 for details). During the same season all inoculated trees developed severe symptoms of ash wilt disease, whereas all control trees remained symptomless.

Two additional trees, one control and one inoculated tree, were sampled in June 1990, i.e. in the second growing season after treatment in early July 1988. The inoculated tree had developed severe symptoms of ash wilt disease in 1988 but recovered (i.e. was free of symptoms) in 1989 and 1990. The control tree was symptomless in all three years.

Sampling, sections and staining

Samples were collected from all trees at several distances above and below the point of inoculation, both from the side of inoculation and from the opposite side of the stem. Samples were fixed in FAA (formalin-acetic acid-alcohol). Sections, 20-25 μm (transverse) and 15-20 μm (radial and tangential) thick, were made with a sledge microtome. They were examined either unstained or after staining with safranine and astra blue or acridine-chrysoidine and astra blue to help differentiate between tissues. In some cases selective staining was used for identifying lignin (phloroglucinol in HCl), starch (potassiumiodide: IKI), lipids (Sudan III) or polyphenols (toluidine blue).

6.3 Results

6.3.1 Trees sampled in the dormant period after the season of treatment

General pattern (Fig. 6.1)
Near the point of wounding each of the 17 trees had formed a barrier zone of axial parenchyma. The parenchyma generally was preceded by a zone of remarkably thin-walled libriform fibres. Control trees (wounded but not infected) continued growth after formation of the barrier zone. In and directly behind the barrier zone often again macropore vessels were formed resulting in the appearance of a false growth ring (Fig. 6.2). Vessels near the inoculation side were occluded by tyloses and yellow-brown depositions. In inoculated trees, however, the growth ring formed in the year of inoculation remained narrow in most cases (Fig. 6.3), whereas many vessels were occluded. Occluded vessels were not restricted to the inoculation side.

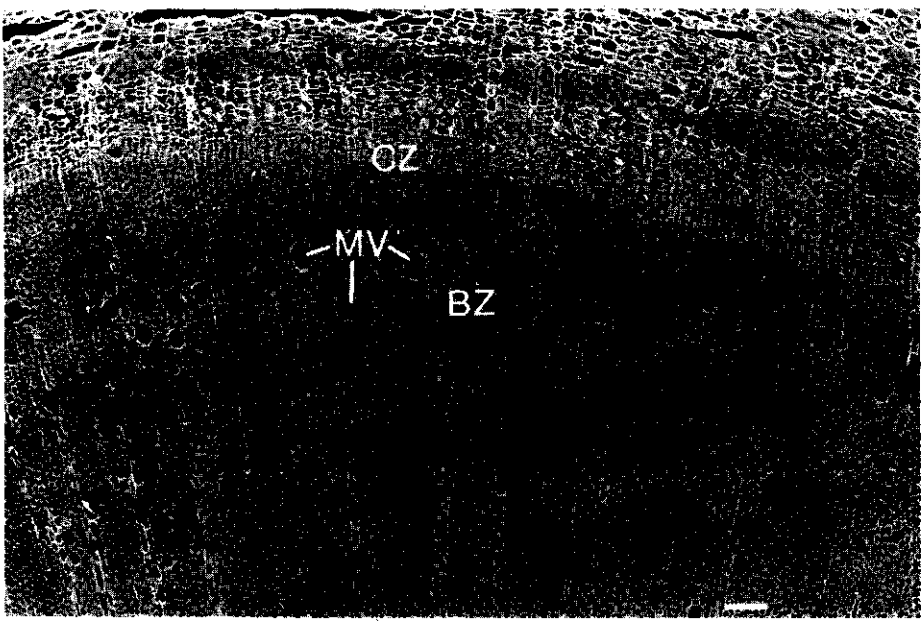


Fig. 6.1 General pattern in stemwood anatomy in wound-inoculated ash tree: macropore vessels (MV) in false growth ring, barrier zone (BZ) and thin-walled fibres at both sides of the barrier zone. (CZ cambial zone). SEM transverse section 10 mm above the point of inoculation. Bar 100 μm.

Barrier zone

This zone consisted of several layers of parenchyma cells in a regular radial pattern (Fig. 6.4). The cells were of the same form and dimensions as the normal axial wood parenchyma and were filled with starch. Staining with phloroglucinol in HCl showed the lignin content in the parenchyma cell wall of the barrier zone to be higher than in the normal axial parenchyma and similar to that in the marginal parenchyma cell wall. Ray parenchyma cells were swollen where they contact the barrier zone parenchyma. Within the barrier zone macropore vessels were present. Tangential sections displayed an abnormal form and arrangement of the vessel members of these vessels. The vessel members were very short, often almost spherical, and appeared to be arranged staircase-like instead of on top of each other (Fig. 6.5).

The barrier zone was widest near the point of wounding, the radial number of cell layers becoming less with increasing distance from the wound in tangential as well as longitudinal direction. In trees inoculated with *V. dahliae* the barrier zone was wider and extended further than in the control trees (Table 6.1).

Table 6.1 Width (radial number of cells in widest part) of barrier zone.

Location[1]	Inoculated trees[2]	Control trees[3]
+200	0.5	0
+ 50	0.7	0.8
+ 30	1.7	0.8
+ 23	2.3	1.5
+ 15	3.3	1.8
+ 10	4.3	3.3
+ 2	5.5	5.3
ip	*[4]	*[4]
- 3	7.5	6.3
- 8	6.3	3.5
- 14	5.0	3.0
- 22	3.7	*[4]
- 30	3.0	2.8
- 50	2.3	*[4]

[1] Vertical distance to point of inoculation (ip) in mm.
[2] Averages for three inoculated trees.
[3] Averages for two control trees.
[4] Data not available.

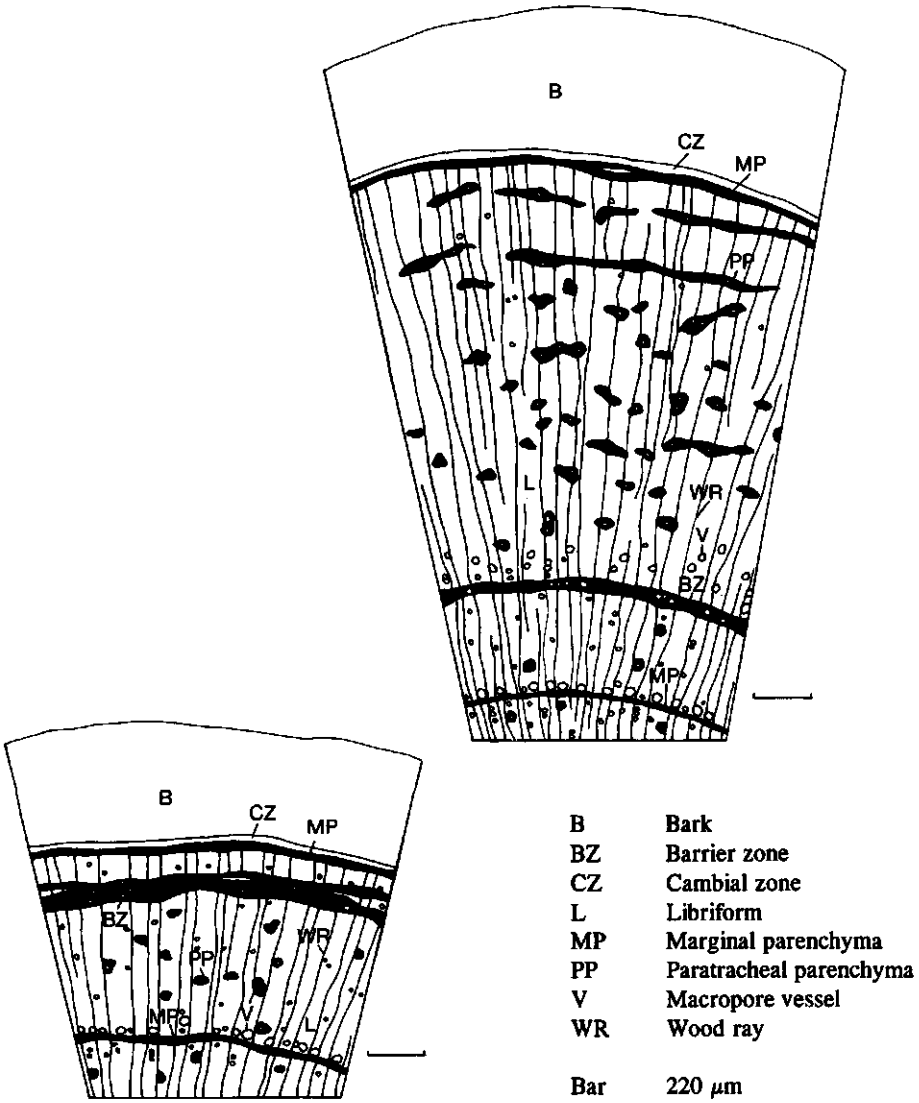
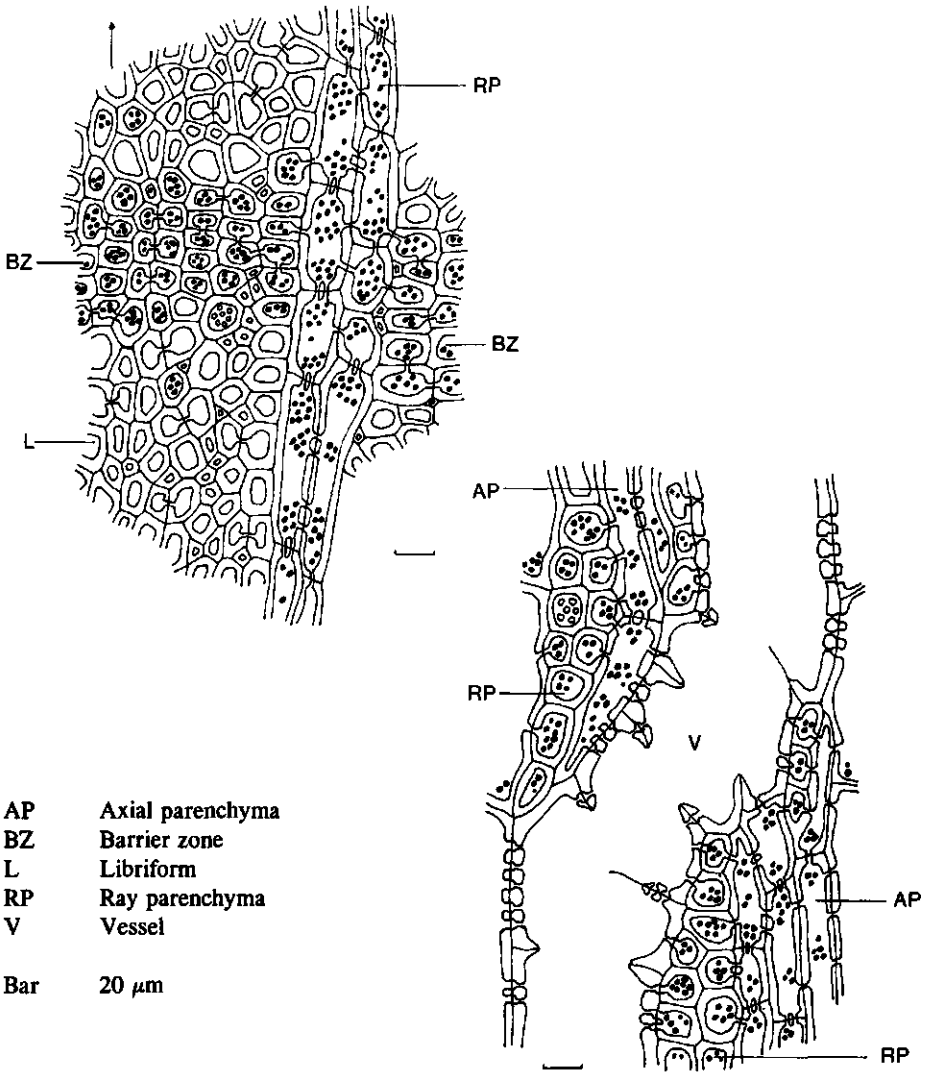


Fig. 6.2 (Top) False growth ring in the stem of a control tree (wounded but not inoculated). Transverse section 2 mm above the wound.

Fig. 6.3 (Bottom) Narrow growth ring in tree that was stem-inoculated with *V. dahliae*. Transverse section 2 mm above the wound.



AP	Axial parenchyma
BZ	Barrier zone
L	Libriform
RP	Ray parenchyma
V	Vessel
Bar	20 μm

Fig. 6.4 (Left) Barrier zone in stem-inoculated tree. Transverse section 1.5 cm above the inoculation wound at the side of inoculation (→ direction of cambium).

Fig. 6.5 (Right) Macropore vessel in the barrier zone: abnormal form and arrangement of vessel members. Tangential section 3 mm below the inoculation point at the side of inoculation.

Vessels

Many vessels in the injured growth ring were occluded by thin-walled tyloses and yellow-brown granular or smooth deposits (Fig. 6.6 and 6.7). Staining with Sudan III or toluidine blue failed to reveal the identity of these deposits. In control trees (wounded but not infected) the occluded vessels were restricted mainly to the tissues near the inoculation site. In wounded and infected trees their number was much higher and they were not restricted to the area of wounding. In these trees they were frequently present in all sections taken above the point of inoculation.

Vessel distribution within the growth ring was changed in wounded trees as well as in wounded and infected trees. In trees from both groups large earlywood vessels and much smaller latewood vessels already had been formed at the time of treatment. After treatment, first a barrier zone was formed and then the trees reverted to formation of macropore vessels, partly in the barrier zone but also in the wood formed immediately outside the barrier zone (Fig. 6.1 and 6.2).

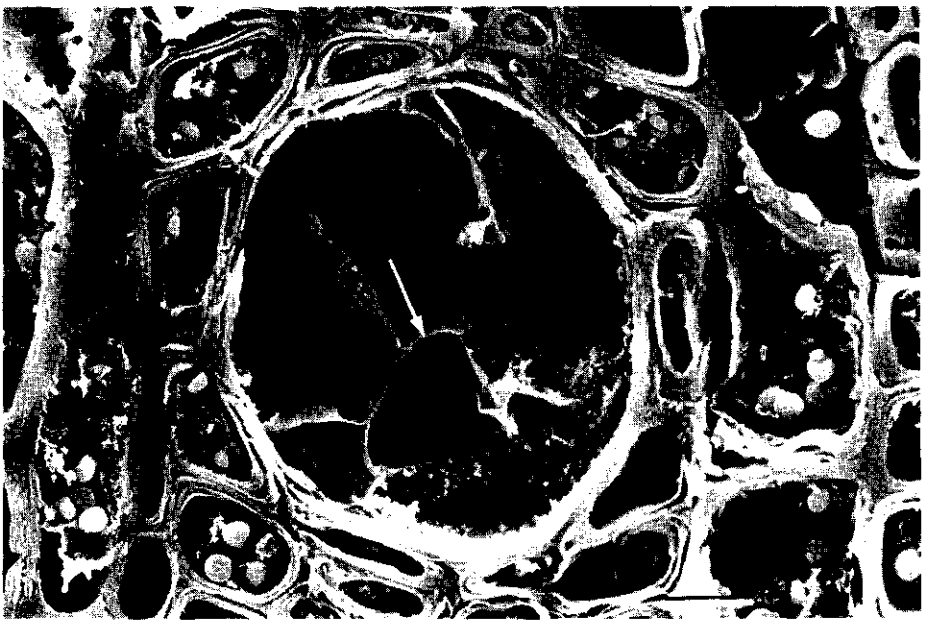


Fig. 6.6 Thin-walled tyloses (→) in a macropore vessel of an inoculated tree. SEM transverse section 20 mm below the inoculation wound. Bar 10 μm.

Ground tissue

In the tissues formed before the barrier zone a sudden transition from the normal non-septate libriform fibres with rather thick walls and small lumina to thin-walled fibres with larger lumina appeared (Fig. 6.1). This effect was strongest in the trees infected by *V. dahliae*. The thin-walled fibres were much less regularly arranged than the normal fibres formed before. Several types of septate fibres, sometimes with granular contents were observed and near the barrier zone more and more parenchymatous elements were present (Fig. 6.8). Tangential sections showed this 'transition parenchyma' to be different from the normal axial parenchyma. The cells were longer and had thinner walls (Fig. 6.9).

When secondary wood formation was continued after formation of the barrier zone (wounded trees), the first-formed libriform fibres were thin-walled but gradually the walls became thicker and finally normal latewood was produced.

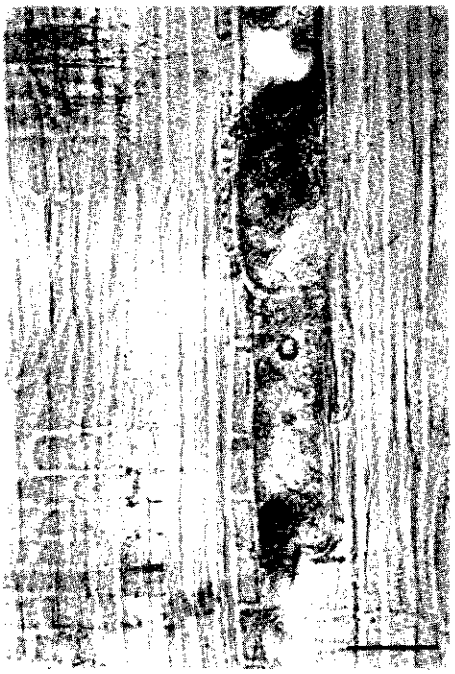


Fig. 6.7 Granular deposits in an occluded vessel (V) in the injured growth ring. Radial section, 35 cm above the inoculation wound. Stained with acridine-chrysoidine and astrablue, bar 50 μm.

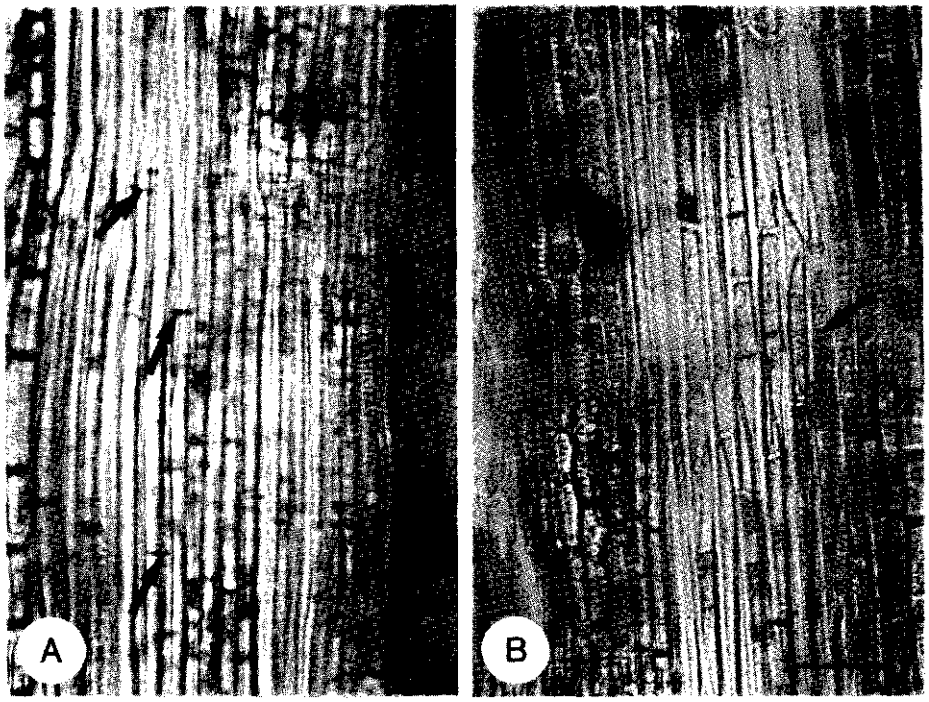


Fig. 6.8 Ground tissue inside the barrier zone: A. Thin-walled, septate fibres (→). Radial section, 140 cm above the inoculation wound. B. Transition parenchyma with granular contents (→). Radial section, 95 cm above inoculation wound. Both sections stained as in Fig. 6.7, bar 50 μm.

6.3.2 Trees sampled in the second growing season after treatment

The most striking characteristic of samples from the inoculated, recovered tree was the brown discoloration of the growth ring formed in the season of inoculation (1988). Toluidine blue to demonstrate polyphenols, however, did not stain this area and all parenchyma cells still contained starch. A second striking aspect of this tree was the large number of vessels occluded with gum-like deposits in the 1988 growth ring. The percentage of such vessels increased with height above the inoculation wound (percentages were determined in sections used for Table 6.1); also on the opposite side of the stem. The control tree (wounded but not infected) did not show these symptoms in the 1988 growth ring.

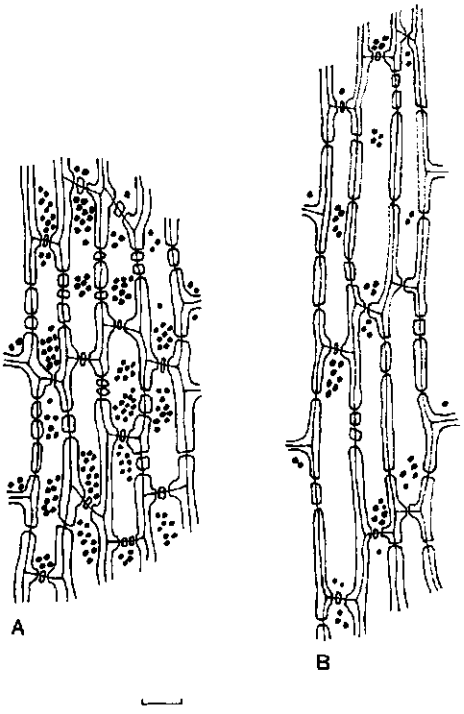


Fig. 6.9 Two morphological types of axial parenchyma observed in stem-inoculated trees. A. Parenchyma of the barrier zone (morphologically identical to normal axial parenchyma). B. Transition parenchyma produced before formation of the barrier zone, longer cells with thinner cell walls. A and B tangential sections, bar 20 μm.

The growth rings formed by both trees in 1989 were very similar, they only differed in the number and size of the earlywood vessels. In the inoculated tree 12 earlywood vessels were present per mm^2 with an average diameter of 62 μm, whereas in the control tree these figures were 16 per mm^2 and 85 μm, respectively. In the 1990 ring the differences between both trees were almost nil with 10 and 11 earlywood vessels per mm^2 and average vessel diameters being 103 and 108 μm, respectively.

6.4 Discussion

The wood anatomy of *F. excelsior* is characterized by a ground tissue of non-septate libriform fibres and a ring-porous vessel arrangement (Braun, 1970; Grosser, 1977). The axial parenchyma is concentrated in closed sheaths around the vessels (vasicentric, 1-2 cell layers wide) and in marginal bands (1-3 cells wide) at the margins of a growth ring. The rays are usually 2- to 3-seriate, composed only of procumbent cells. Earlywood vessels may be up to 350 μm wide, latewood vessels up to 50 μm (Grosser, 1977). Burggraaf (1972) reported an average diameter of 80 to 170 μm for the earlywood vessels of ash.

In the examined trees the maximum diameter of the earlywood vessels was even lower (up to 108 μm). This, however, is probably a juvenile characteristic. The same holds for the short tangential bands of axial parenchyma that were often found in samples from all examined trees. Generally the most important immature aspects in juvenile wood are smaller dimensions of all cells, a changed distribution of vessels and parenchyma, and a less pronounced ring-porous arrangement of the vessels (Braun, 1970; Jane, 1970). Eggenberger (1981; cf. Kuçera and Bariska, 1982) also reported that in ash the dimensions of the macropore vessels increase with age of the tree. Except for these juvenile characteristics, the general wood anatomy of the examined samples corresponded with the normal anatomy of *F. excelsior* as described by Braun (1970), Grosser (1977) and Schweingruber (1990).

The examination of wood sections of wounded control trees showed that in ash trees wounds are compartmentalized by the mechanisms as described by the CODIT-model (Shigo and Marx, 1977). Near the site of wounding the vessels were occluded (wall 1) and a barrier zone (wall 4) was formed, consisting of several layers of parenchymatous cells filled with starch. After formation of the barrier zone, growth of control trees was resumed with the formation of macropore vessels resulting in the appearance of a false growth ring.

In trees wound-inoculated with *V. dahliae*, the same mechanisms were operative. Infection by *V. dahliae* obviously stimulated the formation of the barrier zone which was more extended. But even in inoculated trees, it was limited to the environment of the wound. However, isolation experiments at the end of the season of infection (Chapter 4) demonstrated that the fungus was distributed throughout the infected plant above the inoculation site.

Hence, the reported recovery of wound-inoculated ash trees (Chapter 7) is not due to the formation of a barrier zone that completely seals off the infected part of the stem from the new, uninfected wood that is formed after infection.

The normal anatomy of *F. excelsior*, however, includes a marginal parenchyma band between each two growth rings. This parenchyma strongly resembles that of the barrier zone in wounded ash trees. The cells are of the same shape and in both cases the lignin content of the cells is higher than that of the other axial parenchyma. Therefore the marginal parenchyma in ash trees can be seen as a built-in barrier zone which may be effective in preventing outward spread of vascular pathogens.

Examination of sections from a recovered tree confirmed this view. In the infected growth ring the number of occluded vessels increased with the distance above the inoculation wound, indicating an increasing number of infected vessels. Beside the marginal parenchyma band no barrier zone was found in the infected growth ring, except near the inoculation wound. The almost normal anatomy of the next growth ring showed that the fungus was effectively contained within the inoculated growth ring. The small diameter of the earlywood vessels in this growth ring is a normal characteristic of trees under stress and has also been reported for elm trees recovering from Dutch elm disease (Banfield, 1968).

The vascular system in ash has been termed 'closed', i.e. the vessel networks in subsequent growth rings are interconnected (Braun, 1970). Hence for the marginal parenchyma to be effective as a barrier zone, it is necessary that the latewood vessels near the end of the infected growth ring are occluded. This was indeed observed in the recovered tree. One year after infection virtually all latewood vessels in the infected growth ring were occluded, in contrast to those in control trees. The occlusions were most easily seen in longitudinal sections under a binocular. In radial sections generally only part of the vessels showed occlusions because the vessels were not occluded over their entire length.

Two types of vessel occlusions were present in *Verticillium*-infected growth rings; thin-walled tyloses in the macropore earlywood vessels and gum-like, often granular material in the small latewood vessels (cf. the illustrations in Miller and Hiemstra, 1987). Both types of vessel occlusions are common in trees infected by vascular pathogens (Beckman, 1964; Sutic and Sinclair, 1991). Bonsen (1991) reported that the tyloses in *Fraxinus* are no real tyloses but merely a kind of balloon-like gum structures. However,

because he calls them ash-tyloses ('Eschen-thyllen') and because they are generally referred to as tyloses, in this work this term is used.

