Verticillium Wilt in Trees

Detection, Prediction and Disease Management

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Abstract

Verticillium dahliae, the causal agent of verticillium wilt, is the cause of high losses in a number of crops, especially nursery trees. Existing methods for quantification of *V. dahliae* microsclerotia in the soil were compared. The distinction of *V. dahliae* and *V. tricorpus* was studied on two semi-selective media. The morphology was highly dependent on the medium. Discriminating morphological characteristics were successfully used to identify isolates. There were no virulence differences on trees between the two *V. dahliae* VCGs that occur in the Netherlands. Biological soil disinfestation reduced *V. dahliae* in the soil by 85% and *Pratylenchus fallax* nematodes by 99%, through the creation of anaerobic conditions. The relationships between soil inoculum densities and verticillium wilt in *Acer platanoides* and *Catalpa bignonioides* showed that up to 5% diseased plants occurred at 1-2 detected microsclerotia per g soil. Diseased plants often recovered, but had a higher chance of becoming diseased again.

Voor Judith,
Hanneke en Christianne

Voorwoord

Het onderzoek beschreven in dit proefschrift is begonnen in januari 1997, als AIO-project 'Detectie en schadedrempels bij *Verticillium dahliae*', bij de leerstoelgroep Ecologische Fytopathologie van de vakgroep Fytopathologie van de Landbouwuniversiteit. Het onderzoek werd gefinancierd door het Productschap Tuinbouw, en is geformuleerd naar aanleiding van de problemen met verticillium-verwelkingsziekte in de laanboomteelt. Toen de leerstoelgroep Ecologische Fytopathologie wegens een reorganisatie werd opgeheven is het project, tezamen met de gehele bodemschimmelgroep, eind 1999 verhuisd naar bij de leerstoelgroep Biologische Bedrijfssystemen van Wageningen Universiteit. Vanaf maart 2000 zijn de werkzaamheden in verbrede vorm voortgezet in het kader van het EU-project '*Verticillium wilt in trees*', eveneens bij Biologische Bedrijfssystemen. Op deze plaats wil ik de financiers van beide projecten hartelijk danken voor het in mij gestelde vertrouwen.

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Contents

Chapter 1

General Introduction

Verticillium dahliae Kleb. is a soil-borne fungus that causes wilt in a wide range of plants (Schnathorst, 1981). Among susceptible plant species are agricultural crops (cotton, potato), vegetables (artichoke, eggplant, pepper), fruits (apricot, avocado, cocoa, grapevine, olive, strawberry), flowers (chrysanthemum), fibre and oilseed crops (cotton, flax, sunflower), and various woody ornamentals (Table 1; Pegg and Brady, 2002).

In the Netherlands, verticillium wilt is the cause of high losses in tree nurseries, where many species of economic importance are susceptible (especially many maple species and lilac) (Table 1). Yearly, about 1.5 – 3% of the marketable yield is lost because of verticillium wilt (J.A. Hiemstra, pers. comm). In greenhouse chrysanthemum (Ispahani et al., *in prep.*), and field-grown strawberry (Lamers et al., 2001) losses up to 70% are reported on some farms. In potato, *V. dahliae* causes early dying, and though symptoms are less obvious and sometimes remain unnoticed in the presence of other pathogens, yield losses up to 25% have been reported (Bollen et al., 1989; Haverkort et al., 1989; Locke, 2001, Nagtzaam, 1998). During senescence of the potato crop, the pathogen forms massive numbers of microsclerotia in root and, primarily, shoot tissue. If plant debris becomes incorporated in the soil, soil inoculum builds up rapidly, especially if the rotation is narrow. Similarly, but in smaller quantities, microsclerotia can be formed in petioles of tree species (Hiemstra, 2000; Rijkers et al., 1992) (Figure 1). The microsclerotia can survive in soil for several years and the soil inoculum level can be maintained by the presence of other susceptible plant species (Mol, 1995).

In the Netherlands, most agricultural fields have a potato growing history and the majority of them may be infested with *V. dahliae*. Tree nurseries are often established on fields with a potato cropping history, as a result of expanding of business and to escape fields that had unacceptably high levels of verticillium infestation due to growing of susceptible tree species. This often results in high economical damage: unlike the yield loss in potato, the wilt symptoms in trees are clearly visible and directly affect the quality and the price of the product. Moreover, symptoms already occur at low inoculum densities. These two aspects cause the damage threshold (the inoculum density at which damage starts to occur) to be low. On top of the direct losses, extra efforts are needed by the tree growers to remove the wilted tree directly. This is important to prevent build-up of soil inoculum through fallen leaves (Hiemstra, 2000; Rijkers et al., 1992). Moreover, when disease incidence is severe, the field, which is sometimes owned by the growers or rented for a longer period, is not suitable for growing susceptible plant species. This has already led to court cases about whether or not the soil was already infested when it was first sold or rented to the grower. Thus, verticillium wilt is the primary reason for growers to identify new locations for their business, which again raises the risk of selecting verticillium-infested sites.

Verticillium symptoms may depend on the tree species involved. Generally, three main types of effects may be observed: leaf symptoms (wilt, discoloration, defoliating), vascular symptoms (discoloration of the xylem and plugging of vessels) and decline (stunting and die-back) (Hiemstra, 1998). Wilt can occur rapidly or more chronically and may affect

the entire tree or one to several branches (Piearce and Gibbs, 1981; Himelick, 1968). Affected trees may die, but often they are also able to recover from disease through a new flush of leaves some weeks after defoliation, or by the formation of adventitious shoots, especially from the stem base. Recovery is possible, because of the limited lateral connections between xylem vessels and because of the production of secondary xylem (Shigo, 1984). Recovery is important in, for example, olive orchards, where recovery from infection with mildly virulent strains can keep stems intact and the farmer can regain production over time (Jímenez-Díaz et al., 1998; Tjamos, 1991). Limited damage in olive trees does not warrant the removal of trees, as they become productive only after many years. Also, removing individual ornamental trees from gardens and parks is often not needed if they recover well. However, recovery is of minor value for tree nurseries, because die-back causes loss of ornamental value and because *V. dahliae* in the recovered tree might be still viable and capable of causing new wilt symptoms.