The zone with thin-walled fibres, septate fibres and long thin-walled parenchyma cells formed inside the barrier zone seems to indicate a gradual transition from the formation of libriform fibres to the formation of axial parenchyma strands. First the libriform fibres become thin-walled, then they become septate and strands of oblong parenchyma cells appear, and finally the zone with short, strongly lignified parenchyma fibres is formed. According to Rademacher *et al.* (1984), this indicates that instead of libriform, parenchyma is formed that has retained some structural and chemical characteristics of the libriform.

The cambium changes to formation of live protective tissues at the expense of dead tissues providing strength (libriform) and conductivity (vessels). The strongly deformed macropore vessels observed in the outer part of the barrier zones, apparently are a general characteristic of barrier zones formed after wounding, as they have been reported before in *Acer rubrum* (Mulhern *et al.*, 1979) and *Liquidambar styraciflua* (Moore, 1978).

The above results confirm that the defensive reactions of wounded ash trees correspond with those described by the CODIT-model. They also showed that the vascular pathogen *V. dahliae* strongly stimulates formation of the barrier zone, 'wall 4' of CODIT, in such trees. However, although the fungus could spread throughout the plant, the barrier zone was limited to an area around the inoculation wound. As far as it is anatomically visible, the barrier zone is apparently mainly related to wounding of the plant. Hence in this case wall 4, in contrast to suggestions by Tippett and Shigo (1981) and by Bonsen *et al.* (1985), seems to play a limited role in compartmentalization of the infection.

Walls 1 (plugging of the vessels) and 3 (rays) were neither effective, as the fungus was able to colonize the whole infected growth ring above the point of inoculation. In inoculated trees the failure of wall 1 might be thought to result from the direct introduction of large numbers of conidia into the injured vessels. However, this wall also failed in naturally infected trees. Wall 3 is bound to fail because of the vessel network passing these walls, which offers the pathogen a by-path.

The above does not implicate that the concept of compartmentalization does not hold for ash trees infected by *V. dahliae*, but the most important component is wall 2 (marginal parenchyma bands). Thus, in relation to vascular infections, the xylem anatomy of *F. excelsior* is both its weak point

and its strong point. On the one hand, the ring-porosity makes ash very vulnerable to vascular infections (Braun, 1982). But on the other hand, the marginal parenchyma forms a very effective built-in barrier zone. Together with the closure of the adjacent micropore vessels, the presence of this parenchyma makes complete recovery possible after infection by a vascular pathogen because the cambium is efficiently protected.

Recovery of infected trees is also strongly enhanced by the fact that in ring-porous trees the vast majority of the water is transported in the earlywood vessels of the youngest growth ring. This system is replaced every year and therefore plugging of the vessels resulting from vascular infection in one year does not affect water transport in the next year, provided that the cambium survives. This is greatly enhanced by the presence of marginal parenchyma in every growth ring.

6.5 Summary

The pathological anatomy of stem wood of young ash trees (*Fraxinus excelsior*), that were wound-inoculated on the stem with the vascular pathogen *Verticillium dahliae*, was examined and related to the concept of compartmentalization as described by the CODIT-model. Main pathological changes that were observed included vessel occlusion by thin-walled tyloses in earlywood vessels and by gum-like, often granular, deposits in latewood vessels, and formation of a barrier zone of parenchymatous cells near the wound. In wounded, but not inoculated, trees both abnormalities were restricted to the wood near the wound. In inoculated trees, virtually all vessels in the infected growth ring were occluded. Again, although more extended, the barrier zone was restricted to the environment of the inoculation wound. It is concluded that in these trees formation of a barrier zone is essentially a wound-related reaction. Nevertheless, the concept of compartmentalization holds, but formation of a barrier zone is not essential to it. The bands of marginal parenchyma, that are part of the normal wood anatomy of ash, function as built-in barrier zones. In combination with extensive vessel occlusion in the infected growth ring, as observed in inoculated and recovered ash trees, they are effective in containing the pathogen. This allows complete recovery of infected trees, especially because in ring-porous trees like ash the main water transport is by earlywood vessels that are replaced by newly formed ones every year.

Acknowledgements

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7 RECOVERY OF VERTICILLIUM-INFECTED ASH TREES

7.1 Introduction

In Chapter 4 it was experimentally shown that the ash wilt disease is caused by *Verticillium dahliae* Kleb. However, in contrast to naturally infected trees, all inoculated trees recovered, even after showing serious symptoms of wilt and defoliation in the season of inoculation. In order to get more information on recovery of ash trees infected by *V. dahliae* and to investigate the differences in disease development between naturally infected and inoculated trees, it was decided to monitor the development of both groups of trees over several years (experiments 1 and 3). In addition, the development of a second group of naturally infected trees was monitored (experiment 2) and the inoculation experiment was repeated with a new set of trees (experiment 4). In the latter experiment, inoculation was repeated several times during two successive years in order to test the hypothesis that repeated infection might lead to dieback or death of ash trees.

7.2 Material and methods

7.2.1 Naturally infected trees

Experiment 1

In September 1988, 37 healthy (Disease Class 0) and 71 seriously diseased ash trees (Disease Class 3) were selected in several parts of the country. From each tree, three branch samples were taken and examined for the presence of *V. dahliae* (see Chapter 4). The trees ranged in age from 10 to 25 years. They were marked with paint on their stem and a numbered label, and their positions were recorded on maps. At the date of sampling, and again in September 1989 and September 1990, the condition of the trees was recorded using disease classes (DC, see Chapter 2) ranging from 0 (healthy) to 5 (dead).

Experiment 2
In September 1990, 110 healthy ash trees (DC 0) and 60 trees with distinct symptoms of ash wilt disease (DC 3) were selected within a 15-years-old ash stand in the province of Flevoland (Horsterwold section Pz5-a1). The trees received a paint mark on the stem indicating their state of health (green: DC 0, red: DC 3). In October 1991, the trees were rated again. On that date the condition of each tree and its condition in the previous year, as indicated by its paint mark, were recorded.

7.2.2 Inoculated trees

Plants and inoculum
The inoculated plants were three-years-old ash seedlings, planted in rows of 10 trees with 1 metre spacing between trees and rows on an experimental field near Wageningen (Afweg) in April 1988 (see Chapter 4). For all inoculations an isolate of *V. dahliae* was used that had been isolated in the year before from an ash tree with severe symptoms of ash wilt disease. The inoculum was prepared on Czapek-Dox liquid medium (see Chapter 4).

Experiment 3
Throughout the growing season of 1988 half of the plants in 17 successive rows were inoculated at the lower part of their stems by one of two methods: (a) insertion of a large drop of a conidial suspension (10^7 conidia/ml) directly into the xylem vessels of the plant by a horizontal cut through the bark into the xylem: s(suspension)-type; or (b) placement of a clump of fungal material (a spherical mass of hyphae and microsclerotia of 3-5 mm diameter) in between the bark and the wood of the plant through a T-cut: m(mycelial)-type. At each date of inoculation some new rows of plants were treated, finally resulting in 55 s-type and 30 m-type plants. Control plants (55 + 30) were treated with sterile water instead of inoculum. Inoculated and control plants were alternated in the rows (chessboard pattern).

Experiment 4
In June 1989, a second inoculation experiment was started in two plots: one of 50 trees (plot A) and one of 39 trees (plot B). The plots were located about 40 meters apart on the same experimental field (Afweg). Three groups of inoculated plants (groups 1, 2 and 3) and three groups of control plants

(groups 4, 5 and 6), were selected using a table of random digits (Table A3 in Cox, 1958). Inoculated plants were treated with a conidial suspension of *V. dahliae* as described for experiment 3. Control plants were treated with sterile water. The dates of treatment and the concentrations of the inoculum are given in Table 7.1. In 1989 groups 1 and 4 were treated early in the growing season, groups 2 and 5 were treated early in the growing season and again in the second part of the season and groups 3 and 6 were treated only once, in the second part of the growing season. In 1990 part of the trees in the groups 1, 2 and 3 (randomly selected) were inoculated again (treatment numbers 1-1, 2-1 and 3-1). The remaining trees in these groups (treatment numbers 1-0, 2-0 and 3-0) as well as the control trees (treatments 4, 5 and 6) were not treated again.

Table 7.1 Experiment 4: Dates of inoculation (x) and inoculum concentrations (conidia/ml).

Treatment number	Date of treatment			
	20 June 1989	3 August 1989	15 June 1990	13 August 1990
1-0	x	-	-	-
1-1	x	-	x	-
2-0	x	x	-	-
2-1	x	x	x	-
3-0	-	x	-	-
3-3	-	x	-	x
Inoc. conc.	33×10^{6}	42×10^{6}	23×10^{7}	39×10^{5}

Observations

The development of the plants in both experiments was monitored by describing symptom development and measuring growth in 1989, 1990 and 1991. Symptoms were recorded using the same codes as described for experiment 1. Growth of the trees was assessed by measurement of height and diameter at the start of the experiment and again before the beginning of each growing season. Because of the small dimensions of the trees at the start of the experiment, the diameter was measured 10 cm above soil level using a diameter tape with a millimetre scale. The height was measured with a measuring stick with a centimetre scale and later with a telescopic measuring

pole also in centimetres.

All data of height and diameter growth were analyzed statistically using one-way analysis of variance (STATGRAPHICS computerprogramme, STSC Inc.).

7.3 Results

7.3.1 Development of naturally infected trees

Experiment 1

Because of cutting of some trees due to road building activities and because of disappearance of labels, only 116 of the initial 150 trees were left in 1990. The overall results of these trees showed a strong tendency of recovery. The number of healthy trees (DC 0) increased strongly, while the number of trees with distinct symptoms of ash wilt disease (DC 3) decreased dramatically (Table 7.2).

Analysis per 1988 disease class reveals that of the diseased trees:
- 31% were showing symptoms (DC 2 or DC 3) one year later.
- 13% were dead (DC 5) or showing dieback (DC 4) one year later.
- 24% were classified healthy and 32% were classified dubious.

This means that 56% of the diseased trees were without symptoms (DC 0 or 1) one year later. In the next year this increased up to 69%.

The group of 31 trees classified as healthy in 1988 showed a different development (Table 7.2). In 1989, three of these trees were classified as dubious and one tree showed distinct symptoms of ash wilt. In 1990, again two trees were classified as dubious, one tree showed slight symptoms, and even three trees showed distinct symptoms of ash wilt disease.

In order to answer the question from which disease class the 'new' trees with symptoms of ash wilt disease originate, the results were analyzed in another way. For the trees showing distinct symptoms (DC 3) in 1989 and 1990 it was determined from which disease class in the preceding year they originated. It appeared that:
- Of the 14 diseased trees (DC 3) in 1989 one tree had been healthy the preceding year. The other 13 trees were showing recurrent symptoms.
- Of the seven diseased trees (DC 3) in 1990 five had been free of symptoms the preceding year and one was classified as dubious in that year. Only one tree showed recurrent symptoms.

Table 7.2 Experiment 1: Condition in September 1989 and September 1990 of the trees that were healthy (DC 0) and the trees that were showing distinct symptoms of ash wilt disease (DC 3) during the survey in September 1988.

Disease Class (=DC)	DC 0 in 1988 (N=31) Numbers (%) per DC in		DC 3 in 1988 (N=71) Numbers (%) per DC in	
	1989	1990	1989	1990
0 Healthy	27 (87)	25 (80)	17 (24)	39 (55)
1 Dubious	3 (10)	2 (7)	23 (32)	10 (14)
2 Slight symptoms		1 (3)	9 (13)	8 (11)
3 Distinct symptoms	1 (3)	3 (10)	13 (18)	4 (6)
4 Dieback			4 (6)	5 (7)
5 Dead			5 (7)	5 (7)

Experiment 2

The results for the second group of trees show the same trends as described for experiment 1:

- Of the group of 110 trees that were healthy in 1990, 88% were still healthy in 1991, 6% were classified as dubious, 4% showed slight symptoms, and 2% showed distinct symptoms of ash wilt disease.
- In the group of 60 trees with distinct symptoms in 1990 a strong tendency of recovery was apparent. Only 7% of these trees were showing recurrent symptoms in 1991 and 40% (DC 0 + DC 1) were without symptoms of ash wilt. Another 40% of this group either showed dieback (25%) or were dead (15%) in 1991.
- From the 18 trees with foliar symptoms in 1991, 12 trees had shown recurrent symptoms, and 6 trees had been healthy in the year before.

7.3.2 Symptom development of inoculated trees

Experiment 3

The development of symptoms in the year of inoculation (1988) already was discussed in detail in Chapter 4. Here it is sufficient to recall that all dates of inoculation resulted in development of symptoms a few weeks later by almost all plants and that symptom development was faster and much more extensive after inoculation by conidia (s-type inoculation) than after inoculation by

hyphae and microsclerotia (m-type inoculation).

In 1989 only one tree (s-type, inoculated on 2 August 1988) developed symptoms of ash wilt disease. All other trees, control trees as well as inoculated trees, remained free of symptoms during the full growing season.

In 1990 again the vast majority of the trees remained free of disease symptoms. In this year, however, five trees developed symptoms of ash wilt; four trees inoculated in 1988 and one control tree. In three cases (1 s-type and 1 m-type [number M-8] both inoculated on 2 August 1988, and the control tree) wilt and almost complete necrosis or loss of leaves developed in the second part of the growing season (after 1 August 1990). The other two trees (both s-type, inoculated on 12 July and 17 August 1988, respectively) showed symptoms already on 21 June 1990. In the second part of the same growing season, the latter tree (number O-6) developed characteristic regrowth symptoms (very short new shoots with small chlorotic leaves).

In 1991 a similar trend was observed. By the beginning of August, two trees (1 s-type inoculated on 2 August 1988, and the control tree that had shown symptoms in 1989) had developed symptoms of ash wilt. By the end of September, two other trees (1 s-type inoculated on 2 August 1988 and a control tree) also showed symptoms of ash wilt. In this year also dieback and death of trees appeared. At the beginning of the growing season two trees (numbers M-8 and O-6) were dead and of another tree (s-type inoculated on 17 August 1988, without symptoms in 1989 and 1990) survived only in the lower part of the stem.

Experiment 4

In the year of inoculation (1989) symptom development was similar in both plots (Fig. 7.1):

- All trees inoculated early in the growing season (treatment 1) developed symptoms of ash wilt disease, mostly within one month after inoculation. In some trees it took more time before symptoms appeared, but at the end of the growing season all trees in this group were showing distinct symptoms.
- A second inoculation in August of the same season (treatment 2) did not cause more pronounced symptoms.
- After inoculation late in the growing season (treatment 3) symptom development was slower and the symptoms were less pronounced than after early inoculation (treatment 1). About 50% of the trees under treatment 3 developed only slight symptoms or no symptoms at all.
- Control trees never developed symptoms and only one inoculated tree died.

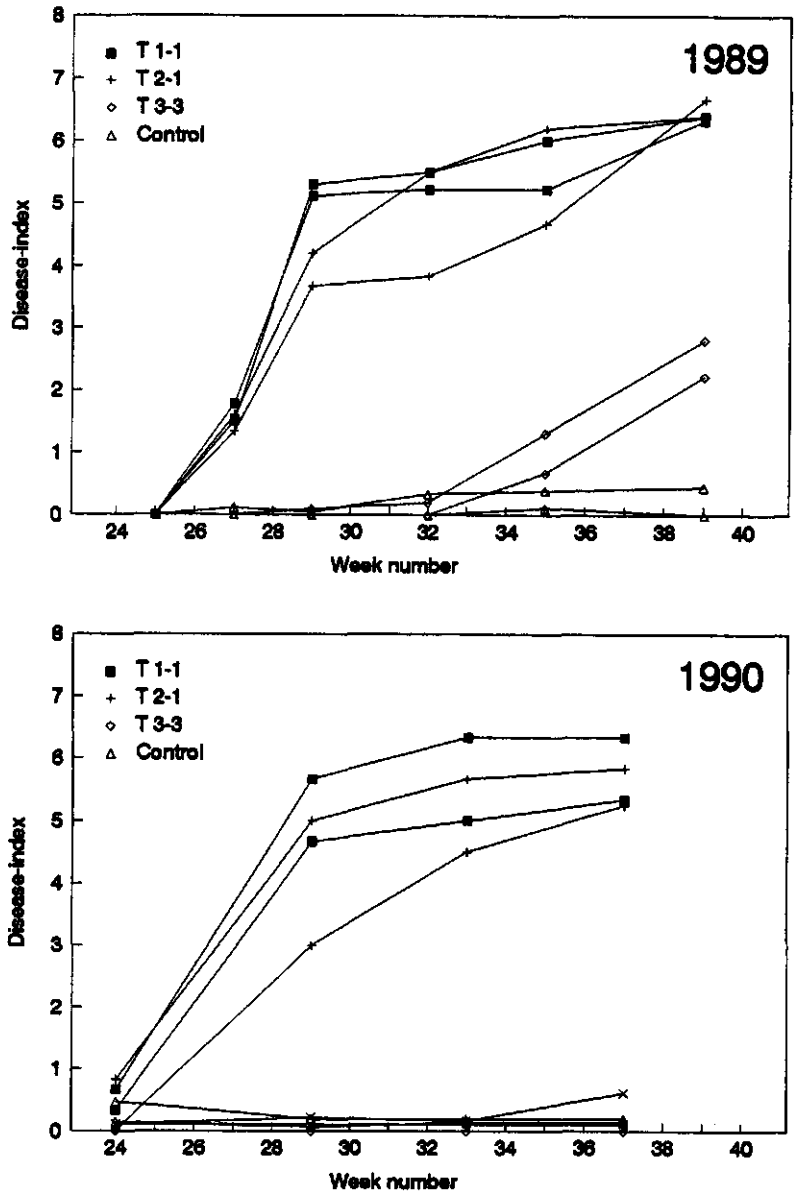


Fig. 7.1 Experiment 4: Average disease index in 1989 and 1990 for all groups of inoculated trees. For each year the lines of both plots are combined in one figure. (See Table 7.1 for the date of treatment and Appendix II for explanation of the disease index).

In the second year of the experiment (1990) symptom development after early re-inoculation showed the same pattern as after the first treatment in 1990, but symptoms were slightly less pronounced (Fig. 7.1). In 1990 inoculation late in the growing season completely failed to cause symptoms. All trees inoculated in 1989 but not re-inoculated in 1990 remained free of symptoms in 1990 and none of the trees died or showed dieback.

In 1991 trees were not treated again. In this year all trees of each treatment seemed to have recovered, as none of them showed any symptoms of ash wilt disease and dead trees or trees showing dieback were completely absent.

7.3.3 Growth of inoculated trees

Experiment 3

This experiment was originally designed only for a practical test of two methods of inoculation, repeated on a new set of plants several times, and for monitoring of symptom development, but not for statistical evaluation of the effect of the date of treatment on growth of the plants. Hence, the plants inoculated on the same date were in rows instead of being randomized. Because of this experimental design, it was not possible to separate the effect of the date of treatment from biasing factors (i.e. block effects). By pooling the plants per treatment type, however, it was possible to compare the effects on growth of the three types of treatment (control, s-type, m-type).

The number of trees on which growth data are based differs from the number of inoculated trees because in 1988 and 1989 several trees were removed for re-isolation experiments (Chapter 4) and anatomical research (Chapter 6). The plants in border rows and plants showing recurrent symptoms after one or more years were also excluded from the calculations. The remaining number of trees per treatment is included in the tables.

It appeared that diameter and height growth of trees inoculated with *V. dahliae* were less than those of control trees. Analysis of the results shows a significant treatment effect on both diameter and height growth in the year of treatment as well as in the next year (one-way ANOVA, $P < 0.05$). In the second and third year after inoculation diameter growth of inoculated plants was still significantly reduced (except for m-type in third and fourth year) but height growth was not (Table 7.3).

The growth reduction depended on the type of treatment. After s-type inoculation growth was reduced more strongly than after m-type inoculation.

Table 7.3 Experiment 3: Growth of inoculated and control trees in four years following treatment.

Treatment[1]	Number of trees	1988-II[2]	1989	1990	1991
			Height growth[3] (cm)		
s-type	25	22 ± 4.7 a	96 ± 6.9 a	167 ± 7.9 a	158 ± 10.5 a
m-type	23	31 ± 5.0 ab	113 ± 7.1 a	187 ± 6.1 a	167 ± 8.7 a
control	55	41 ± 3.9 b	141 ± 4.5 b	178 ± 4.0 a	167 ± 5.2 a
			Diameter growth[3] (mm)		
s-type	25	2.3 ± 0.42 a	11.7 ± 0.73 a	13.1 ± 0.83 a	3.9 ± 0.53 a
m-type	23	3.3 ± 0.46 ab	16.2 ± 1.10 b	13.6 ± 0.92 a	6.5 ± 0.96 ab
control	55	3.9 ± 0.32 b	20.2 ± 0.52 c	17.2 ± 0.76 b	9.1 ± 0.59 b

[1] s-type = inoculated with a conidial suspension of *V. dahliae*.
m-type = inoculated with microsclerotia of *V. dahliae*.
control = non-inoculated trees.

[2] Second part of growing season (after measurement on July 28).

[3] Means ± their standard errors; letters indicate significant differences (multiple range analysis, C.I. 95%).

Experiment 4

During the three-year period of this experiment only one inoculated tree died due to infection by *V. dahliae*. Five others were lost due to other causes (i.e. broken top, girdling by rabbits and caterpillars). Data concerning these trees were excluded from the analysis. The resulting numbers of trees per treatment are shown in Table 7.4 together with means and standard errors for height and diameter growth in 1989.

Table 7.4 Experiment 4: Height and diameter growth[1] in 1989 of young ash trees that were inoculated early, or early and late, or late in the growing season of 1989.

Treatment[2]	Number of trees	Height growth (cm)	Diameter growth (mm)
		plot A	
1	10	25 ± 3.0 a	6.0 ± 0.67 a
2	6	34 ± 9.7 ab	5.0 ± 0.55 a
3	10	50 ± 10.3 ab	12.4 ± 0.75 b
4	6	53 ± 4.8 ab	14.0 ± 1.15 b
5	6	74 ± 12.9 b	13.6 ± 2.16 b
6	6	45 ± 11.7 ab	12.8 ± 0.71 b
		plot B	
1	9	16 ± 3.6 a	3.9 ± 0.62 a
2	10	25 ± 6.1 ab	4.5 ± 0.36 a
3	9	30 ± 7.0 ab	9.2 ± 0.68 b
4	3	41 ± 8.8 ab	9.9 ± 0.51 b
5	3	44 ± 15.5 ab	11.5 ± 4.26 b
6	3	51 ± 6.4 b	12.9 ± 1.67 b

[1] Represented by means ± SE; different letters within one column and plot indicate significant differences (multiple range analysis, 95% conf. int.).

[2] 1 = early inoculation, 2 = repeated (i.e. early and late) inoculation, 3 = late inoculation, 4-6 control groups.

As in the previous experiment, inoculation with *V. dahliae* had a negative effect on both height and diameter growth in the year of inoculation. The size of the effect depends strongly on the date of the first inoculation. Although

not all differences are statistically significant it appears that in both plots:
- The trees of the control treatments (treatments 4, 5 and 6) grew fastest (except for treatment 6 in plot A).
- The trees inoculated early in the growing season (treatments 1 and 2) grew less than trees inoculated in the second part of the growing season (treatment 3).
- A second inoculation late in the growing season (treatment 2) did not cause additional reduction of growth. This agrees with the observation that repeated inoculation within one growing season did not cause more pronounced symptoms (Fig. 7.1).

For the second part of the experiment some treatment groups were pooled, according to the date of the first inoculation with *V. dahliae*, resulting in three groups:
- group 1 (inoculated early in the growing season; i.e. treatments 1 and 2),
- group 3 (inoculated late in the growing season; i.e. treatment 3), and
- group 0 (control trees; i.e. treatments 4, 5 and 6).

In 1990 part of the trees in the first two groups were inoculated again. Table 7.5 presents the height and diameter growth of these groups in 1989, 1990 and 1991.

In 1990 the following results were obtained:
- Repeated early inoculation (treatment 1-1) reduced growth significantly when compared with control trees (treatment 0-0).
- The growth of trees inoculated late in the growing season of two successive years (treatment 3-3) was reduced, but the difference with the control treatment was not statistically significant.
- The growth of trees inoculated only once, early in the growing season of 1989, was still significantly less than that of control trees.
- Trees inoculated only once late in 1989 (treatment 3-0) had recovered completely in 1990 as their growth was equal to that of the control trees.

In 1991 trees were not re-inoculated. The growth in that year, however, showed a pattern similar to that in the year before:
- Growth of trees inoculated early in two successive years (1989 and 1990) was still significantly reduced, except for the height growth in plot B.
- Trees inoculated late in the growing season of two successive years recovered almost completely, as their height growth did not differ from that

of the control trees, and their diameter growth was only slightly but not statistically significantly reduced.
- Growth of trees inoculated once, early in 1989, is still considerably reduced although statistically not significant.
- Trees inoculated once, late in the growing season of 1989, again grew about as much as the control trees.

7.4 Discussion

Disease criteria
The condition of trees can be described using different criteria. The most obvious and in many cases the easiest method is describing the visible symptoms in trees, like discoloration and defoliation, as is often done in forest health surveys (e.g. Commission of the European Communities, 1991). Another method is monitoring the growth of trees. These methods do not necessarily yield the same results (Roloff, 1989; Makowka *et al.*, 1991). In this study both criteria were used to investigate the effect of *V. dahliae* infection on *F. excelsior*. Unfortunately detailed data on the growth of naturally infected ash trees were not available so that discussion of the effect of infection by *V. dahliae* on growth of *F. excelsior* will be limited to inoculated trees.

Disease progress in naturally infected trees
The first and most important conclusion to be drawn is that **ash trees naturally infected by *V. dahliae* have a substantial chance of recovery.** But there is a real but much smaller chance (less than 15% during these experiments) of dieback or death. When trees died, death always occurred within one year after onset of symptoms. Recurrence of symptoms was also observed.

Recovery of trees infected by *V. dahliae* is not uncommon and has been reported before in olive (Wilhelm and Taylor, 1965; Thanassoulopoulos *et al.*, 1979; Blanco-Lopez *et al.*, 1984), apricot (Taylor and Flentje, 1968), peach (Ciccarese *et al.*, 1990) and maple trees (Sinclair *et al.*, 1981). Dieback and death of part of the infected trees is reported by theses authors as well. Thus, disease progress in ash trees infected by *V. dahliae* is similar to that in several other tree species.

Table 7.5 Experiment 4: Height and diameter growth[1] of young ash trees repeatedly inoculated with *V. dahliae*.

T[2]	n	hg 1989-II[3]	dg 1989-II[3]	T[4]	n[5]	hg 1990	dg 1990	hg 1991	dg 1991
					Plot A				
1	16	29 ± 4.0 a	5.7 ± 0.47 a	1-1	7	80 ± 14.6 a	7.7 ± 0.54 a	77 ± 20.6 a	1.9 ± 0.70 a
				1-0	9	119 ± 9.0 ab	11.0 ± 1.10 a	134 ± 15.8 ab	4.8 ± 2.01 ab
3	10	50 ± 10.3 ab	12.4 ± 0.75 b	3-3	5	142 ± 7.1 bc	17.6 ± 1.49 b	161 ± 9.3 b	4.8 ± 1.15 ab
				3-0	5	155 ± 19.0 bc	17.5 ± 1.95 b	162 ± 11.5 b	10.5 ± 3.27 b
0	18	58 ± 6.4 b	13.5 ± 0.81 b	0-0	18	165 ± 7.5 c	17.8 ± 1.08 b	160 ± 9.8 b	9.4 ± 1.20 b
					Plot B				
1	19	20 ± 3.7 a	4.2 ± 0.34 a	1-1	9	49 ± 6.5 a	8.3 ± 0.66 a	106 ± 15.0 a	2.8 ± 0.72 a
				1-0	10	70 ± 11.7 a	11.3 ± 0.80 ab	135 ± 11.1 a	7.2 ± 0.89 ab
3	9	30 ± 7.0 ab	9.2 ± 0.68 b	3-3	4	99 ± 27.8 ab	17.6 ± 3.85 bc	123 ± 26.3 a	10.9 ± 3.04 bc
				3-0	5	134 ± 17.0 b	18.8 ± 1.55 c	126 ± 14.1 a	12.4 ± 0.82 bc
0	9	46 ± 5.7 b	11.4 ± 1.40 b	0-0	9	134 ± 8.6 b	21.4 ± 1.64 c	117 ± 12.8 a	12.6 ± 1.67 c

[1] Represented by means ± SE; height growth (hg) in cm, diameter growth (dg) in mm. Different letters within one column and plot indicate significant differences (multiple range analysis, $P < 0.05$).

[2] Treatment code for 1989: 1 = inoculated early in the growing season (original treatments 1 and 2);
3 = inoculated late in the growing season (original treatment 3);
0 = control trees (original treatments 4, 5 and 6).

[3] Growth in second part of the growing season, i.e. after treatment on June 20, 1989.

[4] Combined code for treatment in 1989 (first figure) and 1990 (second figure); for explanation see note 2.

[5] Number of trees.

The experiments also showed that new cases of ash wilt disease can originate within all disease classes, including DC 0, in the preceding year. This agrees with the findings of Hiemstra *et al.* (1990) who reported that there are no signs of reduced growth in the years before the first symptoms of ash wilt appear. Hence, **it is impossible to predict the new cases of ash wilt disease from the tree's condition the year before**. The same has been reported for *Acer* species infected by *V. dahliae* (Sinclair *et al.*; 1981).

Recovery of inoculated trees

The observed percentage of recovery after inoculation of almost 100% was unexpectedly high. Independent of the method of inoculation (s-type, m-type, repeated inoculation), recurrent symptoms as well as dieback and death of trees were almost completely absent. First it was supposed that this might have been a result of the disappearance of *V. dahliae* during the growing season as reported before by Wilhelm and Taylor (1965) and Vigouroux (1975). In that case, repeated infection might be necessary for recurrent symptoms as well as for dieback or death of *Verticillium*-infected trees to occur. However, the latter does not seem to be the case since repeated inoculation also failed to produce symptoms of dieback (experiment 4). Moreover, re-isolation experiments (Chapter 4) showed that at the end of the season of inoculation the fungus was still actively present in all inoculated trees. Besides, Sinclair *et al.* (1981) isolated *V. dahliae* from 1- or 2-year-old xylem overlaid by healthy uninfected xylem sheets in the stem of young maple trees.

Probably a very high percentage of recovery is possible because the trees were stem-inoculated, leaving the root system largely free of *V. dahliae*. Banfield (1968) reported the same for young elm trees stem-inoculated with *Ceratocystis ulmi* (= *Ophiostoma ulmi*). If the fungus failed to reach the base of the bole of inoculated elms or reached that point only in a few vessels, almost all trees recovered.

Mechanism of recovery

In ring-porous trees like ash, upward water transport almost exclusively takes place in the vessels of the current growth ring (Zimmermann, 1983). Hence, symptom expression after infection by vascular wilt pathogens depends upon the presence of the fungus in the xylem vessels of the most recent growth ring (Manion, 1991). Within these vessels the pathogen can spread rapidly by means of passive transport of conidia and penetration via pits from vessel to

vessel (Banfield, 1941; see Chapter 3).

When the pathogen is confined to the wood present at the date of infection by localization (Beckman, 1989) and compartmentalization (Shigo, 1984) processes and the cambium stays alive, then the tree may recover from infection by producing a new uninfected xylem sheath (Banfield, 1968; Emechebe *et al.*, 1974; Sinclair *et al.*, 1981; Tippett and Shigo, 1981).

However, if *V. dahliae* in the next season can spread to the newly formed xylem sheath, this may lead to recurrent foliar symptoms. The anatomical structure of a tree offers three possible routes by which *V. dahliae* may pass from one sheath of stem xylem to the next (Sinclair *et al.*, 1981): (1) direct growth, (2) growth into xylem traces of buds, thence into new shoots and the subjacent xylem sheet on twigs and branches, (3) radial growth in infected roots followed by upward movement into the current year's stem xylem.

As long as the infected plant is alive, *V. dahliae* is almost completely restricted to the xylem vessels due to its very limited capacity of colonizing healthy parenchyma cells and fibres (Agrios, 1988; see Chapter 3). Therefore *V. dahliae* crosses over to the xylem layer of the next growth period most easily in the roots of a tree because of the much higher percentage of vessels per unit area compared to stem and branch wood (Banfield, 1968).

Hence, **the recovery of all but one of the stem-inoculated young ash trees can be explained by the root system of the plants remaining free of *V. dahliae* and insufficient radial growth of the fungus in the stem for crossing over from the xylem sheath of one growth period to that of the next.** The latter aspect is probably due to the presence of a marginal parenchyma band in each growth ring (Chapter 6)

Recurrent symptoms may also be caused by re-infection (Sinclair *et al.*, 1981). For trees with recurrent symptoms that were inoculated only once, this implies natural infection, i.e. infection from the soil. At the beginning of the inoculation experiment the soil was considered to be free of *Verticillium*. However, during the first year of the experiment it was contaminated with *V. dahliae* through infected petioles falling down from inoculated trees. About 5% of these petioles is known to contain large numbers of microsclerotia of *V. dahliae* (Rijkers *et al.*, 1992).

Hence, recurrent symptoms in inoculated trees in the second and third year of the inoculation experiments (i.e. after initial recovery) probably resulted from natural (re-)infection. The two control trees in experiment 3 that developed symptoms in the third year of the experiment strongly support this hypothesis.

Decreasing susceptibility during the growing season

The spread of *V. dahliae* in infected trees takes place mainly by means of passive transport of conidia (Banfield, 1941; Born, 1974; Emechebe *et al.*, 1975). Data of Van den Ende (1958) and Banfield (1941) show that, after inoculation with a conidial suspension, initial distribution can be very fast and extensive. However, the maximum distance a pathogen can move after inoculation depends on vessel length (Zimmermann, 1983). Hence, the dimensions of the vessels influence the distribution of the fungus.

In the present experiments inoculation was carried out by making an incision in the stem through the bark into the outer xylem (see Chapter 4). Because the incision was only a few millimetres deep, the inoculum was introduced mainly into the vessels directly under the cambium. After early inoculation these were the very large earlywood vessels. In case of late inoculation, however, the inoculum was introduced in the latewood vessels (total diameter growth in the year of inoculation amounted to about 20 mm, see Table 7.5). In ring-porous trees like ash the summer vessels are not only much smaller in diameter but also much shorter in length (Zimmermann, 1983).

Due to these anatomical features, the initial spread of the pathogen after inoculation in the second part of the growing season is limited when compared to early inoculation. This may explain the less pronounced symptoms after inoculation in the second part of the growing season (Fig. 7.1).

A decreased susceptibility to wilt-disease pathogens during late wood formation has been reported for maple (*Acer* spp., diffuse porous) inoculated with *V. dahliae* (Caroselli, 1954; Schreiber and Mayer, 1992) and elm (*Ulmus* spp., ring-porous) inoculated with *Ophiostoma ulmi* (Pomerleau, 1965; MacHardy, 1978).

Effect on growth

The inoculation experiments showed that infection with *V. dahliae* leads to a reduction of the growth of the trees. Growth reduction is strongest after early inoculation. This is self-evident as the main symptoms of the disease (i.e. wilting and defoliation) reduce the photosynthetic capacity of a tree. Reduction of photosynthesis may even occur prior to development of the above symptoms as was reported by Bowden and Rouse (1991), who observed stomatal closure leading to inefficient respiration before foliar symptoms became apparent in *Verticillium*-infected potato plants.

Height growth of healthy trees mainly depends on their genetic characteristics and on the site conditions, whereas the diameter growth depends also on the growing space occupied by the tree (Leersnijder, 1992). Reduced height growth means reduced length growth of the top shoot and the leading branches. Hence, neighbouring uninfected and therefore faster growing trees will take over part of the growing space of infected trees. This may explain why, after one single inoculation of young trees in a closed stand, the diameter growth of inoculated trees lags again significantly behind the year after inoculation, despite the almost normal height growth in the same year.

Effect on intraspecific competition

F. excelsior is a light-demanding species that does not tolerate shading from the moment it reaches its sapling stage. Hence, the reduced height growth of trees infected by *V. dahliae* has important consequences for the dynamics of forest stands affected by ash wilt disease. Especially in dense fast growing young stands, death of many infected trees may be expected despite their initial recovery in the year after inoculation.

Figure 7.2 presents the distribution of the height and diameter of the trees in experiment 3. Early in the season of treatment (July 1988) the distribution of diameter and height were about the same for the three treatment groups (left part of the figure). At the end of the third year after treatment (September 1991), however, the differences between the three groups were conspicuous (right part of the figure). The inoculated trees, and especially those inoculated with a conidial suspension, appear more often in the lower height (Fig. 7.2, top) and diameter classes (Fig. 7.2, bottom) then the control trees.

This means that a relatively high proportion of the inoculated trees will be among the trees that die as a result of suppression. If, for example, all trees shorter than 5 m will die because of suppression within the next few years, then over 40% of the s-type inoculated trees, over 20% of the m-type inoculated trees and only about 10% of the control trees will die (dotted lines in Fig. 7.2). Whether these processes are of similar importance in older forest stands remains to be investigated.

Nevertheless, it can be concluded that **in young ash stands loss of trees affected by ash wilt disease not only results from death caused directly by *V. dahliae* but also from suppression of recovered trees by uninfected neighbouring trees.**

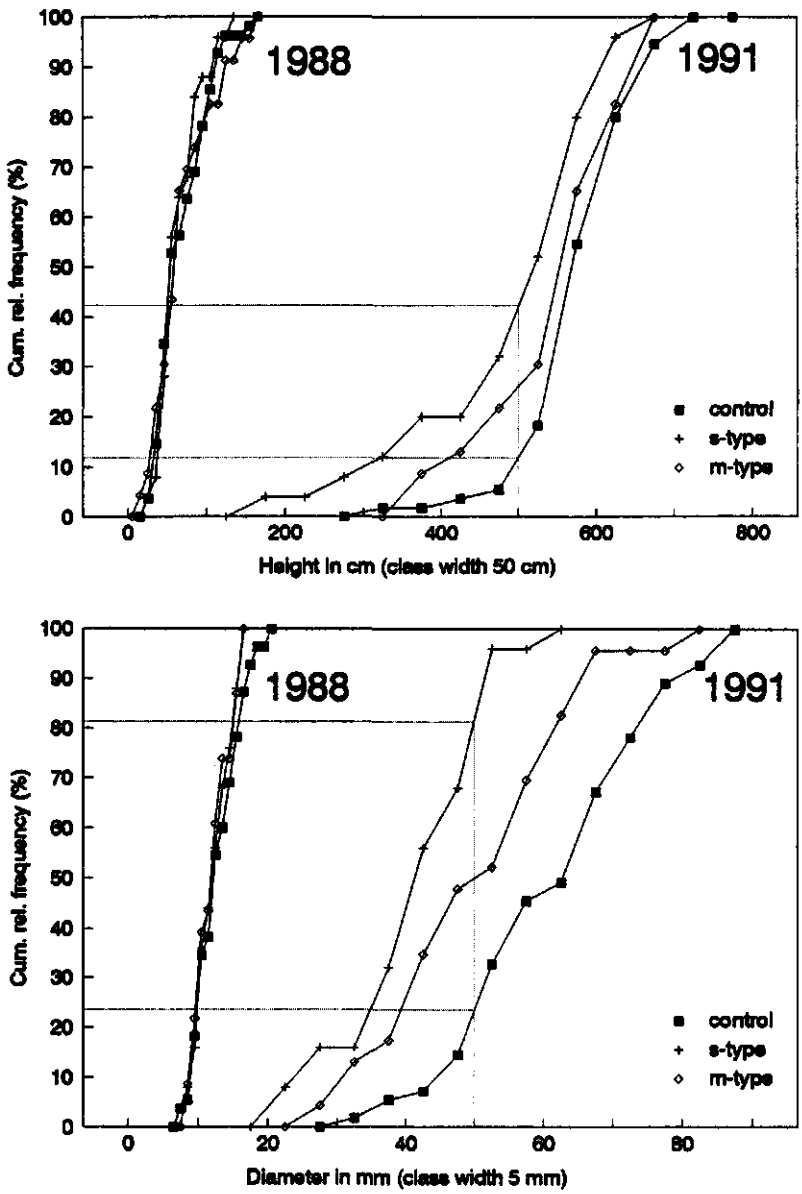


Fig. 7.2 Experiment 3: Cumulative relative frequencies of height and diameter at the time of inoculation in 1988 (to the left) and in September 1991 (to the right) for control trees and trees inoculated with *V. dahliae*. See text for explanation of the dotted lines.

Conclusion

From the above discussion it is obvious that spatial and temporal distribution of the fungus in its host are decisive factors in disease progress. Important factors that affect the distribution of *V. dahliae* in tree hosts are the anatomical features of the tree, its ability to localize and compartmentalize infection and the ability of the fungus to evade these processes.

The experiments discussed in this chapter did not directly address the above factors but were limited to monitoring disease development by means of regular assessment of symptoms and growth of infected trees. Therefore, for a real understanding of disease development and for knowledge about which factors determine recovery or death of infected trees, more research into the spatial and temporal distribution of *V. dahliae* in infected trees and the determining factors is indispensable.

A hypothesis compatible with all observations of the present research is that recovery of infected trees depends on formation of new, uninfected xylem and confinement of *V. dahliae* to the xylem already present at the time of infection. Dieback or death of trees results from the fungus reaching the cambium and killing it before a new sheath of xylem can be formed. Because of effective confinement of *V. dahliae* in the stem and branches of infected ash trees, a new penetration of the fungus into the current xylem is necessary for recurrence of symptoms. This penetration may result from re-infection or from crossing-over of the fungus from infected root xylem into the current xylem sheath.

7.5 Summary

The effects of infection by *Verticillium dahliae* Kleb. on young ash trees (*Fraxinus excelsior* L.) were studied. Disease development of 131 15-years-old forest trees naturally infected by *V. dahliae*, and disease development and growth of 102 inoculated 5-years-old trees in an experimental plot were monitored.

Recovery was common in naturally infected ash trees. About 50% of the diseased trees was without symptoms one year later. This percentage increased in the following years. On the other hand, some of the naturally infected trees suffered from dieback or died. Recurrent symptoms were rare. Inoculated trees never died or died back and only one of 102 inoculated trees showed

recurrent symptoms.

Recovery of infected trees is attributed to formation of new uninfected xylem and confinement of *V. dahliae* to the xylem already present at the time of infection. Dieback and death of infected trees is supposed to be the result of the fungus reaching the cambium and killing it before new uninfected xylem could be formed.

During the inoculation experiments, *V. dahliae* reduced growth of inoculated trees significantly, the magnitude of the reduction depending on the method and date of inoculation. The effect on diameter growth was greater and longer lasting than the effect on height growth. This has an ecologically important implication to forest stands affected by ash wilt disease. Despite initial recovery many of the affected ash trees will die because of suppression by healthy neighbours.

8 INCIDENCE, DISTRIBUTION AND PROGRESS OF ASH WILT DISEASE IN TWO FOREST PLOTS

8.1 Introduction

To assess the impact of a disease on forest stands, information on disease incidence and disease progress, and the spatial distribution of the disease in a forest plot is necessary. An extensive literature survey has revealed only two previous reports of Verticillium wilt in forests. Harrington and Cobb (1984) reported scattered dead and wilted plants of *Ceanothus integerrimus* Hook. & Arn., a common shrub species in disturbed coastal forests in California (USA), throughout most of a logged area of about 125 ha. Morehart *et al.* (1980) reported that in Delaware (USA) Verticillium wilt of *Liriodendron tulipifera* L. was observed in seedlings and occasionally in mature trees in both urban and forest settings. A survey of soil and root samples from 96 woodlands indicated that *Verticillium* spp. were present in 14.6% of the investigated sites. However, *V. dahliae* was isolated only from rhizospheres, whereas *V. albo-atrum* was isolated from root samples as well. Information on the actual disease incidence was not presented.

Further information on Verticillium wilt in tree species is limited to several fruit, shade and ornamental tree species. Most of the information concerns olive (*Olea europaea* L.).

Thanassoulopoulos *et al.* (1979) reported 1% mortality and wilt symptoms on 2-3% of the trees in the most important olive-growing areas of Greece. Disease incidence and intensity was greatest in newly established orchards, where average intensities of 25-30% were reported. Vigouroux (1975) reported that in France especially young olive trees were affected. Blanco-Lopez *et al.* (1984 and 1990) reported disease intensities varying from 0% to about 10% in many olive orchards in Spain. In some cases, affected trees formed patches with disease incidence to about 90%. All authors reported a strong year-to-year variation in disease incidence.

Dochinger (1956a,b) reported an average disease incidence of 3.4% for several maple (*Acer*) species planted as street trees in New Jersey (USA), and Henseler (1986) observed that in parks some individuals of *Catalpa* and *Fraxinus* died because of attack by *Verticillium* spp.

Recovery of at least part of the infected trees was reported for olive (*Olea europaea*: Wilhelm and Taylor, 1965; Thanassoulopoulos *et al.*, 1979;

Blanco-Lopez *et al.*, 1984), apricot (*Prunus armeniaca*: Taylor and Flentje, 1968; Vigouroux and Castelain, 1969), peach (*Prunus persica*: Ciccarese *et al.*, 1990), Catalpa (*Catalpa speciosa*: Carter, 1975) and maple trees (*Acer* spp.: Sinclair *et al.*, 1981). In all cases both dieback and death of part of the infected trees were reported as well.

The above data on disease incidence and recovery of other trees infected by *V. dahliae* do not necessarily apply to ash trees. Besides, disease impact in forest stands strongly depends upon the distribution of diseased and dead trees within those stands. In case of shade trees or trees in orchards every single tree is important. But in forest stands death of a limited number of trees caused by *V. dahliae* may have little or no impact on growth and wood production of the remaining stand. On the contrary, it may even be conceived as a mildly beneficial kind of a natural thinning.

For these reasons it was decided to investigate disease incidence, disease progress and disease distribution in seriously affected ash stands. Two permanent plots were established in different parts of the country and the state of health of the trees in these plots was monitored during the period 1987-1991. This chapter discusses the results and the assessment of disease impact.

Fig. 8.1 Location of the permanent plots in the provinces of Flevoland and Groningen.

8.2 Material and methods

8.2.1 Study sites

Two recently planted ash stands were selected, one in the province of Groningen and one in the province of Flevoland (Fig. 8.1). In each stand a permanent sample plot was established in the summer of 1987. A description of the stands and the plots is provided in Appendix III. At the start of the experiment all trees in the plots received an identification code consisting of a letter indicating the row and a figure indicating the place in the row.

The plot in Groningen (ppGr) measured 18 by 60 metres. It was situated on loamy fine sand overlying heavy clay at 80-120 cm depth. The soil had a pH ($CaCl_2$), of about 5 in the uppermost 25 cm. The mean water table in winter was 40-80 cm, in summer at more than 120 cm below soil level. The stand, situated in the forestry Oldambt (section 25j), was planted on former pastureland in 1973. Two-year-old seedlings of ash of unknown origin were used. The trees were planted in rows with 1 m spacing in the rows and 1.5 m between the rows. Each fourth row mainly consisted of auxiliary species. In 1987 most of these trees were strongly suppressed by the ash trees except some alders (*Alnus glutinosa* (L.) Vill.). In 1984 some ash trees with bad wood production characteristics were removed.

The plot in Flevoland (ppFl) measured 27 by 90 metres. It was situated on a clay soil with mean water table in winter 90 cm and in summer at more than 200 cm below soil level. In this plot the pH ($CaCl_2$) in the uppermost 25 cm of the soil was 8.5. The stand was situated in the Horsterwold forest (section Pz5-a1). This area was reclaimed from the sea in 1968. After the polder fell dry, ripening of the soil was stimulated by sowing reed (*Phragmites australis* (Cav.) Trin. ex Steud.) and drainage of the soil. Later rape seed (*Brassica napus* L.) was grown for a few years. In 1977 two-year-old ash seedlings were planted in rows with 1.25 m spacing in the rows and 1.5 m between rows. Each sixth row consisted of alder (*Alnus glutinosa*) that served as windbreak during the first years after planting. In 1987 many of these alders were dead or suppressed by the ash trees and in 1990 all alders were removed. In the ash rows some other species were interspersed, up to a total of 15%. In 1987 almost all these trees (except a few co-dominant individuals of *Acer campestre* L.) were overtopped by the ash trees. The only silvicultural measure taken since the planting was pruning of some misshapen trees in 1982.

8.2.2 Measurements and processing of data

In the permanent plots tree population dynamics (mortality and social position of trees), disease incidence, disease progress and spatial distribution of the disease were monitored during the period 1987-1991. Each plot was investigated at least once a year, at the end of the growing season. In 1988, 1989 and 1990 the plots were visited several times in order to establish disease progress during the growing season.

Tree population dynamics
Mortality and changes in the social position of the trees were recorded. At each assessment date the trees were classified in five social position classes (Fig. 8.2) based on both crown development and crown position relative to the crowns of adjacent trees and the general canopy. Four classes for canopy trees (classes 0-3) and one for understorey trees (class 4) were distinguished. The classification of the canopy trees was based on the definitions of 'Stammklassen' as given by Kraft (1884) and of 'crown classes' by Ford-Robertson (1971). Mortality was recorded together with disease symptoms (see below).

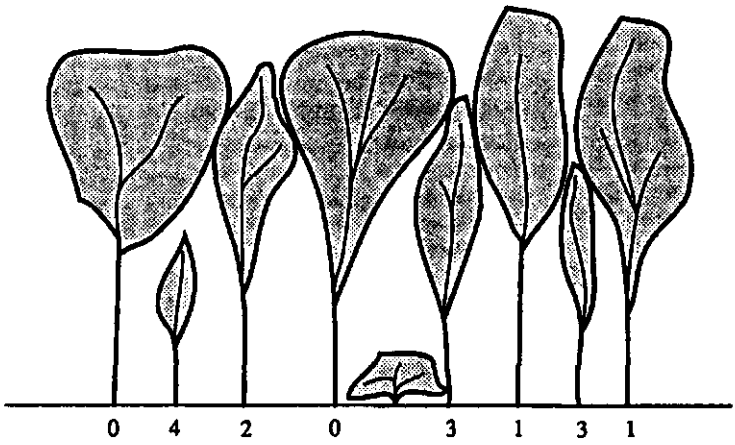
For evaluation of the data, the social position classes 0-2 (as recorded in September of each year) were taken together as dominant trees (i.e. trees with at least part of their crown free) and the classes 3 and 4 were pooled as suppressed trees. Only ash trees were included in this classification.

Disease incidence and progress
At each assessment date the state of health of all trees was recorded using a two-tier symptom code specially developed for describing the symptoms of ash wilt disease (see Chapter 2). The first part of this code, a figure ranging from 0 to 9, indicates the types of symptoms. The second part, one of the letters a to d, indicates the percentage of the tree with symptoms. Trees showing more than one symptom received a combination of symptom codes. This very detailed code enables reconstruction of the disease development in individual trees. For evaluation of stand data, however, a simpler code was needed, as usual in systems analysis. Therefore for each tree all recordings were transformed into disease classes (see Chapter 2) ranging from 0 (healthy) to 5 (dead). Then the numbers of trees per disease class for all dates of assessment were determined.

As ash is a light-demanding species, outshading by neighbouring trees may

lead to loss of leaves, dieback and death of trees, so that the effects of wilt and crowding cannot be distinguished. Therefore it was decided to include only dominant trees (social position classes 0-2) in the analysis of disease incidence and disease progress.

In disease progress two aspects were considered: 1. development of symptoms in diseased trees within the season of symptom onset, and 2. development of the same trees in successive years. Symptom development within a growing season was analyzed by comparing the several recordings per growing season in a year (1988-1990). Disease progress in the period 1987-1991 was analyzed by comparing the numbers per disease class as recorded in September of each year.



Code	Name	Definition
0.	Predominant	Trees with exceptionally large crowns in the upper canopy; the crowns are largely freely-growing.
1.	Co-Dominant	Trees with their crowns in the upper canopy; crowns are less free and tall than the dominant ones but freer and taller than the subdominant ones.
2.	Sub-Dominant	Trees having their crowns in the middle layers of the canopy, with only the leading shoots free.
3.	Suppressed	Trees with their crowns in the lower canopy; the leading shoots are not free.
4.	Understorey	Small trees with their crowns well below the canopy.

Fig. 8.2 Social position classes used during monitoring of tree population dynamics (modified after Kraft, 1884, and Ford-Robertson, 1971).

Spatial distribution of the disease

The position of all trees within the plots was determined exactly and expressed in x- and y-coordinates. Maps were constructed visualizing the data for each assessment date (e.g. Fig. 9.2). For analysis of the spatial distribution of diseased (DC 3) and dead (DC 5), trees these maps were divided into blocks. Several block sizes were analyzed because departure from randomness in a set of samples depends on the size and sometimes on the shape of the sample area used (Greig-Smith, 1983). Because of the rectangular shape of the permanent plots the blocks were not exactly square but rectangular. Each plot was divided into 54 blocks. Because of the difference in total area of the two permanent plots, these blocks were of unequal size in the two plots. In the ppGr-plot the smallest block size was 6 × 3.5 m, in the ppFl-plot it was 9 × 5 m. Larger block sizes were created by pooling two or more adjacent blocks. This resulted in block sizes of 6 × 7, 6 × 21, 18 × 7 and 18 × 21 m in the ppGr-plot and block sizes of 9 × 10, 9 × 30, 27 × 10 and 27 × 30 m in the ppFl-plot.