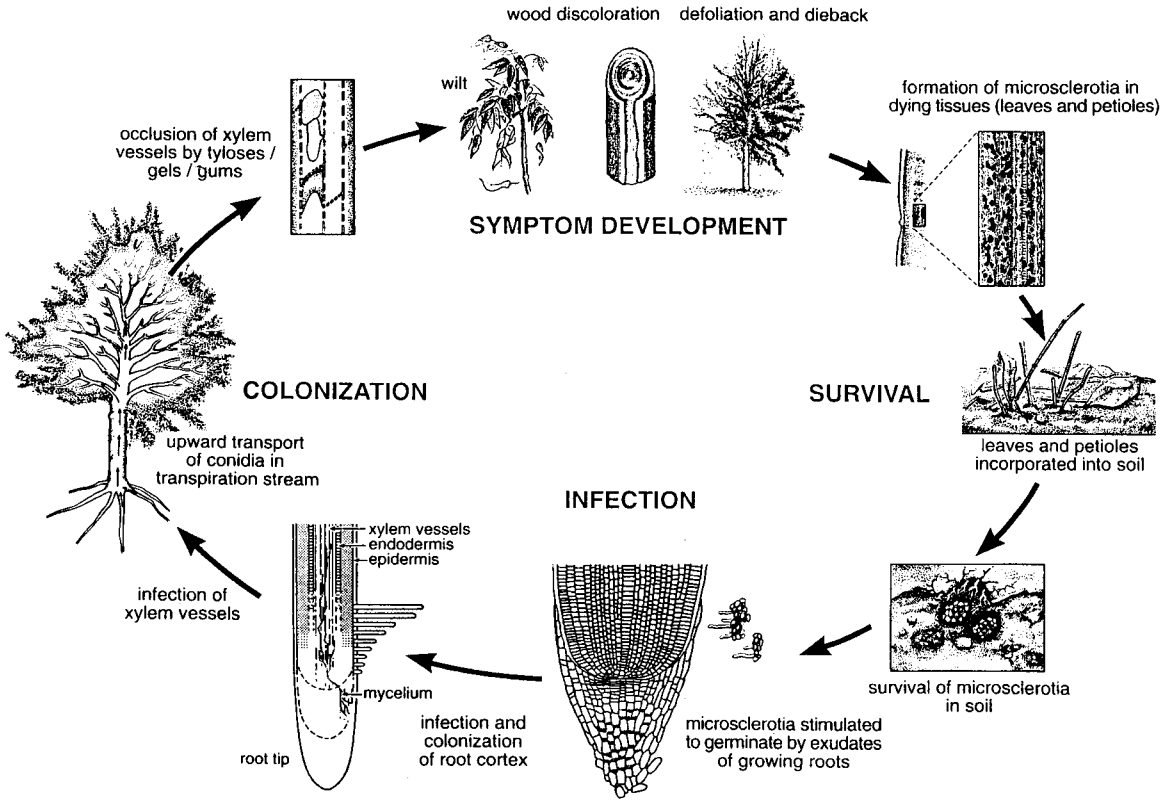

Figure 1. Life cycle of *Verticillium dahliae* in tree hosts (courtesy of J.A. Hiemstra and A.J. Termorshuizen; drawing by P.J. Kostense).

Interactions of *V. dahliae* with plant pathogenic nematodes have been observed, e.g., in potato (Martin et al., 1982; Wheeler et al., 1994) and sugar maple (*A. saccharum*) (Dwinell and Sinclair, 1967). In the presence of root-infecting nematodes (e.g., *Pratylenchus* spp. and *Meloidogyne* spp.), damage caused by *V. dahliae* generally increases. The precise mechanism of this interaction is not known. There are indications that it is caused by systemic, biochemical effects (e.g., Bowers et al., 1996), or by local, mechanical damage effects (e.g., Corbett and Hide, 1971). Thus, nematodes generally lower the damage threshold.

Table 1. Important woody species susceptible to *Verticillium dahliae* (Bisiach et al., 1982; Hiemstra, 1998; Schnathorst and Goheen, 1977).

Genus	Species
Forest and landscape trees	
Acer	*campestre, ginnala, macrophyllum, mono, negundo, nigrum, palmatum, pennsylvanicum, platanoides, pseudoplatanus, rubrum, saccharinum, saccarum, tataricum*
Catalpa	*bignonioides, bungei, speciosa*
Cercis	*canadensis, siliquastrum*
Fraxinus	*americana, excelsior, nigra, pennsylvanica, quadrangulata*
Robinia	*pseudoacacia*
Tilia	*americana, cordata, euchlora, glabra, parvifolia*
Ulmus	*americana, campestris, carpinifolia, fulva, glabra, montana, parvifolia, procera*
Fruit trees and shrubs	
Coffea	*arabica* (coffee)
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*
Rubus	*pennsylvanica, persica* (peach), *allegheniensis, idaeus, occidentalis, rosaefolius, ursinus*
Theobroma	*cacao* (cocoa)
Vitis	*vinifera* (grape)
Ornamental shrubs	
Berberis	*thunbergii, vulgaris*
Buxus	*koreana, microphyllum, sempervivens*
Cornus	*alba, florida*
Cotinus	*coggygria*
Erica	*australis, persoluta*
Ligustrum	*amurense, vulgare*
Rhus	*aromatica, glabra, typhina*
Ribes	*sanguineum*
Rosa	*acnina, multiflora* and others
Syringa	*vulgaris*
Viburnum	*burkwoodii, lantana, lentago, tinus, tomentosum*

Management of verticillium wilt in trees varies on the species and the use of the trees. The following management aspects of verticillium wilt in tree cultivation have been suggested (Tjamos and Jiménez-Díaz, 1998) (1) choice of planting site free of *V. dahliae*; (2) disinfestation of *V. dahliae*-infested soil with fumigants, soil solarization, green amendments or biological soil infestation; (3) use of *V. dahliae*-free planting material; (4) use of resistant cultivars or rootstocks; and (5) cultural practices: avoid intercropping with *V. dahliae*-susceptible crops; minimise cultivation practices that damage the roots; avoid contaminated equipment; and avoid irrigation that may disseminate the pathogen. Disinfestation with soil

fumigants becomes increasingly limited due to negative environmental side-effects. Soil solarization is successfully applied to control *V. dahliae* in areas with a Mediterranean climate (Katan et al., 1976). Solarization may in some cases even be applied in a standing crop, as has been suggested for olive plantations (Tjamos, 1991). More recently, effects of broccoli incorporation into the soil (Subbarao et al., 1999) and of biological soil disinfestation through the fermentation of fresh organic matter in soil (Blok et al., 2000) were suggested to control *V. dahliae*.

The overall objective of this thesis is to arrive at a better prediction of verticillium wilt in nursery trees. Because of the low damage threshold in nursery trees, specific knowledge is needed about inoculum density – disease severity relationships in tree species. In literature, no information is available about these relationships in tree species. The low damage threshold in tree species also requires a quantification method with a low detection limit. Although many quantification methods have been published, they have never been compared properly with respect to their detection limits. Also, there are numerous publications that deal with virulence differences of different isolates of *V. dahliae* to one or several plant species. However, the variation within *V. dahliae*, with respect to virulence to woody hosts in general, has never been tested. This thesis tries to fill these important gaps in the knowledge of managing verticillium wilt in trees.