For all block sizes the numbers of dead and diseased trees per block were determined. For each of the resulting frequency distributions, the variance to mean ratio (relative variance or index of dispersion, $s^2 / \bar{x}$) was calculated. This ratio was used for analysis of the type of distribution of diseased and dead trees (i.e. random or non-random). If a frequency distribution equals a random distribution (Poisson distribution) this ratio is equal to 1. For a contagious distribution (i.e. aggregated or clumped) it is greater than 1, whereas for a regular distribution it is less than 1 (Greig-Smith, 1983; Fowler and Cohen, 1990).

Departure from randomness was tested by comparing the calculated ratio (observed ratio) with the expected ratio 1 (for a Poisson distribution) using Student's t-test. The t-values were calculated according to the equation:

$$t = (\text{observed variance to mean ratio} - 1) / \sqrt{(2/(N-1))}$$

in which N is the number of blocks (Greig-Smith, 1983). The calculated t-values were compared to critical values of Student's t distribution as provided by Table 12 in Rohlf and Sokal (1981).

8.3 Results

8.3.1 Disease incidence

Invariably the highest numbers of trees with distinct symptoms of ash wilt disease (DC 3) were recorded at the end of the growing season. During the early assessments (June and beginning of July) only a few diseased trees were recorded. From then on their number steadily increased with a strong increase in August and September (Table 8.1). The number of trees with slight symptoms (DC 2) showed the same tendency. Major dieback (DC 4) as well as death of trees (DC 5) mainly became visible during bud break in the year after symptom appearance (i.e. it developed either in the season of symptom appearance or during the next resting period). However, in some trees with a partly dead crown at the beginning of a growing season, the dead part of the crown gradually enlarged during the growing season.

In both plots the average yearly percentage of trees with distinct symptoms of ash wilt disease (DC 3) in the period 1988-1991 was less than 10% (Table 8.2). If the trees with slight symptoms (DC 2) are also included, the average yearly disease incidence in both plots was still below 15% (i.e. 15% of the dominant trees). However, considerable differences were observed between the two plots. In the ppGr-plot the disease incidence (DC 2 + 3) was rather stable with a maximum of 18% in 1990 and a minimum of 11% in 1988 and 1989. In the ppFl-plot the yearly percentages fluctuated much more strongly with a maximum disease incidence of 24% in 1988 contrasting with 6% in 1989 and 1990. The highest disease index occurred in different years in the two plots.

Table 8.1 Number of trees with distinct symptoms of ash wilt disease (DC 3) during the growing seasons of 1988, 1989 and 1990 in both plots.

Plot	1988[1]		1989[2]			1990[3]		
	July	Sept	June	July	Sept	July	August	Sept
ppGr	2	16	7	8	16	5	12	15
ppFl	4	132	2	4	8	3	7	10

[1] Dates of assessment for ppGr: 19/7 and 20/9; for ppFl: 28/6 and 28/9.
[2] Ditto ppGr: 8/6, 19/7 and 14/9; ppFl: 6/6, 13/7 and 7/9.
[3] Ditto ppGr: 20/7, 16/8 and 13/9; ppFl: 17/7, 14/8 and 12/9.

The average yearly mortality (Table 8.2) was about 1% of the dominant trees in both plots. In the ppGr-plot it fluctuated from 0.0% in 1990 to 2.9% in 1989, in the ppFl-plot from 0.2% in 1989 and 1990 to 2.1% in 1988.

Table 8.2 Disease incidence (% of dominant trees) in both plots in September of the years 1987-1991.

Disease class[1]	1987	1988	1989	1990	1991	mean[2]
			ppGr-plot[3]			
0	-[4]	68.5	63.2	60.0	61.8	63.4
1	-	13.6	19.3	19.4	22.4	18.7
2	-	2.2	1.2	8.5	8.6	5.1
3	9.1	8.7	9.4	9.1	4.6	7.9
4	3.2	5.4	4.1	3.0	2.0	3.6
5[6]	-	1.6	2.9	0.0	0.7	1.3
			ppFl-plot[5]			
0	-	54.4	87.4	84.6	70.7	67.5
1	-	18.8	8.8	8.8	18.9	12.7
2	-	5.5	4.1	4.1	4.3	3.4
3	3.2	18.6	1.7	1.7	5.3	6.2
4	1.3	0.6	0.7	0.7	0.4	0.5
5[6]	-	2.1	0.2	0.2	0.4	0.8

[1] Meaning of the codes (see also Chapter 2): 0 = healthy, 1 = dubious, 2 = slight symptoms, 3 = distinct symptoms, 4 = dieback, 5 = dead.

[2] Average value for the period 1988-1991.

[3] Dates of assessment 15/10/87, 20/9/88, 14/9/89, 13/9/90 and 4/10/91.

[4] Not recorded.

[5] Dates of assessment 13/10/87, 28/9/88, 7/9/89, 12/9/90 and 3/10/91.

[6] Only dead trees that were alive in the preceding year are counted, for the cumulative number of dead trees see Table 8.5.

8.3.2 Disease progress

In most cases the condition of diseased trees quickly worsened, leading to substantial defoliation a few weeks after appearance of the first symptoms. Not always the whole crown was affected. A few trees were observed with severe wilt and defoliation in part of their crown, another part still being healthy. In some cases this was the situation at the end of a growing season even when symptoms had started early.

If serious defoliation took place early in the growing season, some trees showed regrowth. Very short new shoots with small and chlorotic leaves grew from the terminal and major lateral buds on some or all defoliated branches (see Chapter 2, Fig. 2.4). These leaves never reached normal sizes and therefore these trees were still classified as diseased (DC 3).

Each year slightly different symptoms were recorded on a small number of trees. They showed dieback and often also small chlorotic shoots in part of their crown at the beginning of the growing season. During the growing season the dead part of the crown slowly enlarged and often wilt and defoliation developed gradually in the healthy part of the crown. In all cases these trees had been classified as diseased the preceding year.

A surprisingly high percentage of recovery was observed. Analysis of the condition after one and two years of all dominant trees that had been classified in the disease classes 2, 3 or 4 in one of the years 1987, 1988 or 1989 showed that:

- Most of the trees with slight symptoms (DC 2) were free of symptoms (DC 0 or 1) one year later. After another year, even more of these trees were without symptoms (Table 8.3A).
- Also many of the trees with distinct symptoms (DC 3) were free of symptoms in the next year (Table 8.3B). In the ppGr-plot the number of healthy trees further increased in the second year, resulting in 65% of the trees originating from DC 3 being free of symptoms after two years. In the ppFl-plot 72% of the DC 3 trees were without symptoms after two years.
- Even in the category of trees with dieback (DC 4), recovery of some trees was recorded (Table 8.3C). In these trees the dead parts of the crown were replaced by new branches formed by the surviving part of the tree.

Another important aspect of disease progress is partial dieback and death in diseased trees. Table 8.3 shows that:
- In the first year after symptom onset, dieback occurred both in trees with slight symptoms (DC 2) and in trees with distinct symptoms (DC 3) in the previous year. The number of trees showing dieback did not increase in the second year after symptom appearance.
- Death of trees occurred in all three categories (i.e. DC 2, 3 and 4). In the second year after symptom onset the number of dead trees in the DC 3 group increased strongly. Analysis of the results showed that this increase almost completely resulted from trees that had shown severe dieback in the preceding year.

8.3.3 Disease distribution

The spatial distribution of all dominant trees with distinct symptoms was analyzed for each year in both plots. The results are summarized in Table 8.4. It appears that in 1988 in both plots clumping occurred at several block sizes. In the other years in the ppFl-plot the distribution of diseased trees was random. In the ppGr-plot clustering was also detected at small block scales in 1987 and 1989, but never at block sizes of 6 × 21 m or higher.

Analysis of the distribution of all dominant trees that were dead at the end of the monitoring period (including the trees that were already dead in 1987) showed that in the ppFl-plot these trees were clustered at all block sizes examined. In the ppGr-plot they were clustered only at some block sizes.

8.3.4 Tree population dynamics

In both plots the number of living dominant trees decreased considerably in the period 1988-1991. Both the number of suppressed (living) trees and the number of dead trees (dominant as well as suppressed) increased (Table 8.5).

Table 8.3 The subsequent development of trees that showed slight symptoms of ash wilt disease (DC 2), distinct symptoms (DC 3) or dieback (DC 4) in one of the years 1987, 1988 and 1989.

Disease Class	Trees in ppGr		Trees in ppFl	
	after 1 yr	after 2 yrs	after 1 yr	after 2 yrs
	A. Trees with slight symptoms (DC 2)[1]			
0	1	4	34	29
1	1	0	7	8
2	1	1	1	4
3	1	0	0	0
4	1	0	1	1
5	0	0	2	3
total	5	5	45	45
	B. Trees with distinct symptoms (DC 3)			
0	17	24	90	97
1	8	7	33	21
2	3	3	7	17
3	7	2	16	0
4	9	5	5	6
5	4	7	13	23
total	48	48	164	164
	C. Trees showing dieback (DC 4)			
0	2	3	0	0
1	2	2	1	1
2	3	6	0	1
3	0	1	0	0
4	12	7	6	3
5	4	4	11	13
total	23	23	18	18

[1] Only trees that were classified in DC 2 in 1988 and 1989, data on 1987 not available.

Table 8.4A Spatial distribution of diseased and dead trees in the ppFL-plot; frequencies per block, number of blocks and the variance to mean ratio's for both plots. N is the number of blocks and P the probability that the observed frequency distribution follows a Poisson (i.e. random) distribution. R means random and C means clustered.

	DC5 1987	DC3 1987	DC3 1988	DC3 1989	DC3 1990	DC3 1991	DC3 87-91	DC3+5 87-91	DC5 1991
Block size 9 × 5 m									
N	54	54	54	54	54	54	54	54	54
Mean	.83	.44	2.44	.15	.19	.52	3.46	4.30	1.26
Variance	.93	.48	3.99	.13	.15	.48	4.56	5.95	1.86
Var/Mean	1.12	1.08	1.63	.87	.83	.93	1.32	1.38	1.47
t-value	.62	.39	3.25	-.68	-.87	-.37	1.62	1.98	2.44
P	>.5	>.5	<.01	>.4	>.2	>.5	>.1	>.05	<.02
Result	R	R	C	R	R	R	R	R	C
Block size 9 × 10 m									
N	27	27	27	27	27	27	27	27	27
Mean	1.67	.89	4.89	.30	.37	1.04	6.93	8.59	2.52
Variance	1.92	1.03	12.03	.22	.24	1.11	12.61	17.48	5.34
Var/Mean	1.15	1.15	2.46	.73	.65	1.07	1.82	2.03	2.12
t-value	.55	.55	5.26	-.97	-1.25	.27	2.96	3.73	4.03
P	>.5	>.5	<.001	>.2	>.2	>.5	<.01	<.01	<.001
Result	R	R	C	R	R	R	C	C	C
Block size 9 × 30 m									
N	9	9	9	9	9	9	9	9	9
Mean	5.00	2.67	14.67	.89	1.11	3.11	20.78	25.78	7.56
Variance	7.50	3.75	47.75	.36	.61	5.86	44.94	64.94	22.28
Var/Mean	1.50	1.41	3.26	.41	.55	1.88	2.16	2.52	2.95
t-value	1.00	.81	4.51	-1.19	-.90	1.77	2.33	3.04	3.90
P	>.2	>.2	<.01	>.2	>.2	>.1	<.05	<.02	<.01
Result	R	R	C	R	R	R	C	C	C
Block size 27 × 10 m									
N	9	9	9	9	9	9	9	9	9
Mean	5.00	2.67	14.67	.89	1.11	3.11	20.78	25.78	7.56
Variance	4.75	5.50	74.25	.61	1.11	4.11	82.44	104.94	21.78
Var/Mean	.95	2.06	5.06	.69	1.00	1.32	3.97	4.07	2.88
t-value	-.10	2.13	8.13	-0.62	0.00	0.64	5.94	6.14	3.76
P	>.9	>.05	<.001	>.5		>.5	<.001	<.001	<.01
Result	R	R	C	R	R	R	C	C	C
Block size 27 × 30 m									
N	3	3	3	3	3	3	3	3	3
Mean	15.00	8.00	44.00	2.67	3.33	9.33	62.33	77.33	22.67
Variance	12.00	21.00	457.00	2.33	2.33	46.33	472.33	632.33	154.33
Var/Mean	.80	2.63	10.39	.88	.70	4.96	7.58	8.18	
t-value	-.20	1.63	9.39	-.12	-.30	3.96	6.58	7.18	
P	>.5	>.2	<.02	>.9	>.5	>.05	<.05	<.02	
Result	R	R	C	R	R	R	C	C	

Table 8.4B Spatial distribution of diseased and dead trees in the ppGr-plot; frequencies per block, number of blocks and the variance to mean ratio's for both plots. N is the number of blocks and P the probability that the observed frequency distribution follows a Poisson (i.e. random) distribution. R means random and C means clustered.

	DC5 1987	DC3 1987	DC3 1988	DC3 1989	DC3 1990	DC3 1991	DC3 87-91	DC3+5 87-91	DC5 1991
Block size 6 × 3.5 m									
N	54	54	54	54	54	54	54	54	54
Mean	.83	.31	.30	.30	.28	.13	1.11	1.94	1.00
Variance	1.08	.45	.48	.44	.36	.11	.89	2.02	1.25
Var/Mean	1.30	1.42	1.61	1.48	1.28	.89	.80	1.04	1.25
t-value	1.55	2.15	3.13	2.48	1.44	-.58	-1.01	.19	1.26
P	>.1	<.05	<.01	<.02	>.1	>.5	>.2	>.5	>.2
Result	R	C	C	C	R	R	R	R	R
Block size 6 × 7 m									
N	27	27	27	27	27	27	27	27	27
Mean	1.67	.63	.59	.59	.56	.26	2.22	3.89	2.00
Variance	3.46	.78	1.10	.71	.64	.28	1.49	4.10	4.08
Var/Mean	2.08	1.24	1.85	1.20	1.15	1.07	.67	1.05	2.04
t-value	3.88	.86	3.07	.73	.55	.24	-1.19	.20	3.74
P	<.001	>.2	<.01	>.4	>.5	>.5	>.2	>.5	<.001
Result	C	R	C	R	R	R	R	R	C
Block size 6 × 21 m									
N	9	9	9	9	9	9	9	9	9
Mean	5.00	1.89	1.78	1.78	1.67	.78	6.67	11.67	6.00
Variance	6.00	2.61	3.19	2.69	2.50	.69	5.00	13.75	6.00
Var/Mean	1.20	1.38	1.80	1.52	1.50	.89	.75	1.18	1.00
t-value	.40	.76	1.59	1.03	1.00	-.21	-.50	.36	0.00
P	>.5	>.4	>.1	>.2	>.2	>.5	>.5	>.5	
Result	R	R	R	R	R	R	R	R	R
Block size 18 × 7 m									
N	9	9	9	9	9	9	9	9	9
Mean	5.00	1.89	1.78	1.78	1.67	.78	6.67	11.67	6.00
Variance	9.75	1.61	3.44	1.94	1.00	.69	6.50	22.25	12.00
Var/Mean	1.95	.85	1.94	1.09	.60	.89	.98	1.91	2.00
t-value	1.90	-.29	1.88	.19	-.80	-.21	-.05	1.81	2.00
P	>.05	>.5	>.05	>.5	>.4	>.5	>.9	>.1	>.05
Result	R	R	R	R	R	R	R	R	R
Block size 18 × 21 m									
N	3	3	3	3	3	3	3	3	3
Mean	15.00	5.67	5.33	5.33	5.00	2.33	20.00	35.00	18.00
Variance	25.00	2.33	26.33	10.33	1.00	1.33	49.00	139.00	31.00
Var/Mean	1.67	.41	4.94	1.94	.20	.57	2.45	3.97	1.72
t-value	.67	-.59	3.94	.94	-.80	-.43	1.45	2.97	.72
P	>.5	>.5	>.05	>.4	>.5	>.5	>.2	>.05	>.5
Result	R	R	R	R	R	R	R	R	R

Table 8.5 Forest dynamics; numbers of dominant and suppressed ash trees in both plots in the period 1988-1991.

Type of tree		Year			
		1988	1989	1990	1991
		ppGr-plot (N = 367)			
Dominant	- alive	181	166	165	151
	- dead	48	53	53	54
Suppressed	- alive	119	121	120	128
	- dead	19	20	21	26
Removed		0	7	8	8
		ppFl-plot (N = 940)			
Dominant	- alive	693	639	604	531
	- dead	60	66	67	69
Suppressed	- alive	87	104	102	132
	- dead	100	131	167	208

8.4 Discussion

Limitations

The above data on disease incidence and progress concern dominant trees (social position classes 0-2) only. Suppressed trees (classes 3-4) may be affected by *V. dahliae* as well, but in the field it is impossible to separate the suppression symptoms accurately from those caused by ash wilt disease. A silvicultural reason for limiting the analysis to the dominant trees is that they largely determine forest architecture. Forest production and yield are also mainly determined by the dominant trees.

Disease class 2 contains the trees with slight symptoms of ash wilt disease (i.e. wilt of less than 60% of the crown, or defoliation or dieback on less than 30% of their crown). In the dense young forests studied, these trees may easily be overlooked, especially if the symptoms are limited to a small part of the upper crown. The same applies to trees with dieback of part of their crown (DC 4). This problem however is not limited to this study, but is inevitably associated with all ground-based surveys for disease symptoms.

Therefore the numbers recorded for disease classes 2 and 4 are probably too low. On the other hand, if these trees are situated next to a dead or severely diseased tree they have a much greater chance of being noticed. Especially in the analysis of the spatial distribution of diseased trees this may lead to errors. For these reasons the analysis of the spatial distribution of diseased trees was limited to trees with distinct symptoms (DC 3) and dead trees (DC 5).

Disease incidence

During the field work the impression was gained that in the category of dominant trees the relatively large and vigourous trees were affected more frequently than the somewhat smaller trees. Analysis of the data on the social position of all trees in the disease classes 2 and 3, however, did not confirm this impression. Disease incidence was about the same for trees in each of the social position classes 0 (predominant), 1 (co-dominant) and 2 (sub-dominant).

The values for disease incidence and mortality rate correspond rather well to the few data on disease incidence in orchards as reported by other authors (Section 8.1). A strong variation in disease incidence as observed in this study has also been reported for Verticillium wilts of other tree species, especially olive (e.g. Thanassoulopoulos *et al.*, 1979; Blanco-López *et al.*, 1984) and apricot: 'mais certaines années, il ne se manifeste pas' (Vigouroux and Castelain, 1969, p. 428).

Many authors attribute the strong year-to-year fluctuation in disease incidence of Verticillium wilts to climatic fluctuations. Relatively high temperatures and low water availability are often thought to stimulate Verticillium wilt. Hence, the data on disease incidence were compared with the average monthly values for temperature and rainfall during the growing season. Data were obtained from climatic stations near the plots. However, no relation between varying disease incidences and variation in precipitation or temperature could be found (Fig. 8.3).

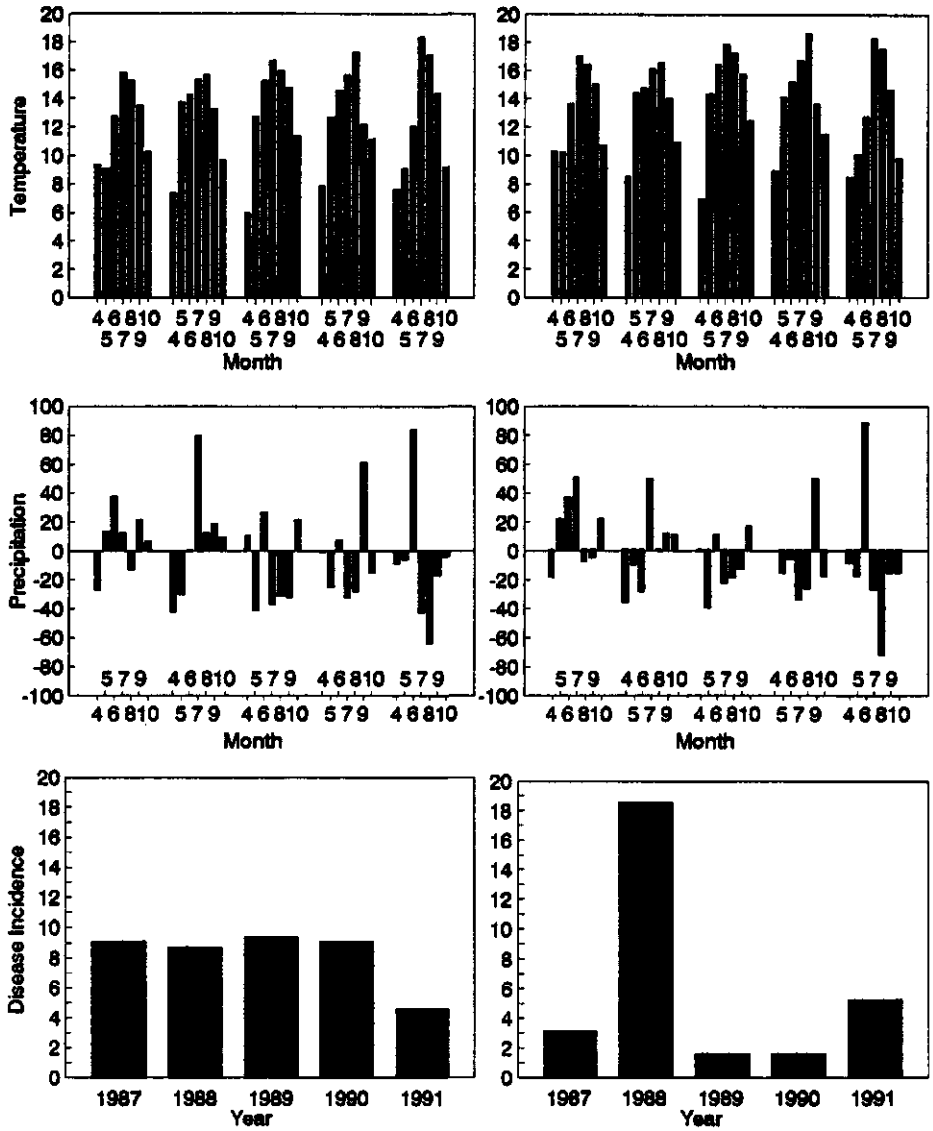


Fig. 8.3 Disease incidence per year, average monthly temperature, and rainfall per month (departures from normal) in the growing seasons of the years 1987-1991 for both plots (climatological data from KNMI, 1987-1991).

In literature the relations between the incidence of Verticillium wilt of trees and climatic factors are not quite clear either (Caroselli, 1957; Morehart and Melchior, 1982). On the one hand, moisture stress, as a result of drought periods, is reported to increase wilt symptoms in maple and tulip trees (Caroselli, 1957 and 1959; Morehart and Melchior, 1982). On the other hand, in olive, irrigation is known to increase incidence of Verticillium wilt (Schnathorst, 1981; Blanco-López *et al.*, 1984). Smith and Neely (1979) found the highest relative susceptibility of several tree species in periods with relatively high rainfall. Finally Bell and Mace (1981, p. 449) state that 'High moisture levels generally decrease resistance (i.e. against wilt diseases), except for some Verticillium wilts, which are largely unaffected by moisture level.' Dochinger (1956c) also reports that no definite relationship could be established between environmental factors, including rainfall and temperature, and the variations in prevalence of Verticillium wilt of maple species.

These inconsistencies may have several causes:

(1) The effect of an environmental factor is complex since it has an influence on each of the three biotic components involved in the production of the syndrome of wilt diseases, i.e. the pathogen, the host plant and interacting soil micro-organisms (see Chapters 3 and 10), and is complicated even more by interaction with other environmental factors (Katan, 1989).

(2) Differences may be caused by the host species used or by the use of different experimental procedures (Morehart and Melchior, 1982).

(3) The large daily temperature amplitude and the large fluctuations in daily temperature per month make it difficult to determine the influence of temperature on disease incidence, because these fluctuations vary strongly at a local scale and are hardly ever completely recorded. Similar problems are related to determining the influence of the amount of rainfall within a certain period.

(4) Effects of low or high amounts of rainfall are biased by adjustment of the water table for agricultural purposes in the surrounding areas.

These factors certainly affect the relation between disease incidence and rainfall and temperature in the plots. Besides, the period of one month for assessment of wheather data seems too long. Comparison with data over 10-day periods probably would give better results. Also climatic data collected in the plot should be used instead of average data for a larger area, as was done in this study. The use of physiological time-degree days (i.e. an accumulation of daily temperatures above a threshold value) as used by

Pullman and DeVay (1982) instead of Julian days also might improve the results.

Disease progress

In literature, several types of symptoms and disease progress in Verticillium wilts of tree species have been described. In *Acer* species, Piearce and Gibbs (1981) and Phillips and Burdekin (1982) discern three types: acute wilt, chronic wilt and overwinter attack (i.e. trees which had appeared healthy in the autumn fail to produce leaves in spring). In olive also both acute and chronic symptoms have been reported (Vigouroux and Castelain, 1969).

The symptoms in ash trees in forest plots generally appeared to be of the acute form with rapid wilting of the whole crown or at least most of it. In contrast to *Acer*, where in case of acute symptoms the leaves often remain on the tree (Piearce and Gibbs (1981), wilt in ash in most cases is followed by premature leaf fall. Gradual increase of symptoms, especially of dieback has also been observed but should be considered as a second phase in disease progress, perhaps a secondary complication, as it was only observed after acute symptoms in the preceding year.

There are three possibilities one year after acute symptoms: complete recovery, partial dieback, sometimes with recurrent symptoms, or finally death of a tree. This study has again shown that **in Verticillium wilt of ash, recovery of diseased trees is very common** (see also Chapter 7). Out of 212 trees with distinct symptoms examined in the period 1987-1989, 73% was free of symptoms in the next year; only 11% showed recurrent distinct symptoms (DC 3). This percentage is only slightly higher than the average percentage of trees with distinct symptoms in both plots in the same period. These results concur with the claim by Piearce and Gibbs (1981) that Verticillium wilt of tree species is essentially an annual event.