Determining the quantity of soil infestation with *V. dahliae* is common practice nowadays (Anonymous, 1997; Locke, 2001; Locke and Buck, 1997). However, the performance of all current quantification methods is subject to a great amount of variation, because of e.g., soil-type (Termorshuizen et al., 1998). This means that, currently, prediction of disease on the basis of pre-planting quantification is generally unreliable. Moreover, quantification methods used in practice have a detection limit of approx. 1-2 microsclerotia g^{-1} soil (Termorshuizen et al., 1998), while a significant amount of disease can occur at such levels (e.g., 5% disease incidence in strawberry (Harris and Yang, 1996)). Within the framework of this thesis, attempts were undertaken to improve the performance of detection assays. They were however in general insufficiently successful to publish new protocols. Attempts undertaken included various adaptations of the medium, the sieving protocol, and the construction of a new device to dry-plate infested soil. Some of the results are included in Chapter 2, where the current status of methods to quantify soil-borne inoculum of *V. dahliae* is reviewed.

Some of the variation in the quantification of soil-borne *V. dahliae* may be due to poor recognition of small colonies appearing on the soil plates (Termorshuizen et al., 1998). In some areas in the Netherlands, *V. tricorpus* occurs commonly on dying potato stem tissue (Lamers and Termorshuizen, 2000). Plating potato stems onto semi-selective media caused problems with respect to identification of *V. dahliae* and *V. tricorpus*. Chapter 3 describes a detailed study on the differentiation between *V. dahliae* and *V. tricorpus* in pure culture and on soil plates using various semi-selective media.

Recently, Vegetative Compatibility Groups (VCGs) were recognised among isolates of *V. dahliae*. Five VCGs (VCG 1-5) have been recognised in the USA, but only the validity of VCGs 1, 2 and 4 were confirmed in repeated studies (Bell, 1995; Joaquim and Rowe, 1990; Strausbauch et al., 1992). Grouping of VCGs varies for USA and Europe. In the Netherlands, only two VCGs have been recognised (Hiemstra and Rataj-Guranowska, 2000, 2003), VCG NL-1 and VCG NL-2, which comprise the American VCG 3 and 4 and VCG 1 and 2, respectively (Hiemstra and Rataj-Guranowska, 2003; Rataj-Guranowska and Hiemstra, 2000).

Some VCGs show ecological specialisation. For example, all cotton-defoliating strains are VCG 1 (Daayf et al., 1995) and a difference in pathogenicity in potato among VCGs has been reported (Omer et al., 2000). Chen (1994) reported that, in the USA, most isolates obtained from woody hosts belong to VCG 1. For the Dutch tree nursery industry it is important to know whether there is variation in pathogenicity among the two VCGs occurring in the Netherlands (Hiemstra and Rataj-Guranowska, 2003; Rataj-Guranowska and Hiemstra, 2000). Therefore, two inoculation experiments were carried out involving 6 isolates and 9 tree species (Chapter 4).

Blok et al. (2000) published a novel method to control various plant pathogenic soil-borne fungi and nematodes, referred to as Biological Soil Disinfestation (BSD). The method implies the induction of anaerobic conditions in soil through the incorporation of fresh organic matter into the soil followed by covering the soil with plastic. The method has shown good performance, but the long-term effects, especially needed for such perennial crops as grown in tree nurseries, were unknown. The effects of BSD on verticillium wilt and the fate of *V. dahliae* and disease symptoms were followed over a 4-year growing period at two locations (Chapter 5).

Tree growers that select new locations for tree nurseries want to know the maximum *V. dahliae* soil inoculum density that can be tolerated for their crops. Reliable information is needed about damage thresholds (Chapter 6). In most tree nurseries, verticillium wilt problems occur to some extent. The action undertaken by the grower is to rogue symptom-bearing plants immediately. The poly-etic effects of *V. dahliae* are therefore not known. Are trees adjacent to diseased trees more likely to become infected than trees standing further away from diseased trees? In addition, diseased trees may recover from verticillium wilt (Hiemstra, 1995). Is this recovery permanent (i.e., is it induced resistance), or have recovered trees an equal chance of becoming diseased again compared to trees that did not become diseased in the first year? The phenomenon of recovery may also depend on the soil inoculum density of *V. dahliae*. If the chance of becoming diseased again after recovery relies on new root infections, this chance likely depends on the amount of soil inoculum. These questions are dealt with in Chapter 6.

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Chapter 2

Quality of methods to quantify microsclerotia of *Verticillium dahliae* in soil

Mini review

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Abstract

Existing methods used to quantify microsclerotia of *Verticillium dahliae* in soil are reviewed. Most quantification methods are soil-type dependent, but are useful for disease prediction within certain soils. The major factor determining the accuracy of dry plating methods is the amount of soil plated per Petri dish. Wet plating methods are less sensitive to higher amounts of soil, especially when the fraction smaller than 20 μm is removed by wet sieving. Despite general assumptions, wet plating methods do not have lower detection limits than dry plating methods. Dry plating methods are less variable at higher inoculum levels, but more variable at low inoculum levels. Bioassays are helpful tools in answering specific research questions, but are not convenient for large scale use. Molecular quantification techniques are promising, because they are not hampered by antagonistic effects, but data on their disease predictive abilities are still largely lacking. Suggestions are given for a better comparison of techniques, and some original results are presented to illustrate certain arguments.

Abbreviations: CFU – colony forming units.

Introduction

The soil-borne fungus *Verticillium dahliae* causes severe wilt in many herbaceous and woody plant species. Among them are agricultural crops (cotton, potato), vegetables (artichoke, eggplant, pepper), fruits (apricots, avocado, cocoa, grapevine, olive, strawberry), flowers (chrysanthemum), fibre and oilseed crops (flax, sunflower), and woody ornamentals (ash, catalpa, maple, lilac, rose). *V. dahliae* is widely distributed throughout the subtropical and temperate zones, but is generally absent from tropical lowlands (Pegg and Brady, 2002). Crop losses of 13-25% have been reported in potato (Bollen et al., 1989; Haverkort et al., 1989; Locke, 2001; Nagtzaam, 1998), or even higher when nematodes were also present (Martin et al., 1982), and up to 90% in maple and olive (Goud et al., 2000; Harris, 1998; Jiménez-Díaz et al., 1998).