This may also explain the increasing disease incidence in the course of a growing season. *Verticillium dahliae* overwinters by means of microsclerotia in the soil or in tissues that were killed by the fungus (see Chapter 3). The microsclerotia in the soil germinate under the influence the exudates of young growing roots. As root growth proceeds, the number of microsclerotia encountered increases. Hence, the number of possible infections increases with time. The increasing temperatures early in the growing season allow the decomposition of the dead plant parts of the previous season, resulting in increased inoculum densities in the soil (Huisman and Gerik, 1989). This may affect disease incidence since disease incidence generally is known to be

related to inoculum density in the soil.

Because of the strong variation in disease incidence and because the present study covers only five years, it is impossible to conclude whether in the monitored plots the disease is still increasing, has reached its maximum level or is decreasing. Thus no any prediction of future disease incidence in the plots is yet possible.

Disease distribution

The results of the spatial analysis show that in the ppFl-plot (Table 8.4A) the trees affected by ash wilt disease have a contagious distribution. In most individual years the distribution of trees with distinct symptoms (DC 3) may be random (Fig. 8.4A), but over a period of 5 years the distribution of these trees (Fig. 8.5) is clearly clustered at almost all block sizes. At the start of the 5-year period dead trees were randomly distributed, but at the end of this period (Fig. 8.6) they were clustered at all block sizes. If the dead trees and all trees that showed distinct symptoms in the period 1987-1991 are taken together, the resulting distribution (Fig. 8.7) is clustered at all but the smallest block sizes.

In the ppGr-plot (Table 8.4B) clustering is much less distinct. Diseased trees (Fig. 8.4B) only appear clustered at some block sizes in some years. If all diseased trees in the five-year period are taken together (Fig. 8.5), no clustering is detected at all. The dead trees are clustered at some block sizes before as well as after the 5-year period (Fig. 8.6). And if the diseased and dead trees are taken together (Fig. 8.7) clustering is detected at one block size only.

Summarizing, **the distribution of dead and diseased trees is one of patches of higher density superimposed on a general distribution at lower density.** The higher density patches are not sharply delineated. Usually patches with relatively many dead and diseased trees also include some healthy trees. This result confirms earlier observations of the disease occurring both in small diffuse groups of diseased and dead trees, and as individual trees in forest plots (Hiemstra 1987a, Hiemstra *et al.*, 1990).

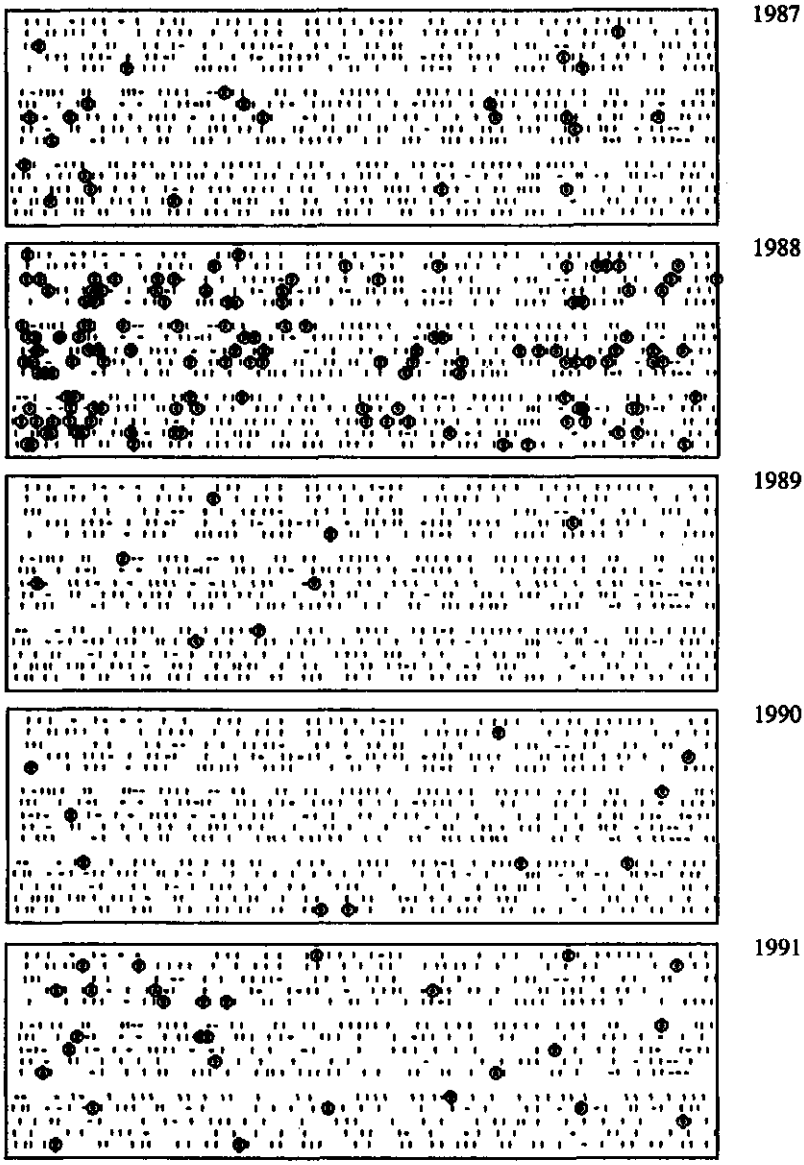


Fig. 8.4A Spatial distribution of the diseased trees (DC 3) in the ppFl-plot in five years (horizontal dash = dead tree, vertical dash = healthy tree, circel = diseased tree).

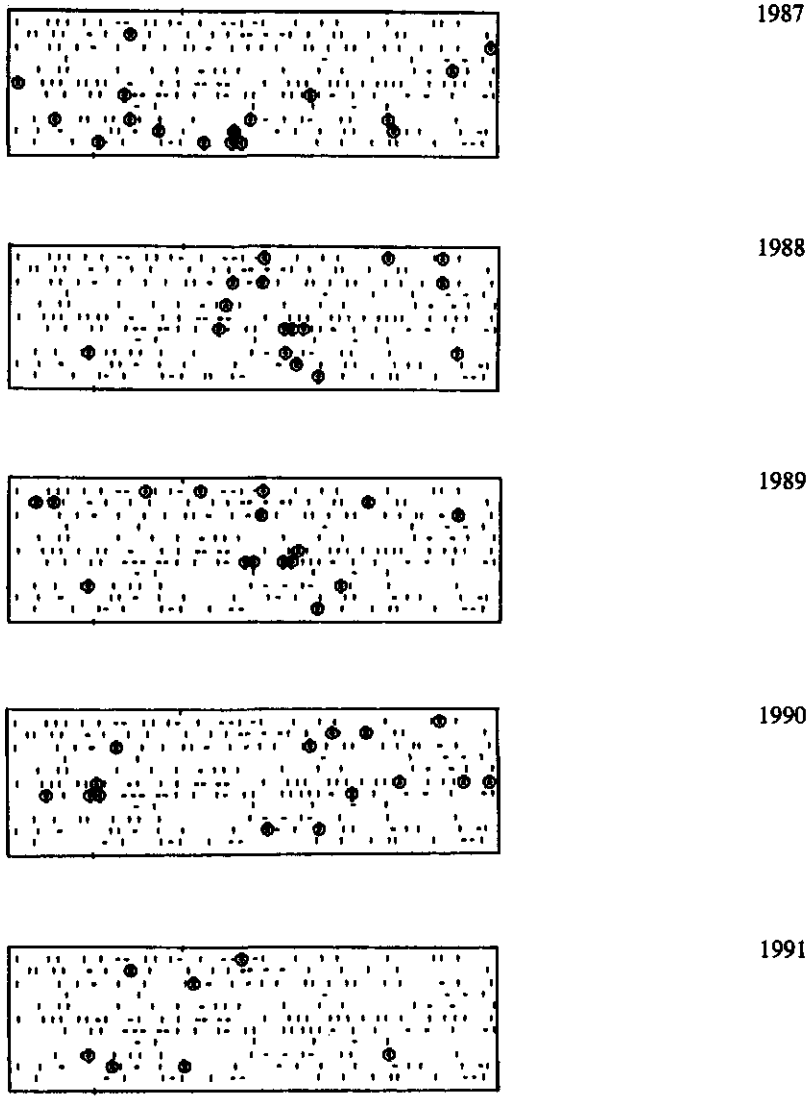


Fig. 8.4B Spatial distribution of the diseased trees (DC 3) in the ppGr-plot in five years (horizontal dash = dead tree, vertical dash = healthy tree, circel = diseased tree).

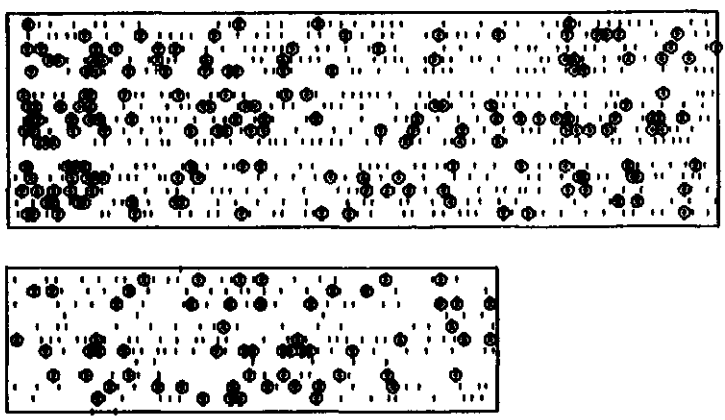


Fig. 8.5 Spatial distribution of all diseased trees (DC 3) in both plots in the period 1987-1991.

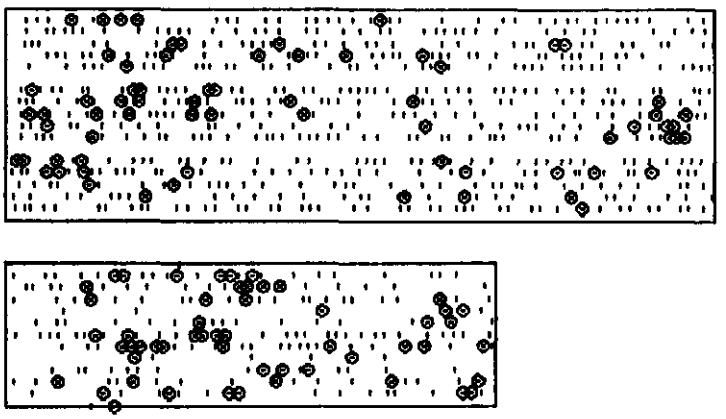


Fig. 8.6 Spatial distribution of dead trees (DC 5) in both plots in the period 1987-1991.

This type of distribution is very commonly found in the field (Greig-Smith, 1983). In this case, it is probably caused by a non-random distribution of soil inoculum of the fungus. For many hosts it has been shown that incidence of wilt caused by *Verticillium dahliae* is strongly related to the density of microsclerotia in the soil (see Chapter 3). In the case of Verticillium wilt of

ash the microsclerotia are spread by infected petioles from diseased trees (Rijkers *et al.*, 1992). Most of these petioles fall down near the tree from which they originate, but some wind-blown petioles may fall at least 20 to 30 m away from the base of that tree (personal observation with some individuals of ash in a maple stand of the same age as the permanent plots). After the petioles fall on the forest floor they start to decompose and are incorporated in the top soil layer by the activity of worms (Chapter 3, see also Fig. 10.1) and other soil organisms. The microsclerotia will play a role as secondary inoculum.

After the appearance of the first diseased trees in a stand, this mechanism certainly will lead to an initial distribution of secondary soil inoculum which is non-random but arranged along gradients. Hence, it can explain both the occurrence of newly diseased trees at some distance from previously infected trees, and the occurrence of clustering of diseased trees around earlier affected ones. If the distribution of secondary inoculum goes on for several years, the soil in the whole stand will become infested with microsclerotia from secondary inoculum sources. High disease incidence will accelerate this process and after some years the pattern cannot be distinguished from a random distribution any more. This may explain why in the ppGr-plot clustering was less conspicuous. This plot is situated in an ash stand that was among the first stands from which the disease was reported and the disease incidence, especially in the early years, was rather high.

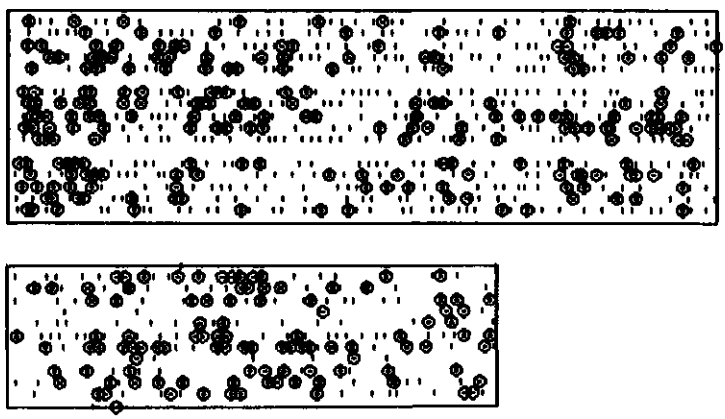


Fig. 8.7 Spatial distribution of all trees that were dead in 1987 and all diseased trees (DC 3) in the period 1987-1991.

Tree population dynamics

In both plots the decreasing number of dominant trees was mainly determined by suppression of trees as a result of outshading by neighbouring trees, which is a normal phenomenon in stand development. Ash wilt disease apparently had much less effect. This was most clear in the ppFl-plot.

In this plot in the four-year period 162 trees (i.e. 23% of the original number) disappeared from the category of living dominant trees (Table 8.5). Of these trees only nine (i.e. about 5%) were killed by ash wilt disease, the others disappeared because of outshading. In the ppGr-plot the situation was complicated by a thinning operation, executed in the autumn of 1988. In this plot 30 trees (i.e. 17% of the original number) disappeared from the category of living dominant trees: six trees were cut down, another six were killed by ash wilt disease, and 18 (i.e. 60%) were suppressed by the remaining dominant trees.

Hence, **the number of dominant trees in both plots was more strongly affected by mortality resulting from competition for light than by death of trees caused by ash wilt disease.** The difference between both plots in mortality of trees because of suppression probably is the result of the much lower initial density of trees in the ppGr-plot (1650 per ha versus 2650 per ha in the ppFl-plot). The removal of six predominant trees during the thinning operation in 1988 in ppGr further contributed to this effect. Table 8.5 shows that only in the second year after the thinning operation the number of suppressed trees increased again. In the first year it was almost stable.

Disease impact

The monitoring has shown that many wilt-affected ash trees recover completely within a year. As growth of these trees continues in the next year there is no permanent damage from a forester's point of view, except perhaps some loss in wood increment. The real damage, if there is any, would result from the death of dominant trees. Because of the low mortality rate caused by ash wilt disease in both plots (0.8 and 1.3%, respectively) this seems to be no real problem.

The distribution of the dead trees, however, is of great importance. A small number of dead trees, scattered through the stand, is no silvicultural problem. In even-aged stands the number of trees always and inevitably decreases with age. Large groups of dead trees, however, cause silvicultural problems because they diminish stand production and stability. Although some clustering of dead trees has been observed in both plots, large openings in the

canopy did not occur because of the small and diffuse nature of the groups of dead trees. The two plots were deliberately chosen in stands severely affected by ash wilt disease. This indicates that in young ash stands, like the two chosen plots, large openings in the canopy will occur only in extreme cases. If the disease goes on when the stands become older this may become a problem. However, so far this has not been reported.

Hence, **in young stands like the two plots, the disease should be seen as a slowly acting natural thinning agent rather than as a threat for the stand.** From an ecological point of view the disease in these stands may even have beneficial effects. Diversity is increased because of the new ecological niches resulting from the death of large dominant trees (Cosijn *et al.*, 1983; VanderKamp, 1991). Especially the increased light level on the forest floor under defoliated and dead trees and the standing dead wood are important determinants of this aspect of the disease.

8.5 Summary

This chapter addresses the question whether or not ash wilt disease might become a serious problem in forest management. A permanent plot was selected in each of two ash forests, one in the province of Groningen and one in the province of Flevoland, both seriously affected by ash wilt disease. Invariably the highest numbers of affected trees were recorded at the end of the growing season. In both plots less than 10% of trees with distinct symptoms of ash wilt disease were observed yearly. Because of a high percentage of recovery, the average yearly mortality was only about 1% of the dominant trees. No relation between varying disease incidences and variation in precipitation or temperature could be detected. The clustered distribution of dead and diseased trees is supposed to be related to the distribution of secondary inoculum through infected petioles. It is concluded that the number of dominant trees in both plots is much more affected by mortality resulting from competition for light than by death of trees caused by ash wilt disease. Hence, in young stands like the two plots, ash wilt disease should be seen as a slowly acting natural thinning agent rather than as a threat for the stand.

9 DISTRIBUTION AND IMPACT OF ASH WILT DISEASE IN THE PROVINCE OF FLEVOLAND

Co-author: J.B. van der Pas[1]

9.1 Introduction

The Dutch province of Flevoland is a large polder area reclaimed from the IJsselmeer, the former Zuiderzee, in 1957 and 1968 (Bos, 1984). Between 1957 and 1990 approximately 12.000 ha of the 97.000 ha reclaimed land have been afforested. Ash (*Fraxinus excelsior* L.) is an important tree species in this area not only for wood production but also for its amenity in roadside and recreational plantings. There are approximately 1100 ha of ash plantations in the Flevopolders (CBS, 1985).

The geographic range of ash wilt disease is only partially known but a nationwide survey (Hiemstra, 1987a; Hiemstra *et al.*, 1990; see Chapter 2) has demonstrated that Flevoland is among the areas where the disease incidence is highest with estimated infection rates of up to 20%. The present study aimed at quantifying the disease impact in forest stands in the province of Flevoland by means of aerial assessment of disease incidence and loss of trees. As the symptoms (sudden wilting, withering and early loss of leaves; see Chapter 2) are most apparent in late summer, this study was executed in September.

9.2 Materials and methods

The technical aspects are only briefly described. The remote sensing techniques used, including image processing and image analysis, have been discussed in detail by Van der Pas *et al.* (1991).

Selected stands

Pre-flight ground surveys in the forests of Flevoland showed that ash wilt disease was present in ash stands in each forest. The disease severity varied

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both among forests and stands. Because the age of the stands varies with the forest, four forests of different age classes were selected for the aerial survey (Fig. 9.1). Within these forests a total of 11 flight strips were selected. The sampling fraction from each age class of stands was kept about equal, providing a self-weighing sample (see Cochran, 1963). The flight strips covered a sample of approximately 30% of the ash stands in the selected forests (Table 9.1). This sample was considered to be representative for the ash stands in Flevoland.

Aerial photography

Photographs were taken using a helicopter equipped with a camera and a colour-infrared (CIR) film. The flight strips and a sample plot for ground truth (see below) were vertically photographed from 1000 feet height (305 m) with 70% overlap. Excellent weather conditions (no clouds, almost no wind) prevailed during photography of the flight strips which took place from 3 to 6 September 1989 between 11.00 a.m. and 2.00 p.m.

Ground truth

Data from a permanent research plot in the 'Horsterwold' (ppFl, see Chapter 8) were used to relate the symptoms observed on the CIR-photographs to disease classes of ash wilt disease. On 7 September 1989 each individual tree in the plot was classified into one of six disease classes ranging from healthy to dead (see Chapter 2, Table 2.2). The trees were also classified into four classes relating to their social position (relative crown size; see Fig. 8.2).

These data were visualized in a ground truth map (Fig. 9.2). On this map the tree crowns are depicted by circles of which the size and colour correspond to the social position class and the disease class, respectively. Average tree crown diameters were estimated during the densitometric analysis of the aerial photographs by counting the number of pixels in the crown of several trees of each class on the scanned images (see Van der Pas *et al.*, 1991). This resulted in average crown diameters of 2.5, 2.1 and 1.4 metre for the social position classes 0, 1 and 2, respectively. These crown diameters were used in constructing the ground truth map shown in Fig. 9.2.

This map is an abstraction of the original ground truth array of the individual tree crowns in the sample plot. The disease classes 0 (healthy) and 1 (dubious) were merged to produce a new class referred to as healthy (i.e. without symptoms of ash wilt disease). Disease classes 4 (dieback) and 5 (dead) were also merged resulting in a new class referred to as dead (i.e. at

least part of the crown dead). Because the map was meant to be superimposed on the aerial photographs, only the trees in the upper layer of the canopy (social position classes 0, 1 and 2; cf. Fig. 8.2) were depicted on the map.

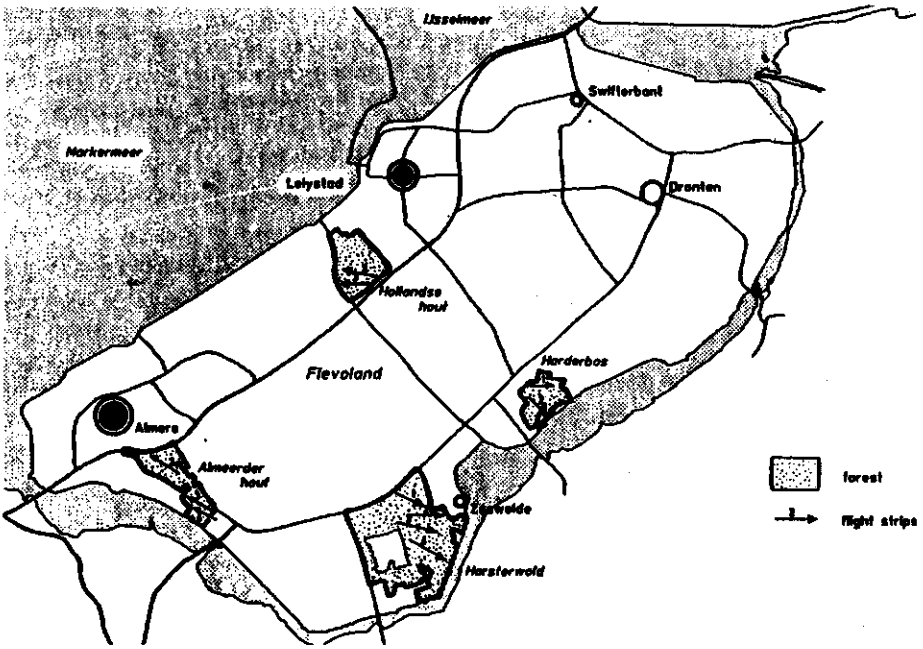

Fig. 9.1 Forests in the province of Flevoland selected for aerial survey in 1989.

Table 9.1 Data on forests and ash stands selected for areal photography.

Forest name	Total area (ha)	Total ash area (ha)	Sampled ash stands		
			Area (ha)	Number	Planted in
Hollandse Hout	680	40	14.9	7	1971-1972
Harderbos	316	22	11.7	4	1972-1974
Almeerder Hout	1198	66	21.2	10	1976*
Horsterwold	2266	123	25.4	11	1977-1978**
Total	4460	251	73.2	32	

* One stand planted in 1978.
** One stand planted in 1973 and one in 1976.

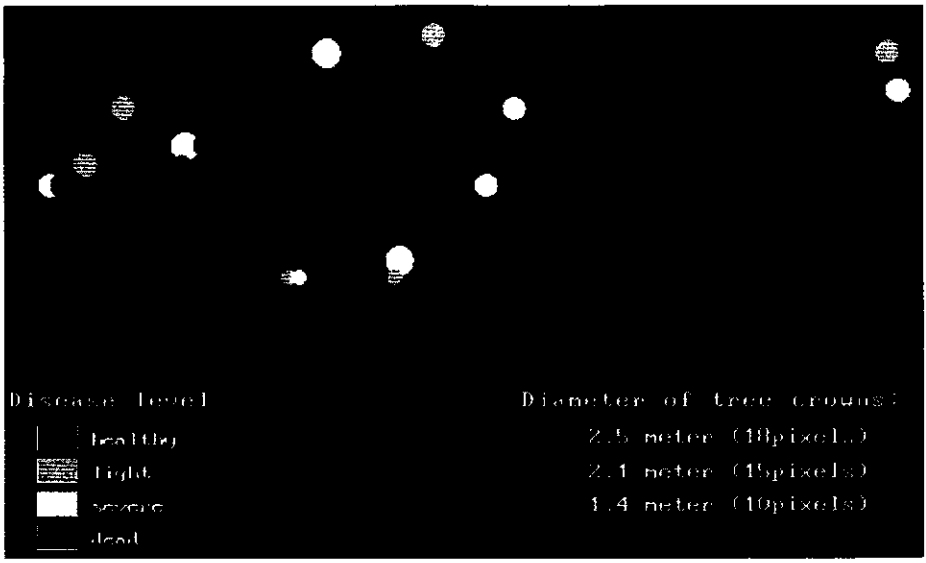

Fig. 9.2 Ground truth map of the permanent sample plot in the Horsterwold (section Pz5-a1) representing condition (disease class) and tree social position on 7 September 1989. Reprinted with permission from: Van der Pas *et al.*, 1991.

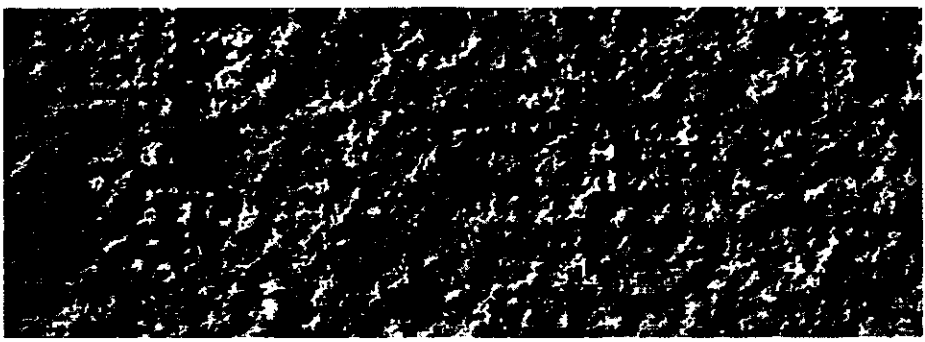

Fig. 9.3 Permanent sample plot in the Horsterwold (section PZ5-a1) Colour composite of 1000 feet red and infrared images. Red hues indicating healthy leaves; blue-green hues indicating diseased or dead leaves. Reprinted with permission from: Van der Pas *et al.*, 1991.

Visual assessments

Visual assessments were performed by viewing the CIR photographs of the flight strips stereoscopically using a Topcon stereoscope 8 x magnification. First the aerial picture and the ground truth map of the sample plot were used to calibrate for identification of damaged trees. Then the disease incidence in the flight strips was assessed by two observers and the scores were averaged. Of each stereopair of pictures the trees were classified into four classes: healthy, diseased, dead and canopy gap. Disease incidence of each stand was recorded by estimating the areas of each class using standard dot grids. After identification on the photographs, random ground checks were made to verify the accuracy.