Microsclerotia, the surviving structures of *V. dahliae*, which are formed in senescing plant tissues, may persist in soil for many years in the absence of hosts (Pegg & Brady, 2002). Quantifying the density of microsclerotia in soil is important for disease prediction and for assessing the effect of control measures. Many methods have been described for quantifying *V. dahliae* in soil, but despite huge efforts, no reliable method exists: the best method detects only 50% of the microsclerotia. Moreover, methods are highly soil-type dependent and large differences are observed between quantification performed by different laboratories

(Termorshuizen et al., 1998), even when the same method is used (Termorshuizen et al., unpublished data). In this review, we will try to uncover what are the reasons for these differences and give suggestions for a better comparison of methods. Existing methods will be grouped according to working mechanisms and discussed with respect to their quality of performance, convenience and disease predictive abilities. Quality of performance includes: (1) the detection percentage (i.e., the nearness to the true value, also called accuracy or recovery); (2) the detection limit (i.e. the lowest density in the soil that can be detected, also called sensitivity or threshold); and (3) the variability (i.e. the variation between measurements; the reverse of precision). Convenience includes the expertise, facilities, time and money needed to perform a given method. The relation between measured inoculum densities and observed disease or yield loss of the crop of interest shows the usefulness of a method in practice. This relation is affected by the microbial, chemical, and physical factors in the soil, so a quantification method that is affected by the same soil factors could be better with respect to disease prediction than a method that detects 100% of the microsclerotia.

Some of the references in this review apparently refer to *V. albo-atrum*. This is because many researchers have regarded *V. dahliae* as a sub-group within *V. albo-atrum*. Strains were referred to as 'microsclerotial' and 'dark mycelial' strains. After 1976, there was a general international agreement that earlier references to 'the microsclerotial form of *V. albo-atrum*' actually referred to *V. dahliae* (Pegg and Brady, 2002).

Throughout the text, results will be presented of own experiments on optimisation of existing quantification methods or refining steps. Until now, one inter-laboratory comparison has been published (Termorshuizen et al., 1998), with erratum (Termorshuizen et al., 1999), which will be discussed thoroughly and partly re-analysed. The aims of the present study were: (1) to review the published methods used to quantify microsclerotia of *V. dahliae* in soil; (2) to discuss their quality of performance, convenience and biological relevance; (3) to give suggestions for a better comparison; and (4) to optimise existing quantitative detection methods.

Methods to quantify *V. dahliae* in soil

Methods to quantify *V. dahliae* in soils can be divided into plating methods (dry and wet plating), bioassays, molecular methods, and immunoassays. Plating methods have often been used in risk analysis studies and therefore they will be treated here extensively, together with published refinement steps and media.

Plating methods

Dry and wet plating involve spreading a known amount of soil onto a Petri dish containing agar media semi-selective for *V. dahliae*, followed by incubation, which enables the microsclerotia to germinate and form colonies in the medium. Prior to plating, the soil sample has to be air dried for 2-4 weeks to kill conidia and mycelial fragments of *V. dahliae* (Butterfield and DeVay, 1977). After incubation, the soil is washed off and the developed colonies containing newly-formed microsclerotia can be counted under a dissecting

microscope. The observed inoculum level is expressed as the number of colony forming units (CFU) per unit of weight (or volume) of dry soil.

Media

To obtain detectable colonies of *V. dahliae* on agar plates, microsclerotia plated onto the agar need to germinate, show hyphal growth and form new microsclerotia. Germination of microsclerotia in soil is inhibited through the process of microbiostasis, which is overcome by excess availability of carbon and nitrogen sources occurring in root exudates (Emmatty and Green, 1969; Olsson and Nordbring-Hertz, 1985; Powelson, 1966; Schreiber and Green, 1963). However, rich media are not useful for soil plating, because other fungi will overgrow *V. dahliae*. Use of poor media amended with undefined medium ingredients such as soil extracts (Harris et al., 1993; Menzies and Griebel, 1967) and cellophane extract (Taylor, 1969) have been advocated. Care should be taken in using undefined compounds in media, as these diminish reproducibility. Our results of effects of adding root exudates of eggplant (*Solanum melongena*) or smoketree (*Robinia pseudo-acacia*), both hosts of *V. dahliae*, to a poor agar medium to improve germination of the microsclerotia, indicated that the media do not lack a stimulant (unpublished data). Rather, germination or growth of *V. dahliae* into the agar medium may be inhibited by competing micro-organisms. To favour growth of *V. dahliae*, semi-selective carbon sources, such as pectate (polygalacturonic acid) (Green and Papavizas, 1968; Zehsasian, 1966) or ethanol (Nadakavukaren and Horner, 1959), that can only be metabolised by relatively few other fungi, are added to the agar medium. In addition, Tergitol NP-10 (NPX) has been used to restrict fungal colony size (Huisman and Ashworth, 1974a) and antibiotics to prevent bacterial growth. Data obtained in our own laboratory show that the brand of agar used can greatly affect quantification. When Oxoid technical No-3 agar (Oxoid, UK) was replaced with BDH technical agar (BDH, UK), significantly lower counts were obtained. Similar problems were observed with sodium polypectate (Sorensen et al., 1991). Currently the use of polypectate is problematic as the most frequently used brands (Bulmer, UK and Sigma, USA) have become unavailable recently, and other brands need to be optimised for pH before use (R. Hooftman, pers. comm.; Kabir et al., 2001).

Dry plating

All published dry plating methods use an Andersen Air Sampler (Thermo Andersen, Smyrna, Georgia, USA) to distribute the soil on the medium (Butterfield and DeVay, 1977). The sampler was designed for sampling and subsequent analysing the microbial community of air (Andersen, 1958). It was first used by Buxton and Kendrick (1963) for analyses of soil and later modified for quantification of *V. dahliae* propagules in soil (Butterfield and DeVay, 1977; DeVay et al., 1974; Harrison and Livingston, 1966). Soil is drawn by an air current through two sieve plates with 400 pores of 1.18 (top) and 0.83 mm (bottom), and impacted in 400 regular spots on Petri dishes placed below the sieve plates. The top Petri dish is changed after each subsample and the bottom ‘catcher’ Petri dish is changed after the final subsample. Several researchers prefer to use one Petri dish (one-tiered), placed beneath the sieve plate with 1.18 mm pores, which is removed after each subsample (e.g., Nicot and Rouse, 1987a; Paplomatas et al., 1992).

As an alternative to the Andersen Air Sampler, another dry plating method was used by us, viz., sprinkling by hand. A known amount of air dry soil is put on a watch glass and carefully sprinkled randomly over a Petri dish from approximately 10 cm height by ticking against the watch glass. A sheet of white paper under the Petri dish catches any soil that falls outside the Petri dish. No significant differences were observed between the number of CFU g^{-1} soil detected by the Andersen Air Sampler method and the dry sprinkling method, but the sprinkling method took five times longer.

Wet plating

Previously published wet plating methods can be divided into two procedures. The first procedure spreads the entire sieved (see Soil handling and refinement procedures) soil sample over a number of agar plates, The second procedure suspends the soil in water and spreads small aliquots of this suspension over a number of agar plates. The first procedure is often confusingly referred to as 'wet sieving' (Ashworth et al., 1972b; Huisman and Ashworth, 1974a), and the second procedure is often erroneously referred to as 'soil dilution' (Nicot and Rouse, 1987a), even though no dilution series is made (Isaac et al., 1971; Rush et al., 1992). The main difference between the two methods is the amount of soil (equivalents) plated per Petri dish, which is about 1.0 g for the wet sieving method (Ashworth et al., 1972b; Huisman and Ashworth, 1974a; Nicot and Rouse, 1987a) and about 0.1-0.5 g for the soil dilution method (Nicot and Rouse, 1987a; Harris et al., 1993).

Both wet and dry plating require basic laboratory facilities to prepare the medium, an incubator and a dissecting microscope. Time needed for plating the soil sample and counting the colonies varies between methods, but usually around 30 samples or more can be plated in one day and counted during several days to one week. Time needed for incubation is 2-6 weeks (Termorshuizen et al., 1998).

Soil handling and refinement procedures

Milling and mixing. Milling of the soil sample to release microsclerotia from soil particles is necessary for clay-type soils. Severe milling, however, may break microsclerotia into smaller fragments, resulting in inoculum densities that are up to five times greater than the actual densities in the field. Prolonged drying of the soil before milling may even aggravate this effect (Ashworth et al., 1974). Milling procedures that led to the highest amount of microsclerotium breakage (as assessed by plating different size fractions) were mortar and pestle (e.g., Schnathorst and Fogle, 1973), followed by high speed (20,000 rpm) micromill (Harrison and Livingston, 1966) and low speed (<200 rpm) milling to 2 mm size (Ashworth et al., 1974; Harris et al., 1993). Butterfield and DeVay (1977) used a revolving jar mill to reduce soil particle size, which did not increase apparent inoculum levels. An extra advantage of this latter technique was that the samples became well mixed, which is very important when working with small sub-sample sizes necessary for dry plating. Wet plating methods use larger subsamples and mixing is less critical than with dry plating methods. Breaking soil aggregates with a 1% sodium hexametaphosphate solution before wet plating had no effect on quantification (Harris et al., 1993). Breaking down heavy clay soils with sodium pyrophosphate in the soil suspension was used occasionally (Nagtzaam, 1998).

Sieving. Microsclerotia can be separated from larger or smaller organisms by wet or dry sieving. The diameter of microsclerotia in naturally infested soils varies between 11 and 125 μm, but larger aggregates may occur (Ashworth et al., 1972b; DeVay et al., 1974). Microsclerotial size distribution varies strongly between soils, probably as a result of cropping history. For example, the fraction smaller than 38 μm has been reported to be < 2% in potato soils (Smith and Rowe, 1984), but 30-68% in cotton and sunflower soils (Butterfield and DeVay, 1977; Camporota and Rouxel, 1977).

Wet sieving with nested sieves (with pore sizes of e.g., 125 and 20 μm) consists of vigorous washing with tap water to break down soil aggregates, manually or with a shaking machine (Harris et al., 1993; Huisman and Ashworth, 1974a). Dry sieving of small amounts of soil is also possible (Ashworth et al., 1972b; Camporota and Rouxel, 1977), and larger amounts can be sieved with an Alpine air current dry sieving device (Hosokawa Alpine Inc., Augsburg, Germany), which creates a continuously moving air current on the sieve surface to prevent pore occlusion. The sieve is covered with a lid and only one sieve can be used at a time. Soil particles are not broken down by dry sieving, which makes the procedure less efficient than wet sieving: more microsclerotia were lost during dry sieving and they were concentrated in a larger volume of soil, viz., 25 (sand) to 33% (clay) of the original soil sample for dry sieving and 15% (sand and clay) for wet sieving (unpublished data).

Sedimentation and flotation techniques. Sedimentation and decanting have been used to separate microsclerotia from smaller soil particles and conidia of soil fungi (Evans et al., 1967). Flotation techniques use the specific density of the microsclerotia to separate them from the bulk soil. A soil sample is suspended in a 65% (w/w) or saturated solution of sucrose (Harris et al., 1993; Huisman and Ashworth, 1974b) and centrifuged. The supernatant containing most of the microsclerotia is decanted, sieved, resuspended in tap water and plated. The flotation step can be repeated to minimise losses (Huisman and Ashworth, 1974b), though Harris et al. (1993) observed no significant improvement after this second step. Ben-Yephet and Pinkas (1976) described a flotation technique for loess soil, using 50% (w/v) caesium chloride and a separatory funnel. This eliminated centrifugation and resulted in a recovery of 55%, with low variation.

Sedimentation and flotation techniques can easily be combined with other refinement procedures such as wet sieving (Huisman and Ashworth, 1974b). An advantage of flotation techniques is that counting the *V. dahliae* colonies is quicker, because the colonies formed are often larger due to less competition from other fungi as a result of low amounts of soil being plated and/or a nutrition effect of the sucrose (Harris et al., 1993). A disadvantage of flotation techniques is that they are time consuming and do not always lead to improved detection percentages or detection limits (Harris et al., 1993; Termorshuizen et al., 1998).

Breaking of dormancy. Kapulnik et al. (1985) described the use of methionine to break fungistasis and to significantly improve detection percentages of dry plating. 10 g of dry soil was incubated for 1 week at 33°C with 15 mg of dl-methionine (Paplomatas et al., 1992). Rinsing for 10 s in 0.5% NaOCl was used to relieve microsclerotia from copper-induced fungistasis (Ashworth et al., 1976), but this appeared not to improve detection in soils without copper-induced fungistasis (Harris et al., 1993).

Bioassays

Bioassays for quantification of *V. dahliae* in soil have not been used on a regular basis and have remained at an experimental stage (Evans et al., 1974; Soesanto, 2000). Sometimes, they have been used for special purposes, such as selection of plants resistant to the pathogen (Palloix et al., 1990) or the evaluation of biocontrol agents (e.g., Nagtzaam et al., 1997). However, bioassays are not truly quantitative, unless serial dilutions of the soil are made with clean soil, and disease or infection is analysed using most probable number statistics (Maloy and Alexander, 1958). During bioassays, soil is planted with susceptible plants (e.g., eggplant (*Solanum melongena*) or *Arabidopsis thaliana*) or resistant plants (thorn apple (*Datura stramonium*)). After growing these plants at standardised conditions for 3-8 weeks, roots are washed and plated on a semi-selective medium. After incubation, the percentage of the root length infected (Soesanto, 2000) or number of colonies per unit root length (Evans et al., 1974; Nagtzaam et al., 1997) can be counted. Alternatively, on susceptible plants, disease can be scored or the amount of systemic infection can be assessed by plating stem pieces or xylem sap, but these measurements are less sensitive (Nagtzaam et al., 1997; Soesanto, 2000). Assessment of the amount of newly formed microsclerotia in senescing shoot tissue was the most sensitive measure, but also more variable than root plating (Soesanto, 2000).