9.3 RESULTS AND DISCUSSION

9.3.1 Ground truth

An inherent problem with the aerial assessment of tree crowns in a closed canopy is the location of individual tree crowns in relation to the ground truth. Ideally the parameters of crown projections should be used but this is impracticable in young, densely stocked stands. Hence, in this study ground truth was related to the position of the foot of the stems although this may lead to anomalies due to stem deflection and deformation of tree crowns. In Fig. 9.2, at variance to the real situation, all crowns in the same social position class are depicted as if they were of the same size and perfectly circular. Therefore some gaps appear in the canopy map where crown coverage in the field proved to be almost 100%. Despite these restrictions the map proved to be adequate for identifying the severely diseased trees on the aerial pictures of the sample plot (Fig. 9.3).

9.3.2 Disease incidence

Disease incidence is 'the frequency of occurrence of a disease; commonly the proportion of plants affected in a given population' (FBPP, 1973). In this study the percentage of diseased trees in a stand was estimated from the area occupied by those trees.

Attempts to subdivide the diseased trees into categories of light symptoms and severe symptoms failed. Per tree no clear distinction could be made of the percentage of the crown that was affected because individual tree crowns could usually not be distinguished on the photos. For the same reason the numbers of diseased and dead trees were not counted but the percentages (area) of these trees in the sampled stands was estimated.

The visual assessments of the photographs which were independently performed by the two observers showed a high degree of agreement. It appeared that all stands were affected and that the average disease incidence (including dead trees and canopy gaps) per forest area ranged from 21% to 28% (Table 9.2). In individual stands it ranged from 15% to 28%.

The total average disease incidence was 25%, including 14% diseased, 4% dead and 7% canopy gap. The canopy gaps were included in the percentage diseased since they were assumed to originate from trees killed by the disease. This was confirmed by subsequent ground checks. The dead stems in the gaps (Fig. 9.4) often indicated the sudden death of a dominant tree.

Table 9.2 Average disease incidence of ash wilt disease in four forest areas in the province of Flevoland. Forest stands arranged in groups according to location or stand ages.

	Sampled stands		Disease incidence			
	N	Area (ha)	Healthy	Diseased	Dead	Gap
Forest						
Hollandse Hout	7	14.9	79	10	3	8
Harderbos	4	11.7	76	10	4	10
Almeerder Hout	10	21.2	72	16	4	7
Horsterwold	11	25.4	77	13	5	5
Period of planting						
1971-1972	6	13.7	77	10	4	10
1973-1974	6	15.2	79	11	4	7
1975-1976	10	20.5	72	16	4	7
1977-1978	10	23.8	77	13	5	5

Fig. 9.4 Dead ash stem in a gap indicating sudden death of a vigorously growing dominant tree caused by ash wilt disease.

The highest disease incidence was found in the two youngest age classes. However, the differences were small and the range of ages was very limited due to the young forest history of this area. Hence, it is impossible to judge the possibility of a decreasing disease incidence with age as has been suggested by some forest managers. But, as the crown size increases with the age of a tree, an average percentage of 25% of the area having symptoms means that there are fewer diseased trees in older stands than in younger ones (but only in number, not in percentage!). The decreasing total number of trees per hectare may lead to the impression that disease intensity decreases with age.

The larger crown size in older stands perhaps also accounts for the proportion of gaps observed on the photographs being higher in the older age classes. Because of the larger crown size in these age classes, canopy closure after death of a tree is slower than in younger stands. Consequently on pictures of older stands, gaps formed in several years may be visible, whilst on photos of younger stands only the most recently formed gaps will be visible.

9.3.3 Disease impact

A central problem in tree and stand growth and its reduction by diseases is the estimation of the potential yield with total absence of the disease. Estimation of yield losses caused by ash wilt disease is further complicated by several factors:

1. There are few if any good volumetric records for growth of ash under the age of 30 years on these sites (Van der Pas and Hoogesteger, 1989).
2. When only a limited number of trees in a stand are killed, growth compensation occurs, since more space is available for the remaining healthy trees (the essence of most thinning systems).
3. Not all affected trees die, some of them show dieback of part of their crown, many others recover (see Chapters 6 and 8).
4. Development and behaviour of the disease with aging of the stand are still unknown.

The present investigation only permits gross approximations of disease impact on the Flevoland forests by extrapolation. In doing so, some arbitrary decisions had to be made.

For affected ash stands disease impact may be estimated by assuming that the disease progresses linearly. Hence, assuming a relatively high mortality (cf. Chapter 8) of 25% every 10 years and 8000 healthy trees per hectare at the start of the rotation, there will be approximately 1400 and 800 healthy trees left per hectare at the end of the rotation of, respectively, 60 and 80 years, provided that ash wilt disease is the only mortality factor. If the dead trees are randomly distributed both in space and time, then there will be ample trees left at the end of the above rotation lengths. Because of growth compensation by the remaining trees, there will be no loss of wood production. Under such conditions ash wilt disease only generates an inexpensive natural thinning and may be welcomed from that point of view.

The above situation is an optimistic view. In contrast, a more pessimistic view may predict premature collapsing due to clustered mortality followed by a blow-down.

These two extremes demonstrate the order of magnitude of possible losses by ash wilt disease. However, large clusters of dead trees have not been observed on the images and a blow-down of ash stands resulting from large openings in the canopy caused by ash wilt disease so far has not been reported at all. Hence the first situation seems much more realistic than the second one. However, because of the lack of older stands in the area under investigation it is impossible to draw conclusions on the impact of ash wilt disease in older ash stands. For this purpose monitoring of sample stands should be continued together with additional research on older stands in other areas.

At this stage it may be concluded that **the effect of ash wilt disease in young ash stands in Flevoland resembles that of a natural thinning.**

However, in contrast to most thinnings executed by the forest management it will not increase the stem quality in the remaining stand as it has no selective character. In fact the indiscriminate killing of dominant trees by ash wilt disease may be one of the causes of undesirable development of subdominant trees in ash stands in the Flevopolders as reported by Van der Pas and Hoogesteger (1989), and of the quality of future trees (i.e. selected trees) not coming up to expectations, as reported by Oosterbaan and Dik (1991).

9.4 Summary

This chapter describes the results of a detailed forest disease appraisal study on ash wilt disease in the province of Flevoland using low-altitude aerial photography. It was envisaged to evaluate disease incidence and the extent of the damage. Disease prevalence was established by ground surveys prior to the selection of the sample areas for the photo flights.

A representative stratified random sample of sites, age classes and disease levels was obtained by selection of 11 flight strips in four forests. Colour infrared photographs of the flight strips and of a permanent sample plot were taken from 1000 feet with 70% overlap using a helicopter.

Ground truth data from the sample plot were used to calibrate for identification of diseased trees. The colour infrared photographs were visually

assessed and average disease levels are given for each forest and age class. It was found that 75% of the ash trees were healthy, 14% diseased, 4% dead and 7% was canopy gap. Attempts to subdivide diseased trees on the images into categories of light symptoms and severe symptoms failed. Disease impact at the end of the rotation was estimated assuming linear progress of the disease in time. It is concluded that in the young stands in Flevoland, the effect of ash wilt disease so far largely resembles that of a natural thinning.

Acknowledgement

The author thanks Drs. R.J. de Boer (GEOSAT international) for providing the Figures 9.2 and 9.3.

10 FINAL DISCUSSION AND CONCLUSIONS

In the present chapter the data obtained on ash wilt disease presented in the Chapters 4 to 9 are integrated and related to the preliminary conclusions reported in Chapter 2 and to the literature data presented in Chapter 3. In a first section, symptoms, cause and name of the disease are discussed. Then disease development is discussed in general (10.2.1), and the disease cycle of ash wilt disease is described (10.2.2). After this 'portrait' of the disease, recovery of infected trees is discussed (10.2.3). Consequences for forest management (10.3), the need for further research (10.4), and an epilogue (10.5) addressing the concern about yet another devastating vascular wilt disease as illustrated by the first lines of the introduction conclude the chapter.

10.1 Symptoms, cause, and name of the disease

In Chapter 2, based on field observations, a disease syndrome including sudden wilt or withering of the foliage followed by defoliation and dieback or death of affected trees was described. The hypothesis was put forward that this disease is caused by *Verticillium dahliae* Kleb. In Chapter 4 this hypothesis was verified following the four steps known as Koch's postulates. The pathogen was shown to be consistently associated with the disease (step 1), it was isolated and grown in pure culture (step 2), when inoculated into a healthy tree it reproduced wilt and defoliation (step 3), and finally, it could be re-isolated from the experimentally infected trees (step 4). Hence, it was conclusively proven that the ash wilt disease is caused by *V. dahliae*.

However, whereas inoculated trees suffered severe wilt and defoliation, dieback or death of trees as described in Chapter 2 never occurred among them. This was attributed to the differences in the way of infection between naturally infected trees infected through the roots, and inoculated trees infected through a stem wound (Chapter 4). This hypothesis is corroborated by the results of the monitoring experiments described in the Chapters 7 and 8. In the years after inoculation, all stem-inoculated trees recovered, even after repeated inoculation in two successive years. On the other hand, in later years on the same experimental field, dieback or death of some of the trees occurred after a natural infection from the soil. This infection was confirmed again by isolation of *V. dahliae*.

Naturally infected trees showed the same symptoms of wilt and defoliation as inoculated trees (Chapter 7). It was also shown that dieback or death of dominant trees in young forest stands is always preceded by wilt and defoliation (Chapter 8). Hence, dieback and death of affected trees are also part of the disease syndrome of ash wilt disease, but they only occur after natural infection, i.e. infection through a root.

Because of wilt being the main symptom, the name 'ash wilt disease' (Dutch: 'essenverwelkingsziekte') had been proposed by Miller and Hiemstra (1987) even before the cause of the disease was known. Now, with the cause of the disease known, this name still is apropriate because the diseases caused by *V. dahliae* generally are called 'wilt diseases' (FBPP, 1973) or in Dutch 'verwelkingsziekten' (NPV, 1972).

Hence, it can be concluded that the disease syndrome described in Chapter 2 is caused by *Verticillium dahliae* Kleb. The proper name for the disease is 'ash wilt disease' (Dutch: 'essenverwelkingsziekte').

The disease syndrome of ash wilt disease largely corresponds with symptoms reported for Verticillium wilts of other tree species. Wilt, defoliation and dieback or death are very common in Verticillium wilts (Chapter 3). Elongated cankers on the stem of affected ash trees as reported by Hoogesteger and Arnoldussen (1985) and Hiemstra (1987a,b) have been reported before on *Verticillium*-infected tree species, especially maple (*Acer* spp.), by Himelick (1968) and Sinclair *et al.* (1981). Moreover, the absence of distinct vascular discoloration has been reported before for olive (*Olea europaea*) by Wilhelm and Taylor (1965) and for American ash species (*Fraxinus* spp.) by Himelick (1968).

The absence of discoloration of the xylem in ash trees infected by *V. dahliae* implies that for a reliable diagnosis of ash wilt disease isolation of the pathogen is required. Samples from young, recently wilted branches are the most suitable material for this purpose (Chapter 4).

10.2 Disease development

10.2.1 Disease triangle and levels of integration

Disease symptoms are the visible result of a complex set of interactions between many factors on different levels of integration. Generally these factors are categorized into three components of disease; host factors, pathogen factors and (biotic and abiotic) environmental factors. Their interactions have often been visualized in a 'disease triangle', whose sides represent the three components of disease (e.g. Agrios, 1988). Variation of each component will influence disease severity in individual plants and in a plant population. In case of a specific combination of a particular pathogen and its host, variation in environmental factors is the only important component left to cause variation in disease intensity and even in the type of symptoms.

In the present book, emphasis has been mainly on the host component. The development of diseased trees and distribution of the disease in forest plots in particular has received much attention. During the research it became clear that besides the three components of the disease triangle a fourth dimension, time, is needed for explaining disease development. In fact, as the disease triangle is not static but changes with time, it should be replaced by a series of succeeding triangles. Especially for woody hosts which, in contrast to many agricultural crops, live for many growing seasons or years, this time dimension is too often neglected.

In trees and forest ecosystems interactions take place at many different levels. To start with the affected tree, interaction between the tree and a wilt pathogen like *V. dahliae*, because of the size of the pathogen, mainly takes place at cell and tissue level. However, the main symptoms, i.e. wilt and defoliation, become visible at organ level (leaves). The outcome of the disease, growth reduction or even death of infected trees, appears at organism level and finally influences species composition and the relative position of individuals within eco-units (eco-unit level).

The above levels are levels in an hierarchy based on architectural (morphological) criteria (cf. Oldeman, 1990a). Population levels, i.e. numbers, are also important. Infection usually takes place through a root (Chapter 3). Hence, the soil inoculum density (i.e. the population of microsclerotia of *V. dahliae*) is an important factor for disease incidence. In this context the root system might be interpreted as a highly organized

population of root tips surrounded by a population of microsclerotia of *V. dahliae*. Further, each tree root is surrounded by populations of soil organisms many of which (e.g. nematodes, mycorrhiza, antagonistic fungi and bacteria, protozoa, arthropods) directly or indirectly influence disease development. Also generally many superficial root infections are needed for one vascular infection to occur. After vascular infection the conidia that are produced by the primary vascular infection, cause many secondary infection sites, which in turn produce large numbers of conidia.

Hence, for understanding disease and disease development, a combination of the knowledge gathered at several levels of integration is necessary. In this process the time dimension is essential.

10.2.2 Disease cycle

Germination of microsclerotia

Abiotic environmental factors, such as moisture stress and temperature, strongly affect wilt diseases since they have an influence on each of the three biotic components involved in the production of the disease syndrome, i.e., the pathogen, the host and the biotic environment (Katan, 1989). In *Verticillium* wilts of perennial host plants, like ash wilt disease, an important aspect is the effect on root growth of the host since microsclerotia generally remain dormant until they are stimulated to germinate by root exudates (Chapter 3).

Ladefoget (1939) showed that root growth of common ash is limited to periods with soil temperatures above 8°C (May - first half of November in Denmark). This limits the yearly period in which new infections may occur to the growing season. This period is even further limited by the minimum temperature for mycelial growth of *V. dahliae* which is about 10°C (Caroselli, 1957; Regulski and Peterson, 1983). Because of the incubation period, this implies that newly diseased trees will be visibly ill only after some time elapsed during the growing season and that their number will increase continuously during that growing season. This was indeed observed in the permanent plots (Chapter 8).

If a microsclerotium is stimulated to germinate by a growing root, this need not lead to systemic infection. Generally, superficial root colonization by *V. dahliae* is thought to exceed systemic infections by several thousand-

fold (Chapter 3). Hence, the period with maximum root growth seems most appropriate for new systemic infections to occur. In Denmark maximum root growth of ash on well-drained soils was found to coincide with the rainy periods in summer (Ladefoget, 1939). Ash stands in the Netherlands are generally located on well-drained soils (Chapter 1). This might suggest a positive relation between disease incidence and high precipitation values. However, in both permanent plots no such relation was detected (Chapter 8; cf. Hiemstra, 1987, and Hiemstra *et al.*, 1990). Most probably this is due to the complex nature of the interactions between pathogen, host and environment.

A very important aspect in this part of the disease cycle is the amount and distribution of the soil inoculum. Generally, in Verticillium wilts, disease incidence is related directly to the inoculum density in the soil (Chapter 3). Rijkers (1991) showed that at stand level ('eco-unit') the presence or absence of microsclerotia of the pathogen corresponds with the stand being affected by ash wilt disease or not. However, within affected ash stands, i.e. at a tree level, the work by Rijkers did not show any relation between the disease classes of small groups of trees and the number of microsclerotia in soil samples taken around the stem base of those trees. In samples from small areas (8 m^2) supporting groups of diseased trees, he found from 0 to 102 microsclerotia per 15 g of soil, whereas in areas with healthy trees this number ranged from 0 to 21.

This apparent discrepancy probably is due to a clustered distribution of microsclerotia in the soil of affected ash forests, of which the research by Rijkers (1991) presented some evidence. Around each of three trees, soil samples were taken within 0.5 m from the stem base, one sample at each of four sides of a tree. For one diseased tree, three samples yielded 4, 6 and 7 microsclerotia, but the fourth sample contained 41 microsclerotia per 15 g of soil.

Such a clustered distribution of microsclerotia and the large root volume of individual trees makes the assessment of hypothetical relations between soil inoculum concentration and the condition of individual trees in forest plots difficult. If somewhere in the rooting space of an ash tree, the roots meet an area with many microsclerotia, this results in an increased possibility of vascular infection. At the same time, if such a soil area with many microsclerotia is relatively small, it can easily be missed in soil sampling because of the large rooting volume of each individual tree. And even if such an area is sampled, the method of sampling may lead to a low number of

microsclerotia in the soil sample of a specific tree, because each sample consists of many sub-samples taken around the stem base of each sampled tree.

This dilution effect may explain the lack in consistentency of the relations between the condition of individual trees in forest stands and the inoculum level in the corresponding soil samples. However, it does not imply that at stand level no relation exists between soil inoculum level and disease incidence. Such a relation has been demonstrated in orchards of olive (Blanco-López *et al.*, 1989) and pistachio (Ashworth and Zimmerman, 1976). For forest stands of ash affected by *V. dahliae* not enough data are available as yet for a conclusion on this matter.

Apparently there are also other factors that, with soil inoculum of the pathogen and a susceptible host being both present, determine whether the disease occurs or not. In Chapter 8 it has been shown that in severely infested parts of forest plots not all ash trees became diseased. Diseased trees may also recover and stay healthy for at least several years (Chapter 7) while the soil in which they are growing is still infested by *V. dahliae*. Probably the number of microsclerotia in the soil even increases in the year of infection because of the large numbers of microsclerotia that were shown to be present in leaves from diseased trees (Rijkers *et al.*, 1992).

Invasion of the root

Hyphae from germinating microsclerotia usually contact growing roots at some distance from the root tip. In wilt-immune species, the resulting colonies are restricted to the root surface and the outer cortical layers. In other species, like ash, the fungus is able to penetrate the vascular cylinder and colonize xylem vessels (Chapter 3). During colonization and invasion of the root, the interactions with the environment, especially those with other soil organisms, are of paramount importance.

Two aspects have to be considered; first, the interactions during the superficial colonization of the growing root; and second, the interactions during the actual invasion of the root. The effect of abiotic factors on both the pathogen and other soil organisms may explain why environmental conditions more suitable for *V. dahliae* do not always lead to an increased disease incidence.

The rhizosphere is colonized by many soil organisms. As a result of competition for nutrients or because of released chemical compounds, many of these organisms may limit the ability of pathogenic species to colonize the

root surface (Hornby *et al.*, 1990). Well-known antagonists of *V. dahliae* are the fungus *Talaromyces flavus* (Marois *et al.*, 1984; Fravel *et al.*, 1987) and several *Pseudomonas* bacteria (Gregory *et al.*, 1986; Leben *et al.*, 1987). A special category of soil organisms that may limit disease incidence of soil pathogens are the mycorrhizal fungi (Dehne, 1982; Hwang *et al.*, 1992).

No data are available as yet on the role of root-colonizing organisms in ash wilt disease, except for mycorrhizal fungi. Van der Vegt (1988, see Chapter 2) has shown that in neither permanent plot significant differences in the frequency of VA-mycorrhizal roots between diseased and healthy ash trees did occur. Hence, it was concluded that for ash wilt disease, the mycorrhizal frequency is no major factor explaining disease incidence.

During the actual invasion of the root, another class of soil organisms is of great importance: the nematodes. Although *V. dahliae* is able to penetrate readily into unwounded roots, the small wounds inflicted by plant-parasitic nematodes can greatly enhance root penetration by the fungus (Chapter 3). Moreover, synergistic relationships between *V. dahliae* and root-infecting nematodes like *Pratylenchus* spp. have been known for many years and have been described for several hosts including woody species (Dwinell and Sinclair, 1967; Faulkner *et al.*, 1970; Pegg, 1974).

A survey of nematode populations in root and soil samples collected around healthy and diseased ash trees by Denekamp (1989; cf. Chapter 2) indeed revealed higher numbers of *Pratylenchus* spp. in the roots of trees with symptoms of ash wilt disease. The numbers were not high enough for causing direct damage to the trees but a synergistic action with *V. dahliae* could not be excluded. Also, the number of saprophagous nematodes was higher in soil samples taken around diseased trees. This fits in well with the increased amount of dead organic material resulting from defoliation caused by the disease. Bongers (1985) also found relatively high numbers of plant-parasitic nematodes in soil samples from three ash forest plots affected by ash wilt disease. However, little is known about nematode populations in 'normal' ash forest soils. Therefore, so far, the role of nematodes in ash wilt disease is still unclear.

Colonization of the vascular system

After penetration of the vascular system, *V. dahliae* spreads by means of conidia transported upward with the sap stream. In combination with active growth from one vessel to the next, this mechanism enables extensive and fast

colonization of the above-ground parts of a tree, provided the failure of the resistance mechanisms of the infected tree (Chapter 3).

In *Fraxinus excelsior* this apparently is the case. *V. dahliae* was easily isolated from the last growth ring in topmost branches of symptom-bearing young trees (Chapter 4). The fungus must have reached this height in a few months at most, since the study of disease development (Chapter 7) showed that *Verticillium* wilt of ash trees is generally a one-year event. Because the distance between top and root of the trees, up to 15 m, is much too large to be spanned by continuous mycelial growth, the spread of *V. dahliae* in ash trees must be achieved, at least partially, by passive distribution of conidia. The discontinuous distribution of *V. dahliae* in both inoculated trees and naturally infected trees (Chapter 4), and the almost complete absence of mycelium in samples of infected ash trees that were used for wood anatomical research (Hiemstra, 1989 unpublished data; Schroeijers, 1990) also concur with distribution within a tree mainly by conidia.

Because of the anatomical structure of the xylem of trees, each vascular infection offers a pathogen the opportunity to invade all parts of the vessel system upward from the point of infection. Braun (1982) stated that especially ring-porous trees, like ash, are very vulnerable to colonization by vascular pathogens because of their long and wide vessels and the closely-knitted vessel network (also cf. Zimmermann, 1983). However, this is an over-generalization. Indeed, some of the most notorious wilt diseases do affect ring-porous tree species; e.g. Dutch elm disease of *Ulmus* spp. and oak wilt of several American *Quercus* spp. Verticillium wilt fits perfectly in this category, at least with ash as its host. Still, other ring-porous species like elm (*Ulmus* spp.) are rather tolerant to infection by *V. dahliae* (Chapter 5) or are even not susceptible at all like oaks (*Quercus* spp., Chapter 3).

Thus, although the anatomical structure of vessels certainly is important in the process of disease development in vascular wilts, it is just one factor in a complex set of interactions between the pathogen, its host and the environment. Other important factors are chemical and physical characteristics of the environment inside the vessels and active responses by the host. The latter is emphasized by Beckman *et al.* (1976, p. 93) who state that, in cotton: 'the potential of the host to respond to infections must differ from cultivar to cultivar and be directly responsible for resistance versus susceptibility'.

Symptom development

In Chapter 3 it was concluded that wilt symptoms in tree species caused by *V. dahliae* result mainly from clogging of the vessel network by widespread local occlusions, resulting from defensive actions of the host in response to the presence of the pathogen. Concerning ash wilt disease, the symptoms of the disease (Chapter 4), the distribution of the pathogen in diseased trees (Chapters 4 and 6), and the anatomy of diseased trees (Chapter 7) are in agreement with this concept.

At the moment of symptom expression, *V. dahliae* could be isolated from the stem as well as from the topmost branches (Chapter 4) of diseased trees. Rijkers *et al.* (1992) showed that the pathogen is also present in the petioles of leaves from diseased trees. Since infection starts at the root system, this means that the fungus has managed to travel from the infection site in the root through the vascular xylem all the way up to the most distal parts of the tree crown. Hence, everywhere in the xylem vessels the presence of the fungus may have incited defensive reactions by the host, resulting in widespread local vascular occlusions.

Anatomical research of wood samples from stems and branches of both naturally infected and inoculated trees (Chapter 7) indeed showed many occluded vessels in the most recent growth ring of infected ash trees. Because in ring-porous species, like ash, water transport is concentrated in the large-diameter vessels of the last growth ring, such vascular occlusions inevitably lead to symptoms of water stress, i.e. wilt and early defoliation.

Symptoms of marginal and interveinal necrosis, which Talboys (1958b, 1968) attributed to direct toxic effects of fungal metabolites, are rare in naturally infected ash trees. Hence, in the case of ash wilt disease, there is much, although circumstantial, evidence that supports the concept of vascular occlusion. This process, however, may be mediated by toxic effects of fungal metabolites.

In elm (*Ulmus carpinifolia*), inoculation with *V. dahliae* caused much less extensive symptoms than in ash (Chapter 5). Moreover, in most of the inoculated elm trees the symptoms were limited to marginal and interveinal necrosis, indicating toxicosis rather than water stress. Hence, the relative importance of either vascular occlusion or toxicosis in the process of symptom development seems to depend also on the host species. As Talboys (1968, p. 303) stated: 'The tacit assumption that all vascular diseases form a homogeneous group because their symptoms all arise as a result from water stress is clearly not the whole truth.'

Survival and spread of the fungus

Generally, *V. dahliae* does not colonize tissues other than xylem vessels in the above-ground parts of its host as long as the host is alive. Even in the xylem vessels hyphal growth is sparse. After the host plant has died, the fungus may colonize the moribund tissues and form large numbers of microsclerotia (Chapter 3). For this reason, in annual crop plants like potato and tomato, the plant remains left on a field after harvest may lead to a strong increase of the soil inoculum. The fungus also may be dispersed to other parts of a field or to neighbouring fields by means of wind-blown infested soil particles and infected plant remnants.

In perennial species like olive and maple a similar mechanism may occur. In this case the dead plant body is not the most important source of new inoculum. Many infected trees recover, and, even if they die, the dead tissues are not distributed over significant distances. The leaves falling from infected trees are of much more importance. For several tree species including maple (*Acer* spp.; Zimm, 1918; Townsend *et al.*, 1990), yellow-poplar (*Liriodendron tulipifera*; Morehart and Melchior, 1982) and olive (*Olea europaea*; Tjamos and Tsougriani, 1990) infection of the petioles has been demonstrated. Hence, after incorporation in the soil and decomposition, the leaves from diseased trees may contribute to the soil inoculum. Even more important is that they can contribute to the dispersal of the disease, as the fallen leaves may be blown away over considerable distances.

In ash wilt disease these mechanisms have been clearly demonstrated. Rijkers *et al.* (1992) have shown that petioles of diseased ash trees may contain large quantities of microsclerotia. In ash forest stands the litter is incorporated in the soil by the action of earthworms, even the large ash petioles are pulled down (Figure 10.1). Earthworm numbers in ash stands generally are high. Muys (1991) reported 382 kg/ha in an ash forest on sandy loam in Belgium. This results in a fast and extensive intermixing of the litter into the soil. Decomposition of ash litter is very fast (Sevink *et al.*, 1989). Thus in ash stands the microsclerotia in the petiole tissues of infected trees are likely to contribute effectively to the soil inoculum.