The bioassays of Soesanto (2000) and Nagtzaam et al. (1998) had the lowest published detection limits (viz., 1 microsclerotium g^{-1} soil), but have only been tested with artificially infested semi-sterile soils and not with field soil. The method of Evans et al. (1974) was claimed to have a detection limit of 2-3 microsclerotia g^{-1} dry field soil, but it was probably higher because of underestimation of the natural infestation level, which was estimated by plating.

Bioassays require standardised growing conditions (temperature, soil humidity) and expertise in executing and standardising all steps involved (transplanting, disease assessment, root plating).

Other approaches use measurements of infection in the crops of interest as a measure for soil infestation. Davis et al. (1983) assessed the number of *V. dahliae* microsclerotia in dried stem tissue of potato by plating ground potato stems on semi-selective medium. The increase rate of microsclerotia in the potato stem during the growing season (the slope of the line) was linearly correlated with soil inoculum levels. Similar relationships were found by other authors (Bollen et al., 1989; Nagtzaam, 1998; Nicot and Rouse, 1987b). Alternatively, microsclerotia can be released from potato stems by grinding and dry-sieving (Isaac et al., 1971), blending and wet-sieving (Soesanto, 2000) or enzymatically with cellulase (unpublished data). Some authors plated potato stem sap (Hoyos et al., 1991; Nagtzaam et al., 1997) on agar media, or assessed the concentration of cotton microsclerotia in a suspension by means of light transmittance at 400 nm (Tsai and Erwin, 1975). A similar approach of focusing on disease in the crop of interest is chosen by some tree nursery growers in the Netherlands. When renting land they include a clause in the contract allowing them to leave the land whenever verticillium wilt is encountered in their first growing season. During this first season they plant throughout the field the highly susceptible *Acer ginnala*. They regard this method as being more reliable and indicative for disease than plating methods (W. Bijvoet, pers. comm.). Probably, data obtained are a good indicator for disease prediction in future crops. However, such methods are essentially post-planting and therefore sub-optimal

for high value or perennial crops. Moreover, stem infection of potato is cultivar-dependent (Davis et al., 1983). Because stem colonisation increases during the growing season, multiple samples are necessary to assess the increase rate (Bollen et al., 1989; Davis et al., 1983).

Immunoassays

Enzyme-linked immunosorbent assays (ELISA) have not frequently been used for quantification of *V. dahliae* in soil. Heppner and Heitefuss (1997) developed a double-antibody sandwich (DAS) ELISA. This method uses a specific monoclonal antibody, together with a polyclonal antiserum, which react with soluble proteins from microsclerotia. The method includes making a soil suspension, wet sieving, air drying the 20-125 μm fraction, sonification in an extraction buffer, centrifugation, incubation of the supernatant in microtiter plates and measuring the optical density at 405 nm. The detection limit was 2.4 μg of microsclerotia g^{-1} soil equalling about 1-2 microsclerotia g^{-1} soil. A negative relation with yield of oilseed rape was observed in field plots in the range of 0-240 μg of microsclerotia g^{-1} soil (Heppner and Heitefuss, 1997).

Molecular methods

Molecular methods for quantification of *V. dahliae* in soil are based on detection of species-specific nucleotide sequences, using the polymerase chain reaction (PCR) with specific primers. Most of the methods described in literature use sequence differences in the intervening transcribed spacer (ITS) regions of the rDNA. They were initially developed for pure cultures (Carder et al., 1994; Nazar et al., 1991; Robb et al., 1993) and for detection and quantification (Aspromougos and Schlösser, 2000; Hu et al., 1993; Robb et al., 1994) *in planta* and were adapted for detection (Mahuku et al., 1999; Pérez-Artés et al., 2001; Platt et al., 2000) and quantification (Krishnamurthy et al., 2001; Mahuku and Platt, 2002; Robb and Nazar, 1997) in soil. Other methods use sequence differences in the mitochondrial small rDNA (Li et al., 1994) or genomic DNA (Li et al., 1999). The method of Li et al. (1999) was used for quantification of *V. dahliae* in potato and in silica sand. During quantitative assays, different amounts of a competitor DNA are added, which are amplified by the same primers as the target DNA, resulting in an amplified fragment of a different size (competitive PCR). The two fragments are visible on the gel as two bands. The different amounts of competitor DNA result in different ratios of band intensities between the competitor and the target DNA fragment. Ratios close to 1.0 are used to estimate the concentration of the target DNA (Mahuku and Platt, 2002).

Mahuku and Platt (2002) observed that the log of the estimated amount of target DNA in the range of 10 ng - 100 fg was linearly correlated with the log of the number of microsclerotia in the range of 5-100 microsclerotia. The log of the estimated amount of target DNA in the range of 10 ng - 1 pg was linearly correlated with the log of the number of conidia in the range of 10^2-10^7 conidia. Higher amounts of microsclerotia or higher conidium densities resulted in the saturation of the PCR reaction and a new range of competitor DNA or dilutions were needed to accurately quantify target DNA. The method of Krishnamurthy et al. (2001) accurately detected DNA in the range of 2 to 200 pg, with 2 pg approximately

equalling 60 genomes of *V. dahliae*. The detection limit observed by Li et al. (1999) was 100 pg of pure genomic DNA (50 copies of target DNA). The lowest numbers of fungal structures they were able to detect were 450 conidia and 17-165 microsclerotia. However, microsclerotium size and consequently number of genomes may vary considerably and the consequences for epidemiological interpretation of PCR-based results need further investigation. Most experiments were performed on microsclerotia or conidia added to sterile soil, but Mahuku et al. (1999) and Mahuku and Platt (2002) quantified *V. dahliae* in field soil. They compared their PCR assay with a plating method (wet sieving over a 38 µm screen followed by sucrose flotation and plating onto NPX-medium). The PCR assay generally detected more microsclerotia than the plating method.

Molecular methods can discriminate between *V. dahliae* and *V. tricorpus* (Heinz and Platt, 2000), and can be adapted to detect other plant pathogenic *Verticillium* species (Robb et al., 1994) or groups within *V. dahliae*, such as the cotton defoliating and non-defoliating strains (Mercado-Blanco et al., 2001; Pérez-Artés et al., 2001). PCR assays deliver results in 1-2 days which is fast compared to plating techniques, as lengthened incubation steps are not necessary. Time consuming plate counts are not needed, but molecular methods need larger initial investments on equipment and chemicals. Molecular quantification methods need to be compared with different plating methods, preferably in an interlaboratory comparison, to obtain insight in their relative performance. More data is needed on the relation between amount of DNA detected in soil and disease in the field.