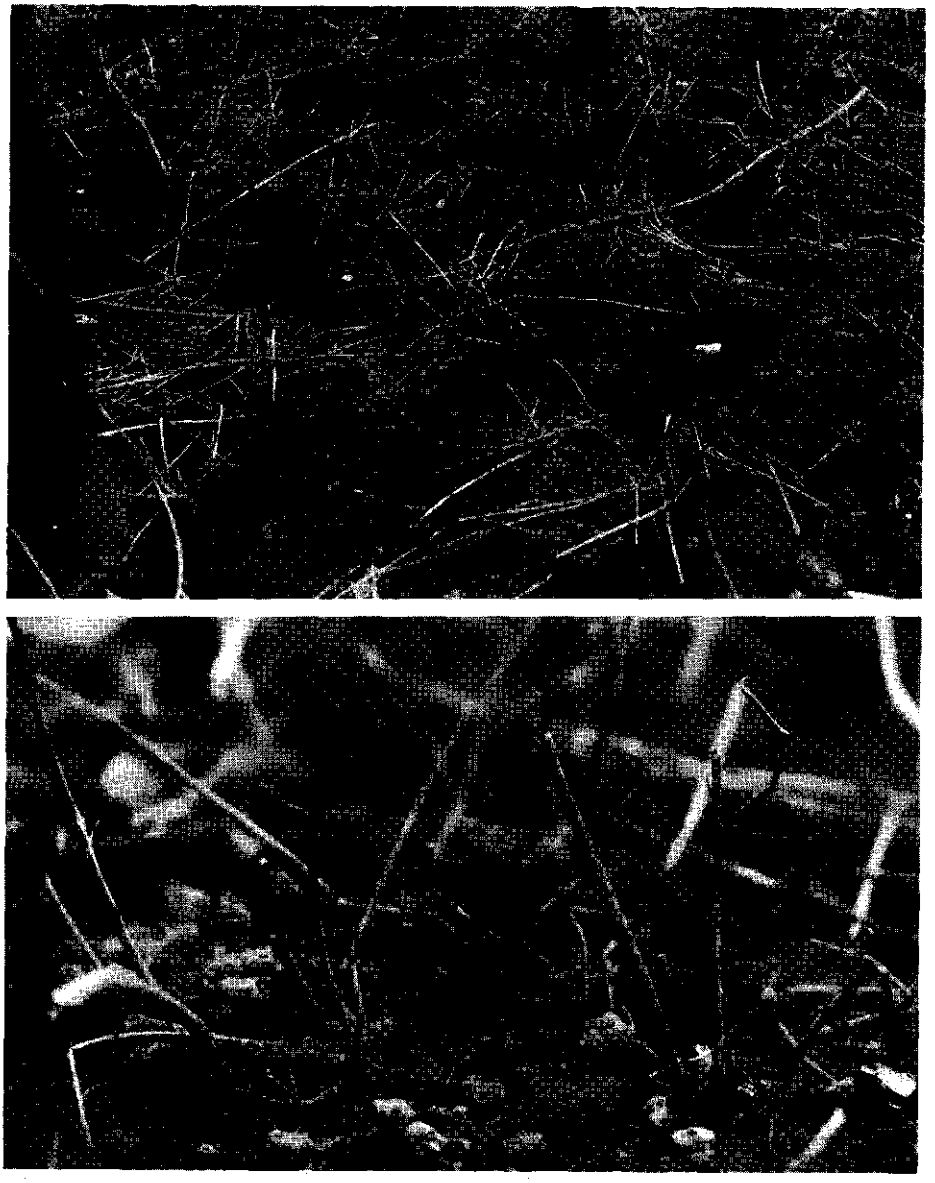

Fig. 10.1 Incorporation of petioles into the top soil layer by the activity of earthworms. Note the almost complete absence of litter except petioles and small branches (top-photo), and the many petioles partly drawn into the soil by earthworms (bottom-photo). Photographs taken by the author in April 1991 in a seven-years-old experimental plot on a clay soil (Wageningen, Afweg).

In combination with wind-blown distribution this has important implications for disease distribution in ash forest stands. In Chapter 8 it is argued that a few scattered diseased trees in a forest stand over a period of several years may lead to contamination of the soil of the whole stand. Finally a more or less diffuse pattern will be the result with areas with high concentrations of soil inoculum around recently diseased trees and areas with lower soil inoculum densities further away from these trees. Such a distribution pattern of the soil inoculum could explain the disease distribution and progress as observed in the two permanent plots, i.e. patches of higher density superimposed upon a general distribution of lower density (Chapter 8).

10.2.3 Recovery of infected trees

Trees do not overcome injuries and infections by healing damaged or infected tissues, but by isolating the damaged part and replacing it by new functional tissues. If the cambium survives and retains its ability to produce healthy secondary tissues, trees often survive xylem invasion by pathogens. Survival of the cambium is possible because of the highly compartmented nature of the xylem. After infection new 'walls' or 'boundaries' are added to the boundaries inherent to the xylem structure (cell walls, last cell layer of each growth ring, sheets of ray cells). In combination they serve to isolate infected tissues from the remaining and newly formed healthy tissues. They compartmentalize the infected tissues. This so-called CODIT concept (Compartmentalization Of Decay In Trees) is functional not only against decay after wounding (Shigo and Marx, 1977) but also in vascular wilt diseases (Shigo, 1984).

The first step in the process of compartmentalization is localization of the pathogen by plugging of infected vessels. In ash trees infected by *V. dahliae* this mechanism apparently is not very successful, since the pathogen manages to spread through the whole vascular system (see above). However, the final step in compartmentalization of an infection, i.e. sealing off the infected tissues, seems to be completed much more successfully. In the growing season after infection the vast majority of infected trees recovers despite development of serious symptoms in the season of infection (Chapters 7 and 8).

Tippett and Shigo (1981) claim that formation of a barrier zone is a consistent feature in trees recovering from vascular wilt diseases. In response to the infection, a zone of living axial xylem elements, usually parenchyma,

would be formed that protects the cambium from being invaded or influenced by the toxic metabolites of the vascular pathogen. In order to be effective, such zones should be continuous all around an infected xylem volume. During wood-anatomical research of ash trees affected by ash wilt disease (Chapter 5) such barrier zones were detected near inoculation wounds, but neither further away from these wounds, nor in naturally infected trees. Hence, it was concluded that recovery in ash trees infected by *V. dahliae* cannot be attributed to formation of a barrier zone only.

However, the anatomy of healthy ash wood includes a sheath of marginal parenchyma between each two growth rings (Grosser, 1977; Schweingruber, 1990). Except for some vessel contacts between latewood vessels of one growth ring and earlywood vessels of the next growth ring (Braun, 1970), these parenchyma layers are continuous around the whole body of previous growth rings. If the vessel contacts between succeeding growth rings are sealed effectively, these layers may serve as built-in barrier zones.

According to Baas *et al.* (1988) the marginal parenchyma is formed at the beginning of each growing season. This may explain why infected ash trees often die in the resting period following the season of infection (Chapters 7 an 8). It also may explain why recurrent symptoms are rare. If infected ash trees manage to start normal xylem formation in the year after that of infection, the pathogen is effectively contained in the old wood and newly formed wood remains free of infection, unless a new root infection takes place.

Here, it also should be stated that **the ring-porous anatomy is not only a risk factor because of its vulnerability to disturbance of the water transport by vascular pathogens or injuries, but also a strong point because of the chances for recovery of infected trees.**

In ring-porous trees water transport is concentrated in the last growth ring that is replaced each year. Hence, if the vessels in the growth ring of the previous year cease to be functional because of defensive reactions against a vascular pathogen, this poses no problem for survival of the tree, since trees with this anatomy are programmed to replace the functional vessels each year. Perhaps, this also explains why dieback is much more common in a diffuse-porous species like *Acer pseudoplatanus*. In diffuse-porous trees the xylem vessels in each growth ring remain functional during several years. Therefore loss of the transport capacity of one (or more) of these rings will also limit the sap stream in the next growing season.

10.3 Impact, implications and control of the disease

Disease impact depends on three factors; disease incidence, mortality rate and debilitation of the affected trees in the years following. In case of ash wilt disease, disease incidence may easily be underestimated. Not only is this caused by difficulties in tracing individual affected trees in dense young forest stands (Chapter 8), but it is also due to the timing of the onset of symptoms. The number of trees with symptoms usually increases steadily during the second half of the growing season. Symptoms of ash wilt disease also resemble the phenomena during natural leaf-fall in autumn. Therefore, if symptoms develop late in the growing season, they may easily be overlooked.

Here an analogy can be drawn with Verticillium wilt of potato, a host plant in which wilt caused by *V. dahliae* has been called 'early dying disease'. In analogy with this name Verticillium wilt in ash might have been called 'early defoliation disease'. The important difference in the outcome of Verticillium wilt in potato and ash is related to their annual and perennial character, respectively. In potato the outcome of the disease is final, i.e. early death of the plant, whereas defoliation in ash in many cases is lethal only for the leaves, not for the tree, because of recovery and renewed growth in the next growing season (Chapters 7 and 8).

Because of the low mortality rate and the rather diffuse distribution of dead trees in affected forest stands, it was concluded in Chapters 8 and 9 that in young ash stands the disease should rather be seen as a slowly acting 'natural thinning agent' than as a threat to the whole stand. From an ecological point of view, the disease in these stands even may have beneficial effects as it increases diversity. Only in extreme cases it may lead to undesirable stem quality of remaining trees or serious reduction of stand productivity because of large canopy gaps. Hence, **ash wilt disease, unlike diseases such as watermark disease in willow, or Dutch elm disease, is no serious problem in management of Dutch forest stands.** Therefore, no control of ash wilt disease in forest stands is necessary. However, in planting ash forests it seems sensible to avoid soils where very susceptible plant species like potato have been grown before.

In other types of plantations, death or dieback caused by ash wilt disease may be more serious. In line and landscape plantations and also in tree nurseries each dead tree means a loss of either production or value, or both. In road plantations, and even more so in gardens and parks, dead trees are not tolerated at all. Unfortunately, there are no therapeutic measures to control

ash wilt disease in these plantations. Soil solarization which may be beneficial (Chapter 3) is impracticable in the Netherlands because of the climate. Soil fumigation in almost all cases is much too expensive and also undesirable because of the environmental effects. Thus the only practicable way for control of the disease is prevention.

This may be achieved by avoiding contaminated soils and by using disease-free planting material, or resistant planting stock. On the long run, the first option is not fully efficacious. Low soil inoculum densities of the pathogen, especially when the distribution of the microsclerotia is clustered (see above), are difficult to detect. The pathogen may be introduced during the long life-span of the trees, at places where at the time of planting the soil and the trees were free of *V. dahliae*. Here, the long life-span of trees makes the situation completely different from that in agriculture with annual crops.

Therefore the use of resistant planting stock would be much more reliable. The problem is that at the moment no resistant varieties of ash exist. However, resistance of planting stock may be improved by selection and breeding, since important variation in susceptibility to Verticillium wilt has been shown to exist within several tree species (Wilhelm and Taylor, 1965; Taylor and Flentje, 1968; Rauscher *et al.*; 1974; Townsend *et al.*, 1990; Morgan *et al.*, 1992).

Ash wilt disease at present is reported mainly in the Dutch coastal provinces, including Flevoland, and the area around the rivers in the central part of the country, although in some other parts of the country considerable ash areas are present. In the affected areas the disease is almost completely restricted to young forest stands and landscape plantings. The restricted geographical distribution may be related to the age of the trees. In the affected areas many small plots of ash have been planted recently. On the one hand, it is often suggested that young trees are more susceptible to attack by *V. dahliae* than older trees. Also some forest managers reported that disease incidence decreased with increasing age of the affected plantation (pers. comm. J. Vis, 1990). On the other hand, the distribution may be related to the previous land-use as most forest and landscape plantings in the affected area were planted on former agricultural land.

A third, and perhaps the most interesting, explanation for the limited distribution of ash wilt disease is that the disease is limited to young forest plots. This explanation is different from the former ones in that it concerns another integration level, not organisms (trees, fungi) or sites (soil, relief) but

whole-stand ecosystems. Young forest plots in this case are forests recently planted on soil that was previously not forested. Due to previous agricultural use, such soils may be severely infested by *V. dahliae*. After afforestation, soil life changes strongly. Perhaps, after some time, the increased content of organic material and the changed soil life may strongly reduce the ecological niche where *V. dahliae* can live. One might speak of 'wilt-suppressive' mature forest soils. However, additional research is necessary to answer this question.

10.4 Further research

In Chapter 1 the aim of the research project, of which the present book is the final report, was formulated as: 'To understand disease so well that we can manage it effectively'. From this aim three research questions were derived:
1. What is the cause of the disease?
2. What is the effect of the disease on individual ash trees?
3. What is the effect of the disease on forest stands of ash?

Each of these questions was answered in one or more of the previous chapters. In combination these answers and additional data presented in the same chapters provide enough information to deal with the disease in daily forest management practice. However, for really understanding the disease further research is necessary. Some of the most important areas of research that may aid in understanding Verticillium wilts of tree species in general and of ash in particular are outlined here.

Three main areas for further research can be distinguished:
1. characterization of the soil populations of *V. dahliae* in forest plots,
2. plant-pathogen interactions,
3. resistance of the host.

At present the geographic distribution of *V. dahliae* in Dutch forests and its role in natural (or semi-natural) forest ecosystems is unknown. Hence, important questions to be answered are: Is *V. dahliae* present in the soils of forest stands in all parts of the country or only in those parts where ash wilt disease has been reported? Is the pathogen limited to certain forest types? What is the composition (genetic types) of the soil populations of the fungus? Does host specialization occur? Is there a consistent relation between soil inoculum concentration and disease incidence, and how should this be

examined?

In the previous section it has already been pointed out that answering these questions may be complicated by the lack of reliable soil-sampling methods for forest stands. Hence, a reliable sampling method has to be developed before the above questions can be tackled effectively. Such a method should take into account the large root volume and the huge numbers of fine roots (potential infection sites) of tree hosts as well as the heterogeneous character of forest soils resulting from the long life-span of trees and the absence of soil cultivation practices.

Genetic characterization of the soil populations is also important as strains of *V. dahliae* may differ in virulence and because recent research has shown that genetic variation in soil populations of this pathogen occurs, as expressed by vegetative compatibility groups (Joaquim and Rowe, 1990, 1991; Okoli *et al.*, 1993).

For a better understanding of disease development (including recovery and resistance) more information on the interactions between perennial hosts and *V. dahliae* is necessary. Much is known about annual hosts but for perennial hosts the information is scarce. Especially on the spatial and temporal distribution of the pathogen in perennial hosts after invasion of the roots little is known. The concept of time—space frames as suggested by Beckman (1964, 1984, 1989) probably offers a good starting point for investigating these aspects.

A related aspect is the effect of biotic and abiotic environmental factors on the plant—pathogen interactions. The differences between old and new forest sites should be studied in more detail. Especially synergistic and antagonistic interactions with other soil organisms need attention. Research into these aspects probably will explain the actual geographic distribution of the ash wilt disease in the Netherlands. Also, it will provide useful information for control of the disease.

The distribution strategy of the pathogen also needs further investigation. Distribution by means of infected leaf petioles already has been demonstrated. So far no animal vector is known. Two comparable vascular wilt diseases of tree hosts, i.e. Dutch elm disease and oak wilt, are distributed by bark beetles. On *Fraxinus excelsior* similar bark beetles occur, of which *Leperisinus varius* is the commonest. This warrants further research.

At present no information is available on resistance levels of different

cultivars of *Fraxinus excelsior*. It has already been mentioned that seedlings of one species may differ in resistance to *V. dahliae*. Therefore, selection and breeding for resistance may provide a solution for the ash wilt problem. Development of efficient screening techniques for resistance will greatly be enhanced by knowledge about the resistance mechanisms. Because 'a study of resistance mechanisms can be fruitful only if we know how the normal processes of pathogenesis elapse' (Elgersma, 1982; p. 143) this again requires research into the plant—pathogen interactions.

In conclusion, the goal to be achieved by further research into ash wilt disease is to understand the plant—pathogen interactions as part of the ecological complex in which they occur. Once this is achieved, it will be possible to develop more effective control measures. It also may provide information useful for selection and breeding of plants more resistant to Verticillium wilt.

10.5 Epilogue

> *Is het dan te veel, als wij spreken over het onheil, dat de iepen bedreigt? En schijnt de vrees niet gewettigd, ..., dat de iep als boom praktisch uit ons land gaat verdwijnen?*
>
> (Is it too much if we speak about the disaster that is threatening the elms? And is not the fear justified, ..., that the elm as a tree will virtually disappear from our country?)
>
> From: Het raadsel der Iepenziekte
> (The mystery of the Elm Disease)
> by S. Broekhuizen, 1937; p. 7.

The above lines stem from the same dramatic description of the effect of Dutch elm disease on elm trees in the 1930's as the lines cited in the introduction of this book. The kind of concern expressed by such words was

the main reason why the present research was initiated. Meanwhile the fear in the above lines has largely come true for elm (Guldemond, 1994). For ash the situation appears to be less dramatic. The present study has shown that indeed 'they are dying'; in some forest stands even 'by the score'. However, it has also been shown that even more trees survive infection by *Verticillium dahliae*. Therefore, no fear is justified that ash will virtually disappear from our country as a result of the ash wilt disease.

This does not mean that the disease is a minor problem. In most forests this may be the case, but in landscape and urban plantations it may be serious, like in shade tree nurseries. The somewhat exaggerated fear of losing the ash as a useful forest tree probably is related to the type of symptoms of ash wilt disease. 'Rapid wilting and death of "normal"-looking trees' .... 'is a dramatic thing to see, and therefore wilt disease epidemics produce a high degree of emotional concern by individuals and communities.' (Manion, 1991; p. 210).

Alarming reports on 'new' disease epidemics in tree species are a regularly returning phenomenon in forest history (cf. Delatour, 1983; Houston, 1987). In some cases after the initial reports, such 'new' diseases have developed into serious problems (e.g. oak wilt, Dutch elm disease, chestnut blight). In many other cases, however, after a few years the epidemics disappeared or decreased to a very low level. Only time can tell in which direction ash wilt disease will evolve. But at present it seems that after initial serious outbreaks of the disease in young forest stands, the disease incidence decreases to low levels without serious problems for the management of these stands. In fact nowadays most forest officers regard the disease in this way, whereas many of them at the time of the initial reports on ash wilt disease were seriously worried about the future of their ash forests.

Both the host, *F. excelsior* L., and the pathogen, *V. dahliae* Kleb., have been present in the Netherlands for a long time. Why ash wilt disease has not become a common disease before has not been revealed by the research presented in this book. But it fits in a series of reports on Verticillium wilts becoming more prevalent in the last decades (Chapter 4). In the USA Sinclair *et al.* (1981) attribute it to distribution of infected planting stock resulting from the establishment of shade tree nurseries on land where infected herbaceous crops once grew. In the Netherlands this practice may also have contributed to distribution of the disease. This is all the more likely, because the presence of only a few infected trees in a plantation may result in contamination of the soil of the whole plantation through infected petioles (Subsection 10.2.2). But the source of infection may as well be in the soil of

the afforested land itself as most of the affected forest plots were planted on former agricultural land.

Besides, management practices and changed environmental factors may also have favoured Verticillium wilts. First generation monocultures, like most of the affected ash stands, usually are affected seriously if they are attacked by a pathogen. Of the environmental factors especially the increased amount of nitrogen-compounds by air pollution may be of importance since high nitrogen levels in *Verticillium*-infected plants stimulate distribution of the pathogen in the plant (Dochinger, 1956a,b; Regulski and Peterson, 1979; Phillips and Burdekin, 1982).

Finally, the origin of the infected plants might influence the disease. Today large quantities of ash seed are imported from Italy and other South-European countries to stock Dutch forest nurseries (P. Schalk, pers. comm.). Generally the use of imported populations which are not completely adapted to the local conditions may increase the chances for pathogens already present (Heybroek, 1992). The introduction of a 'new' genetic type of the pathogen with imported seed cannot be excluded because *V. dahliae* can be transported with seeds of various plants (Chapter 3). For a conclusion on these topics, however, research into the genetics of the host population as well as the pathogen population is necessary.

In conclusion, the present study has shown that ash wilt disease is not as serious as it was feared after the first reports. But before it can be said for Verticillium wilt of ash that 'the acid test of our understanding of disease processes is our ability to predict the progress of disease' (Cowling and Horsfall, 1980; p. 12), much more research has to be done.

GENERAL SUMMARY

Recently, in some parts of the Netherlands a new, so far unknown disease of common ash (*Fraxinus excelsior* L.) caused great concern among owners and managers of broadleaved forests and landscape plantings. This concern instigated a research project of which the present book is the final report.

In **CHAPTER 1**, first a description of *F. excelsior* is given, then the research project is introduced, and finally the design of the other chapters and of the book as a whole are outlined. The three main research questions to be answered by this study concerned (1) the cause of the disease, (2) the effect of the disease on individual trees, and (3) the effect of the disease on ash forest stands.

In **CHAPTER 2** the 'new' disease is delimited. The name 'ash wilt disease' (Dutch: essenverwelkingsziekte) is introduced to replace the vague and unspecific 'dying of ash' (Dutch: essensterven). The main symptoms of this disease are severe wilt and defoliation. Defoliated plants may flush again in the same season. Dieback and death of affected trees is also reported. The disease appears to occur frequently especially in the coastal provinces, the province of Flevoland and the area bordering the rivers in the central part of the Netherlands. In the last section of Chapter 2 the possible causes of the disease are evaluated, leading to the hypothesis that the disease is caused by *Verticillium dahliae* Kleb.

In **CHAPTER 3** the literature on Verticillium wilts of woody species is reviewed. *V. dahliae* is known to be pathogenic to many fruit- and shade tree species, including *Fraxinus excelsior*. However, as far as known, large-scale problems in ash have not been reported before. Also serious Verticillium wilt in forest stands has not been reported before. *V. dahliae* infects its host from resting structures in the soil that are stimulated to germinate by exudates from growing roots. In the root of a host the fungus grows into the xylem vessels. Here conidia are produced that are transported upward by the sap stream. When they become trapped at the end of a vessel, they germinate and penetrate by hyphal growth through bordered pits into the next xylem vessel where the process is repeated. In this way even large trees may become completely colonized within one growing season.

In **CHAPTER 4** it is demonstrated that the ash wilt disease is indeed caused by *Verticillium dahliae* Kleb. In 1988 during a nationwide survey, 150 ash trees, both diseased and healthy ones, were examined. *V. dahliae* was isolated from 63 percent of the diseased trees but never from healthy trees. Stem-inoculation of healthy young ash trees with *V. dahliae* led to development of symptoms of ash wilt disease within six weeks after treatment. Spread of *V. dahliae* after inoculation seems to be mainly upward and absence of dieback symptoms or death of inoculated trees is attributed to the root system remaining free of *Verticillium*.

In **CHAPTER 5** the host specificity and virulence of four *V. dahliae* isolates originating from ash, elm, maple and potato are examined in an inoculation experiment with ash, elm and maple. All isolates proved to be pathogenic on each of the three tree species. In maple, the maple isolate was more virulent than the other isolates. In ash and elm, no differences in virulence were observed. It is concluded that the recent large-scale occurrence of ash wilt disease is not the result of a host-specific or extremely virulent strain.

CHAPTER 6 describes the pathological anatomy of stem wood of young ash trees, that were wound-inoculated on the stem with *V. dahliae*. Main pathological changes included vessel occlusion by thin-walled tyloses in earlywood vessels and by gumlike, often granular, deposits in latewood vessels, and formation of a barrier zone of parenchymatous cells near the wound. It is concluded that in wound-inoculated trees formation of a barrier zone is basically a wound-related reaction. It is argued that the bands of marginal parenchyma, that are part of the normal wood anatomy of ash, function as built-in barrier zones. In combination with extensive vessel occlusion in the infected growth ring, they are effective in containing the pathogen. This enables the observed complete recovery of infected trees, especially because in ring-porous trees like ash the main water transport is by large earlywood vessels that are replaced by newly formed ones each year.

In **CHAPTER 7** the effect of infection by *V. dahliae* on inoculated or naturally infected young ash trees is investigated. Recovery was common in 131 naturally infected ash trees. About 50% of these trees was without symptoms one year later. This percentage increased in the following years. On the other hand, some of the naturally infected trees suffered from dieback or died. Recurrence of symptoms was rare. Inoculated trees never died or

died back and only one out of 102 inoculated trees showed recurrent symptoms. During the inoculation experiments *V. dahliae* reduced growth, especially height growth, of the inoculated trees significantly. This has an ecologically important implication to forest stands affected by ash wilt disease. Despite initial recovery many of the affected ash trees may later die of suppression by healthy neighbours.

CHAPTER 8 addresses the question whether ash wilt disease might become a serious problem in forest management. Distribution and development of the disease were examined during a 5-year period in two permanent plots selected in ash forests seriously affected by ash wilt disease. Invariably the highest numbers of affected trees were recorded at the end of a growing season. The average annual percentage of trees with distinct symptoms of ash wilt disease was less than 10%. Because of a high percentage of recovery, the average annual mortality was only about 1% of the dominant trees. No relation between varying disease incidences and variation in precipitation or temperature was detected. The clustered occurrence of dead and diseased trees is supposed to be related to the distribution of secondary inoculum through infected petioles. It is concluded that the number of dominant trees in both plots is much stronger affected by mortality resulting from competition for light than by death of trees caused by ash wilt disease. Hence, in young stands like the two plots, ash wilt disease should be seen as a slowly acting natural thinning agent rather than as a threat for the stand.

In **CHAPTER 9** the results of an aerial survey of ash wilt disease in the province of Flevoland are discussed. Colour infrared photographs of eleven flight strips in four forests and of a permanent sample plot were used to assess average disease incidences (in % of the total area). It was found that 75% of the stand was healthy, 14% diseased, 4% dead and 7% was canopy gap. Disease impact at the end of the rotation was estimated assuming linear progress of the disease in time. It was concluded that at the end of a rotation damage may range from no growth loss to an undesirable quality of the remaining stand. Only in extreme cases premature collapse of the affected stand may occur. In the young stands in Flevoland, however, the effect of ash wilt disease up to now largely resembles that of a natural thinning.