Biological relevance of quantification methods

Biological relevance is the most important criterion for the quality of a detection method. Does the result of pre-planting quantification correlate with the amount of disease in the crops of interest? At first sight, biological relevance seems greatest in bioassays, where only those microsclerotia are detected that are viable, germinable and capable of infecting the root or the root cortex. Moreover, *Verticillium* is quantified in the presence of many other soil organisms that can have strong competitive (Evans and Gleeson, 1973; Isaac, 1967), interactive (Martin et al., 1982; Wheeler et al., 1994) or antagonistic (Berg and Ballin, 1994; Marois et al., 1982) effects, thus giving insight in the conduciveness or suppressiveness of a soil. The detection percentage might be negatively affected, but the result may be biologically relevant. However, inoculum density – disease incidence relationships are host-dependent, experimental conditions may affect the results, and care should be taken whenever the test plant is another species or cultivar than the crop of interest. When roots are plated after the bioassay, the colonisation of the root cortex may not be related to pathogenicity (Evans et al., 1974; Huisman, 1988). Colonisation of the vascular system is usually related to disease (Huisman, 1988; Soesanto, 2000).

Quantitative detection through soil plating is also biologically relevant, because only those microsclerotia that are viable, germinable and able to form colonies containing new microsclerotia are counted. Competitive and fungistatic effects are partly taken into account on the semi-selective nutrient media, but are likely to be different than their effects in the soil. All agar media described as semi-selective for *V. dahliae* still sustain the growth of a considerable number of other organisms, though bacterial activity is strongly reduced. The importance of competitors and antagonists on agar plates is illustrated in Figure 1, showing

that the more soil is put on a Petri dish, the less colonies of *V. dahliae* are observed, both relative to the amount of soil (CFU g^{-1} soil) and (partly) in an absolute sense (numbers of colonies per plate). Measurements on artificially infested, partially sterilised soil are not biologically relevant. In the expression of inoculum density as CFU g^{-1} soil, biological relevant information is lost, because large microsclerotia form larger colonies, which are more clearly visible than small microsclerotia (Ben-Yephet and Pinkas, 1977; Hawke and Lazarovits, 1994). Chemical soil characteristics can also influence detection. Ashworth et al. (1976) observed a sudden failure of their detection method because of copper-induced fungistasis of the microsclerotia. However, the infection of cotton in the field was also strongly reduced, so detection was still meaningful. In general, all frequently-used plating methods, such as dry plating, wet sieving, and sucrose flotation, have given significant correlations with observed disease (e.g., Ashworth et al., 1972a; Nicot and Rouse, 1987b, Paplomatas et al., 1992), so in principle all are biologically meaningful for specific soils and crops of interest. DeVay et al. (1974) found no significant correlation between inoculum densities assessed by dry plating on soil extract agar with a layer of cellophane and disease in cotton, but possibly many more unpublished negative results exist.

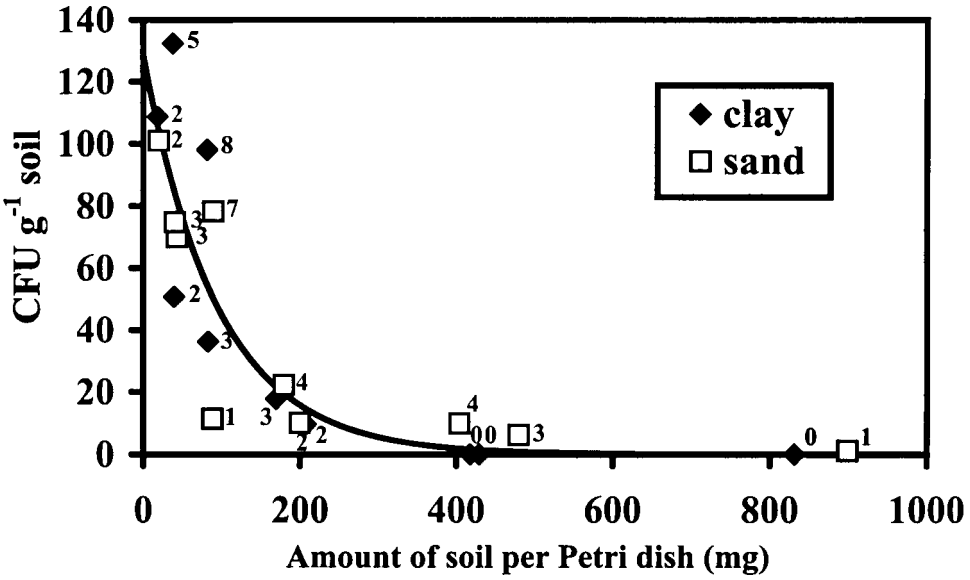

Figure 1. Relation between the detected inoculum density of *V. dahliae* (CFU g^{-1} soil) and the amount of soil per Petri dish. One sandy and one clay soil were used, which were naturally infested with *V. dahliae*. Soils were dry-sieved and only the 20-106 µm fraction was dry plated by hand sprinkling different amounts of soil on modified soil extract agar (Harris et al., 1993), with 50 ppm oxytetracyclin as the single bacteriostatic agent. Each point represents one Petri dish. Figures next to the data points indicate the absolute amount of colonies on that Petri dish. The fitted line shows a significant ($P < 0.0001$) exponential decline model describing the experimental data: the detected inoculum density (CFU g^{-1} soil) = 129 exp.(-0.01 × the amount of soil per Petri dish (mg)). Data for clay and sand were pooled, because 95% confidence limits for separate parameter estimates overlapped.

Molecular quantification methods do not take the soil microbial community into account, though it can interfere during DNA isolation. Primers are only useful when they are *V. dahliae*-specific and therefore quantification depends only on the amount of DNA of

V. dahliae present. This unique advantage can result in detection of dead microsclerotia (Mahuku and Platt, 2002), and could be biologically irrelevant. It has been hypothesised that this problem could be overcome by quantification of mRNA, because those represent recently transcribed genes, and thus represent the active microsclerotia. More data is needed on the relation between PCR-based quantification in the soil and observed disease, to assess the bias due to detection of dead microsclerotia.