In **CHAPTER 10** the results of the research as presented in Chapters 4-9 are integrated and compared to previous results and literature data presented in

Chapters 1-3. It is concluded that the name 'ash wilt disease' is appropriate since diseases caused by *V. dahliae* generally are called 'wilt diseases'. Then in a section on the disease cycle all data are integrated in a detailed portrait of ash wilt disease. It is argued that in integrating information on host factors, pathogen factors and environmental factors (the disease triangle concept) the time dimension is essential, especially in Verticillium wilts of tree hosts, because of the long life-span of trees. Recovery of infected trees is attributed to formation of new uninfected xylem and confinement of *V. dahliae* to the xylem already present at the time of infection. In this context it is stated that the ring-porous anatomy of ash is not only a risk factor because of its vulnerability to disturbance of the water transport by vascular pathogens or injuries, but also a strong point because of the chances for recovery of infected trees. In the section on disease impact it is concluded that ash wilt disease, unlike diseases such as watermark disease in willow or Dutch elm disease, is no serious problem in management of Dutch forest stands. Therefore, in forest stands no control of ash wilt disease is necessary. Finally three main areas for further research are identified: the plant—pathogen interactions, resistance of the host and characterization of the soil populations of *V. dahliae* in forest plots.

SAMENVATTING

Enige jaren geleden veroorzaakte een tot dan toe onbekende ziekte van de es (*Fraxinus excelsior* L.) grote bezorgdheid bij eigenaren en beheerders van loofboombeplantingen en loofbossen in enkele delen van Nederland. Deze bezorgdheid leidde tot een onderzoeksproject waarvan dit boek het eindverslag vormt.

In **HOOFDSTUK 1** wordt eerst een beschrijving van de es gegeven, daarna wordt het onderzoeksproject geschetst, en tenslotte wordt een overzicht gegeven van de daaropvolgende hoofdstukken en van de opzet van het boek. De drie belangrijkste door het onderzoek te beantwoorden vragen hebben betrekking op (1) de oorzaak van de ziekte, (2) het effect van de ziekte op individuele bomen, en (3) het effect van de ziekte op bosopstanden van es.

In **HOOFDSTUK 2** wordt de "nieuwe" ziekte beschreven. De naam "Essenverwelkingsziekte" (Engels: ash wilt disease) wordt geïntroduceerd ter vervanging van de vage aanduiding "essensterven" (Engels: dying of ash). De belangrijkste symptomen van de ziekte zijn verwelking en bladverlies. Hernieuwd uitlopen na vroegtijdig bladverlies komt voor, maar sterfte van enkele takken of van de hele boom is ook gemeld. De ziekte blijkt veelvuldig voor te komen in de kustprovincies, de provincie Flevoland en het rivierengebied in centraal Nederland. De laatste paragraaf van Hoofdstuk 2 bevat een evaluatie van de mogelijke oorzaken van de ziekte. Dit leidt tot de hypothese dat de ziekte veroorzaakt wordt door *Verticillium dahliae* Kleb.

HOOFDSTUK 3 geeft een overzicht van de literatuur over de door *Verticillium* bij houtige gewassen veroorzaakte verwelkingsziekten. *V. dahliae* staat bekend als een schimmel die verwelking kan veroorzaken bij veel fruit- en bosboomsoorten, waaronder *Fraxinus excelsior*. Grootschalige aantasting van de es is echter nooit eerder gerapporteerd, evenmin als ernstige *Verticillium*-aantasting in bosopstanden. *V. dahliae* infecteert zijn waardplanten vanuit ruststructuren in de bodem die kiemen onder invloed van de door groeiende wortels uitgescheiden stoffen. Eenmaal in de wortel dringt de schimmel de houtvaten binnen. Daar worden conidia geproduceerd die met de sapstroom naar boven getransporteerd worden. Wanneer deze conidia vast komen te zitten, bijvoorbeeld aan het einde van een vat, kiemen ze en dringen door middel van hyfen via hofstippels de aangrenzende vaten binnen, waar het

proces zich herhaalt. Op deze manier kunnen zelfs grote bomen binnen één groeiseizoen volledig door de schimmel gekoloniseerd worden.

In **HOOFDSTUK 4** wordt aangetoond dat de essenverwelkingsziekte inderdaad door *V. dahliae* veroorzaakt wordt. In 1988 zijn bij een landelijke inventarisatie 150 essen onderzocht, zieke bomen zowel als gezonde. *V. dahliae* werd uit 63 procent van de zieke bomen geïsoleerd maar nooit uit gezonde bomen. Kunstmatige infectie van de stam van jonge bomen leidde binnen zes weken tot de ontwikkeling van symptomen van de essenverwelkingsziekte. Na de inoculatie lijkt de schimmel zich in de boom voornamelijk naar boven te verspreiden. Het uitblijven van het geheel of gedeeltelijk afsterven van de geïnoculeerde bomen wordt daarom toegeschreven aan het feit dat de wortel van deze bomen vrij blijft van *Verticillium*.

In **HOOFDSTUK 5** wordt de waardplantspecifiteit en de virulentie van vier *V. dahliae* isolaten afkomstig van es, iep, esdoorn en aardappel onderzocht door middel van een inoculatie-experiment met es, iep en esdoorn. Alle isolaten bleken voor elk van de drie boomsoorten pathogeen te zijn. In esdoorn was het isolaat van esdoorn virulenter dan de andere isolaten. In es en iep werden geen verschillen in virulentie gevonden. Hieruit wordt geconcludeerd dat het recente grootschalige optreden van de essenverwelkingsziekte niet veroorzaakt wordt door het optreden van een waardplantspecifieke of meer virulente stam van *V. dahliae*.

HOOFDSTUK 6 beschrijft de anatomie van het stamhout van jonge essen die door middel van staminoculatie met *V. dahliae* geïnfecteerd waren. De belangrijkste pathologische afwijkingen omvatten verstoppingen van de voorjaarsvaten door dunwandige thyllen en van de zomervaten door gomachtige, vaak korrelige afzettingen; en de vorming van een 'barrier zone' bestaande uit parenchymatische cellen nabij de inoculatiewond. Geconcludeerd wordt dat de vorming van een barrier zone in de door middel van staminoculatie geïnfecteerde bomen, in essentie een aan de wond gerelateerde reactie is. Verder wordt betoogd dat de marginale parenchymbanden, die een onderdeel zijn van de normale anatomie van essenhout, functioneren als ingebouwde barrier zones. In combinatie met de uitgebreide vaatverstoppingen beperken ze de verspreiding van de schimmel. Dit maakt het complete herstel van geïnfecteerde bomen mogelijk, mede doordat in ringporige boomsoorten

zoals de es het watertransport voornamelijk plaatsvindt in de grote voorjaarsvaten die ieder jaar vervangen worden.

In **HOOFDSTUK 7** wordt het effect van kunstmatige en natuurlijke infectie met *V. dahliae* op jonge essen onderzocht. Herstel was zeer algemeen bij 131 natuurlijk geïnfecteerde essen. Circa 50% was zonder symptomen in het jaar na infectie. In het tweede jaar na infectie steeg dit percentage nog verder. Aan de andere kant stierven enkele bomen van deze groep geheel of gedeeltelijk af. Herhaalde symptomen in opeenvolgende jaren waren zeer zeldzaam. Gehele of gedeeltelijke sterfte trad echter nooit op na inoculatie en slechts één van 102 geïnoculeerde bomen vertoonde symptomen in het jaar na inoculatie. Uit de inoculatie-experimenten bleek verder dat *V. dahliae* de groei van geïnfecteerde bomen, en dan met name de hoogtegroei, wezenlijk beïnvloedt. Dit heeft een belangrijke ecologische consequentie voor de door de essenverwelkingsziekte aangetaste bosopstanden. Ondanks aanvankelijk herstel, zullen veel van de aangetaste bomen uiteindelijk toch sterven als gevolg van onderdrukking door gezonde buurbomen.

HOOFDSTUK 8 behandelt de vraag of de essenverwelkingsziekte problemen kan veroorzaken voor het beheer van aangetaste essenopstanden. Daartoe zijn de verspreiding en de ontwikkeling van de ziekte in twee proefperken in ernstig aangetaste bosopstanden gedurende een periode van vijf jaar nauwkeurig gevolgd. De hoogste aantallen zieke bomen werden altijd aan het einde van het groeiseizoen waargenomen. Het gemiddelde jaarlijkse percentage bomen met duidelijke symptomen was in beide opstanden minder dan 10%. Vanwege het hoge herstelpercentage was de jaarlijkse sterfte minder dan 1% van de heersende bomen. Een relatie tussen het jaarlijkse ziektepercentage en de hoeveelheid neerslag of de temperatuur kon niet worden aangetoond. Het enigszins groepsgewijs voorkomen van zieke en dode bomen wordt verondersteld gerelateerd te zijn aan de verspreiding van het secundaire inoculum door middel van geïnfecteerde bladstelen. Geconcludeerd wordt dat het aantal heersende bomen in beide opstanden sterker beïnvloed wordt door sterfte ten gevolge van concurrentie dan door sterfte veroorzaakt door de essenverwelkingsziekte. Daarom dient de aantasting in jonge essenopstanden eerder beschouwd te worden als een langzaam optredende natuurlijke dunning dan als een bedreiging voor de opstand.

In **HOOFDSTUK 9** worden de resultaten van een onderzoek met behulp van

luchtfoto's naar het voorkomen van de essenverwelkingsziekte in de provincie Flevoland besproken. Kleuren-infrarood foto's van elf vluchtlijnen en van een proefperk werden gebruikt om het gemiddelde ziektepercentage (in oppervlakteprocenten) vast te stellen. Hieruit bleek dat 75% van de essen gezond was, 14% ziek, 4% dood en 7% van de oppervlakte bestond uit gaten in het kronendak. Het effect van de ziekte aan het einde van de rotatie werd geschat uitgaande van een lineaire progressie van de ziekte. Geconcludeerd wordt dat aan het eind van de rotatie de schade kan variëren van geen produktieverlies tot een ongewenste kwaliteit van de overgebleven opstand. Slechts in zeer extreme gevallen zou de ziekte tot het instorten van de opstand kunnen leiden. In de jonge bosopstanden in Flevoland komt het effect van de ziekte tot nog toe echter overeen met een natuurlijke dunning.

In **HOOFDSTUK 10** worden de onderzoeksresultaten zoals beschreven in de Hoofdstukken 4-9 geïntegreerd en vergeleken met de eerdere resultaten en de literatuurgegevens zoals beschreven in de Hoofdstukken 1-3. Hierbij wordt in de eerste plaats geconstateerd dat de naam "essenverwelkingsziekte" een passende naam is omdat de door *V. dahliae* veroorzaakte ziekten gewoonlijk als "verwelkingsziekten" worden aangeduid. Daarna worden alle beschikbare gegevens geïntegreerd in een gedetailleerde beschrijving van de ziektecyclus. Hierbij wordt betoogd dat bij het integreren van de gegevens betreffende het pathogeen, de waardplant en de omgeving (het concept van de ziektedriehoek) de tijdsdimensie essentieel is, met name voor de door *Verticillium* bij bomen veroorzaakte verwelkingsziekten, vanwege de lange levensduur van bomen. Herstel van geïnfecteerde bomen wordt toegeschreven aan de vorming van nieuw, niet-geïnfecteerd xyleem en het beperken van de verspreiding van de schimmel tot het al op het tijdstip van infectie aanwezige xyleem. In dit verband wordt opgemerkt dat de ringporige anatomie van de es niet alleen een risico-factor is vanwege de gevoeligheid voor verstoring van het watertransport, maar ook een sterk punt vanwege de kansen op herstel van geïnfecteerde bomen. In de paragraaf over de gevolgen van de ziekte wordt geconcludeerd dat de essenverwelkingsziekte, in tegenstelling tot ziekten zoals de watermerkziekte bij wilg en de iepeziekte bij iep, geen serieuze problemen veroorzaakt voor het beheer van aangetaste opstanden. Bestrijding van de ziekte is in bosopstanden dan ook niet nodig. Tot slot worden drie gebieden voor verder onderzoek aangegeven: de interacties tussen de waardplant en de schimmel, resistentie van de waardplant en karakterisering van de populaties van *V. dahliae* in de bodems van bosopstanden.

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Appendix I TOTAL FORESTED AREA[1] AND ASH AREA IN THE 12 DUTCH PROVINCES

Province	Forested area[2]	Ash area[2,3]	Ash area as percentage of forested area	Ash area in province as percentage of total Dutch ash area
Groningen	2,786	261	9.4	5.2
Friesland	9,512	167	1.8	3.3
Drenthe	28,970	84	0.3	1.7
Overijssel	40,079	536	1.3	10.7
Gelderland	95,604	297	0.3	5.9
Utrecht	19,710	446	2.3	8.9
Noord-Holland	11,197	518	4.6	10.4
Zuid-Holland	5,848	502	8.6	10.0
Zeeland	3,276	357	10.9	7.1
Noord-Brabant	74,282	194	0.3	3.9
Limburg	32,824	557	1.7	11.1
Flevoland	9,938	1,080	10.9	21.6
Total	334,026	5,000		100

[1] As forested area are understood complexes of 0.5 ha and more with a crown density of 20% and over; i.e. small woodlots and line or road plantations are not included (CBS, 1985).

[2] Data from CBS (1985).

[3] About 50% of the ash area consists of pure stands, the other 50% is mixed with other broadleaves, mainly oak. In some provinces a substantial part of the ash area consists of coppice or outgrown coppice (60% in Utrecht, 50% in Zuid-Holland and 10-15% in Drenthe, Gelderland, Noord-Holland and Limburg).

Appendix II SYMPTOM CODE, DISEASE INDEX AND DISEASE CLASSES

1 Symptom code

The condition of the trees under investigation was recorded using a two-tier symptom code system (Table II.1). This system was developed especially to rate the symptoms of ash wilt disease. The first element of the code is a figure ranging from 0 to 8 indicating the type of symptoms. The second element is a letter representing the estimated percentage of one individual crown showing the symptoms that are indicated by the preceding figure. Codes may be combined. For example the combination 2a/4b means a tree with 0-30 % of the leaves wilted and 31-60 % loss of leaves.

Table II.1 Codes used for rating the symptoms of ash wilt disease.

Symptom code	Meaning
First code	Type of symptoms
0	Healthy tree, i.e. vigourously growing tree without any disease symptom
1	Tree not healthy but without symptoms of ash wilt disease
2	Tree with wilted leaves
3	Tree with withered leaves
4	Tree with premature loss of leaves
5	Tree with small chlorotic new shoots (after premature leaf loss)
6	Tree with partially dead crown (dieback)
7	Tree with dead crown but living stem
8	Dead tree
Second code	Percentage affected (to be used in combination with codes 2-6)
a	5 - 30 %[1]
b	31 - 60 %
c	61 - 80 %
d	81 - 100 %

[1] The lower limit for symptoms to be recorded was 5%, if less than 5% of the foliage was affected trees received code 0 or 1, depending on their condition.

2 Disease index

In order to be able to compare individual trees and to indicate disease progress, the symptom code data were converted in a disease index ranging from 0 to 10 with increasing severity of symptoms. The differences between index classes are not fixed; the scale only indicates increasing severity of symptoms (ordinal scale). The following schemes were used for conversion of symptom codes into disease indices:

a. Conversion of a single symptom code

The basic principle followed in the conversion of the coded symptom data is that symptoms with a higher code are supposed to indicate lesser health. Also, wilt is considered to be less serious then withering or defoliation. In the latter two cases the leaves are irreversibly out of function, whereas wilt symptoms may be reversible. For single symptom codes this leads to the conversion table as shown by Table II.2.

Table II.2 Conversion of a single symptom code into a disease index.

Symptom code									Disease index
0									0
	1								1
		2a	3a	4a		6a			2
		2b							3
		2c	3b	4b					4
		2d			5c,5d				5
			3c	4c	5a,5b				6
			3d	4d					7
						6b,6c			8
						6d	7		9
								8	10

At first sight the position of symptom code 5 may seem somewhat arbitrary. This type of symptoms, however, only appears after serious defoliation (in most cases symptom code 4d). It is thought to represent an early stage of recovery. Therefore trees with symptom code 5 are given a disease index 1 unit lower (in case of symptom code 5a or 5b) or 2 units lower (5c or 5d) than the disease index representing complete defoliation.

b. Conversion of a combination of two symptom codes

The effect of different symptoms occurring simultaneously is considered to be additive. Hence the disease indices for each symptom are combined with some corrections to keep the resulting disease index comparable to those resulting from single symptom codes. In this operation the same principles are valid i.e. wilt is a less serious symptom than withering or leaf fall, and combination with symptom code 5 results in a disease index diminished by one or two units.

For **combinations of the symptom codes 2, 3 and 4** these principles result in the conversion table shown in Table II.3. In this table only possible combinations are stated. Symptom codes are given in the top row and the left column of the table. The rest of the table gives disease indices for all possible combinations of two symptom codes.

Table II.3 Conversion of a combination of the symptom codes 2, 3 and 4.

	3a	3b	3c	3d	4a	4b	4c	4d
2a	3	5	7	7	3	5	7	7
2b	4	6	7		4	6	7	
2c	5	6			5	6		
2d	6				6			
4a	4	6	7	7				
4b	6	7	7					
4c	7	7						
4d	7							

In case of a **combination with symptom code 5** the disease index resulting from the code other than 5 is diminished by 1 unit in case of combination with 5a or 5b and with 2 units in case of combination with 5c or 5d.

In **combinations with symptom code 6a** this symptom code was given the same weight as symptom code 3a or 4a. Then the above table was used for conversion. In **combinations with 6b, 6c or 6d** the other symptom codes were neglected in the conversion. All combinations with 6b or 6c received disease index 8 and all combinations with 6d received disease index 9.

Combination of three symtom codes
In this case first the codes 3 and 4 were added up (e.g. 3a + 4a = 3b; 3b + 4a = 3c) and then the result was combined with the third symptom code as has been described for combination of two symptom codes. The resulting figure diminished by one unit gives the disease index for this combinations.

3 Disease Class

In many cases a more general classification was needed. Therefore a system of disease classes was used. The disease index can be transformed into the disease class as shown in Table II.4.

Table II.4 Transformation of disease indices in disease classes.

Disease index	Disease class	Meaning
0	0	Healthy[1]
1	1	Dubious[1]
2, 3	2	Slight symptoms
4, 5, 6, 7	3	Distinct symptoms
8, 9	4	Dieback
10	5	Dead

[1] Cf. definitions of symptom codes 0 and 1 in Table II.1

Appendix III DESCRIPTION OF THE PERMANENT PLOTS

1 Permanent plot in the province of Flevoland (ppFl)

Location: Forest complex Horsterwold, section Pz5-a1 (Fig. III.1)

Soil type: Clay soil overlying Pleistocene sand (> 120 cm)
pH($CaCl_2$) in the uppermost 25 cm: 8.5

Soil water table: 90 cm below soil level in winter
200 cm below soil level in summer

Stand data
Trees in rows with 1.25 m spacing in rows and 1.5 m between rows.
Each 5 rows of ash (*Fraxinus excelsior* L.) followed by a row of *Alnus glutinosa* (L.) Vill.
In the ash rows 15 % auxiliary species (*Acer campestre* L., *Ligustrum vulgare* L., *Rhamnus catharticus* L., *Corylus avellana* L.) are intermixed.

Stand history

1968	Polder fell dry and reed (*Phragmites australis* (Cav.) Trin. ex Steud) was sown.
1970	After burning of the reed, oilseed rape (*Brassica napus* L.) was sown.
1977	Afforestation after ploughing (35 cm deep).
1982	Pruning of trees with silviculturally bad form.

Permanent plot

Size:	Length 90 m, width 27 m.
Number of tree rows:	17 (3x5 ash rows and 2 *Alnus* rows)
Total number of trees (1986):	Ash (including dead trees): 940 Other species (including many shrubs): 265
Tree height (1986):	8 m (dominant ash trees). Almost all auxiliary tree were outgrown by the ash trees, only some *Alnus* and *Acer* trees were present in the canopy as co-dominant trees.

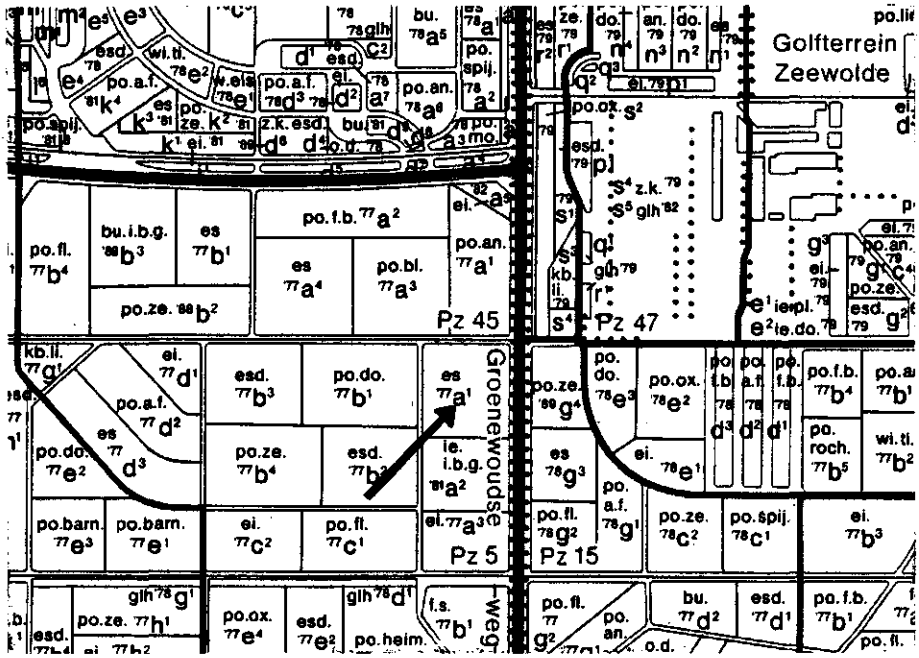

Fig. III.1 Location of permanent plot in the province of Flevoland (ppFl).

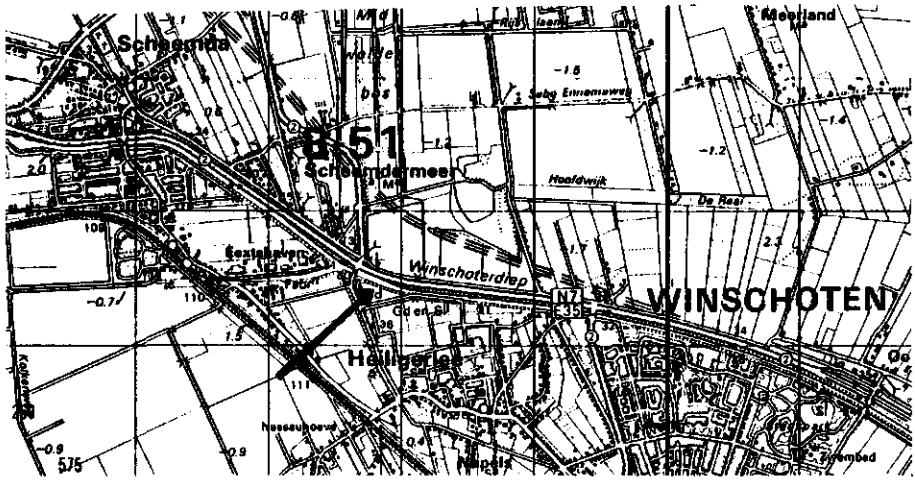

Fig. III.2 Location of permanent plot in the province of Groningen (ppGr).

2 Permanent plot in the province of Groningen (ppGr)

Location: Forestry Oldambt, complex De Hoogte, section 25j (Fig. III.2)

Soil type: Loamy fine sand overlying heavy clay at 80-120 cm depth. pH($CaCl_2$) in the uppermost 25 cm: 5.

Soil water table: 40-80 cm below soil level in winter.
> 120 cm below soil level in summer.

Stand data

Trees in rows with 1 m spacing in rows and 1.5 m between rows.
Each 3 rows of ash (*Fraxinus excelsior* L.) are followed by a row of *Quercus robur* L. (45 %), *Alnus glutinosa* (L.) Vill. (10 %), *Crataegus monogyna* Jacq. (7.5 %), *Salix alba* (7.5 %), *Salix aurita* L. (5 %), *Betula pubescens* Ehrh. (5 %) and *Viburnum opulus* L. (5 %).

Stand history

1973 Planted on former pastureland; planting stock 2 or 3-years-old.
1980 Most *Salix* and *Alnus* trees removed
1984 Ash trees with silviculturally undesirable form removed.

Permanent plot

Size:	Length 60 m, width 18 m.
Number of tree rows:	13 (3x3 ash rows and 4 rows of auxiliary species).
Total number of trees (1986):	Ash (including dead trees): 367 Other species: 95
Tree height (1986):	12 m (dominant ash trees). The few remaining *Salix alba* (16-18 m) and *Anus glutinosa* (14 m) were much taller than the ash trees. *Crataegus monogyna* was present as healthy shrubs, the other auxiliary species were decling or dead.

CURRICULUM VITAE

Jelle Adolf Hiemstra werd geboren op 1 april 1961 te Sneek. Na het behalen van het diploma Atheneum-B aan het Lienward College te Leeuwarden begon hij in 1979 met de studie Bosbouw aan de toenmalige Landbouwhogeschool te Wageningen. Zijn praktijktijd bracht hij door bij de afdeling Bosontwikkeling van het Staatsbosbeheer te Utrecht. In 1987 behaalde hij zijn doctoraaldiploma cum laude. De afstudeervakken omvatten Agrarische geschiedenis, Boshuishoudkunde en Bosteelt. Onderwerp van het laatstgenoemde vak was een inventarisatie van het zogenaamde essensterven in Nederland. Dit leidde tot een door de Landbouwuniversiteit gehonoreerd voorstel voor verder onderzoek. Van 1 mei 1987 tot 1 mei 1991 heeft hij hieraan gewerkt als Assistent in Opleiding (AIO) bij de vakgroepen Bosteelt en Bosoecologie (later Bosbouw) en Fytopathologie. Gedurende deze periode werd in het kader van de ERASMUS regeling van de Europese Gemeenschap een stage van vier maanden doorgebracht bij het Ordinariat für Holzbiologie van de Universiteit van Hamburg. Aansluitend aan de AIO-periode was hij negen maanden werkzaam als toegevoegd onderzoeker bij de vakgroep Bosbouw. In deze tijd werkte hij aan de formulering van het project "Bosontwikkeling" en aan het opstellen van een voorstel voor een door de EG te financieren onderzoeksproject "New Forests on old Farmlands". Daarna werkte hij nog circa een jaar aan de verdere voltooiing van het in dit proefschrift beschreven onderzoek. Vanaf 15 maart 1993 werkt hij als onderzoeker bij de afdeling Siergewassen van het DLO-Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO) aan het project "Verticillium-resistentie bij *Acer platanoides*".