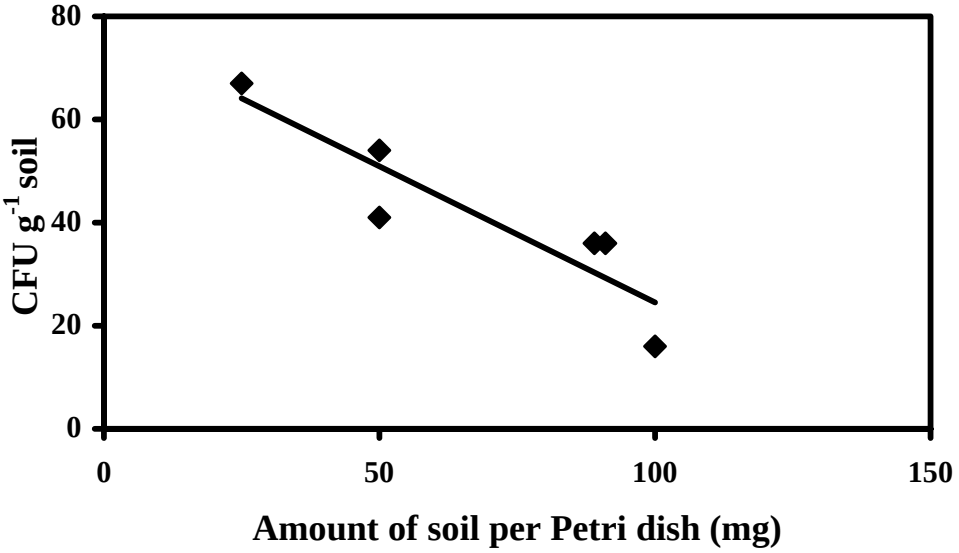

Figure 2. Quantification of *V. dahliae* (CFU g^{-1} soil) in relation to amount of soil per Petri dish. Soil was dry plated with an Andersen Sampler. Data obtained from Termorshuizen et al. (1998; 1999). The fitted line describes a significant ($P = 0.01$) linear regression of the data. The regression equation was: CFU g^{-1} soil = 77.3 – 0.53 × amount of soil per Petri dish; R^2=0.82.

Quality of performance of plating methods

The quality of performance of a method depends on two characteristics, viz., the detection percentage and the detection limit. The detection percentage of a method can only be determined for artificially infested soils. Care should be taken to use different soil types and microsclerotia from different origins, because of presence of a method × soil interaction, as observed by Termorshuizen et al. (1998). They included in their study, one soil which had been artificially infested at 5 and 60 microsclerotia g^{-1} soil. The best methods detected 30-50% of the microsclerotia in this soil. However, because of the observed soil-type dependency of methods, it seems better not to focus on performance of methods with this particular soil type. Rather, the performance of a method tested on all soils (in this particular study 12 naturally and 2 artificially infested) should be emphasised and compared with the performance of the best overall method (Table 1). Methods differed much in their performance (Table 1). Dry plating methods generally had a higher overall mean detection percentage than wet plating methods. Dry plating methods performed best at the lowest amounts of soil (25 mg) per Petri dish and performance decreased with increasing amounts of soil (Figure 2), which is in agreement with our own findings (Figure 1), but contradict earlier findings of Butterfield and DeVay (1977). For the wet plating method this relation was unclear, a finding which is in agreement with those of Harris et al. (1993). The detection limit

Table 1. Detection percentages and detection limits derived from an interlaboratory comparison of methods (a-k) to quantify *V. dahliae* in soil (Termorshuizen et al., 1998; 1999).

	Plating method										
	Wet plating					Dry plating					
	a [1]	b	d	e	f1	f2	g	h	i	j	k
Amount of soil or soil equivalents per Petri dish (g)	1.50	0.20	0.20	0.10	0.27	0.09	0.10	0.09	0.05	0.05	0.025
Number of plates	10	10	10	6	5	5	4	9+1 [2]	5	3	10
Total amount of soil plated (g)	15	2.0	2.0	0.6	1.35	0.45	0.4	0.81	0.25	0.15	0.25
Theoretical detection limit [3]	0.067	0.5	0.5	1.7	0.74	2.2	2.5	1.23	4.0	6.7	4.0
Observed detection (CFU g^{-1} soil) [4]	0.57	6.4	17	26	18	36	16	36	41	54	67
Detection percentage relative to the best method [5]	0.85	9.6	25	39	27	54	24	54	61	81	100
Relative detection limit (CFU g^{-1} soil) [6]	7.88	5.25	1.97	4.38	2.75	4.09	10.5	2.29	6.5	8.3	4.0
Relative detection limit per Petri dish	78.8	52.5	19.7	26.3	13.8	20.5	42.0	22.9	32.5	24.9	40.0
Variability (%) [7]	30	4.4	1.9	1.7	2.0	1.4	1.6	1.3	1.4	1.0	1.0
Ranking detection percentage	11	10	8	6	7	4.5	9	4.5	3	2	1
Ranking relative detection limit per Petri dish	11	10	2	6	1	3	9	4	7	5	8
Ranking variability	11	10	8	7	9	4	6	3	5	2	1

[1] Methods as described in Termorshuizen et al. (1998). In brief: method a: wet sieving (38-130 μm) followed by sucrose flotation, method d: wet sieving (20-130 μm), other methods applied no refinement steps. Method h used a two-tiered Andersen Sampler, other dry plating methods used a one-tiered Andersen Sampler. Methods h and j used an ethanol-sucrose medium, other methods used pectate based media.

[2] 9 Petri dishes and 1 catcher Petri dish.

[3] Calculated from 1 colony observed on the total number of plates.

[4] Overall average of 12 naturally infested soils and 2 (one soil type at 2 densities) artificially infested soils.

[5] Method k had the highest amount of CFU g^{-1} soil and was set to 100%.

[6] The theoretical detection limit divided by the detection percentage relative to the best method.

[7] Standard deviation among Petri dishes of the same soil sample ($\sqrt{s_P^2}$ as described in Termorshuizen et al. (1998) multiplied by 100) divided by the observed detection (CFU g^{-1} soil).

is a measure describing the lowest concentration of CFU in the soil that can be detected. This means 1 colony in the total number of Petri dishes, and is calculated as 1 divided by the total amount of soil on all Petri dishes. Whenever microsclerotia are concentrated in a smaller volume of soil by refinement procedures, 1 is divided by the original amount of soil that is represented by the concentrated soil on the Petri dishes. In this way, methods that use larger amounts of soil (or after refinement: more soil equivalents) have lower theoretical detection limits than methods that use smaller amounts of soil (or soil equivalents). However, these published theoretical detection limits are of no use in practice because they do not take into account the detection percentage. If a certain method is said to have a theoretical detection limit of 0.5 CFU g^{-1} soil, but a detection percentage of 30% (Termorshuizen et al., 1998), the real detection limit would be 0.5/0.3=1.67 CFU g^{-1} soil. Because the real detection percentage is unknown (see above), detection percentages relative to the best method can be used to calculate the relative detection limit of a method (Table 1). Expressed per Petri dish (Table 1) this figure can be used to compare methods properly. By doing so, it becomes clear that, despite of what is generally thought, wet plating methods do not have better detection limits than dry plating methods. Since observing the colonies on the plates is the most time consuming step (about 5 minutes per plate), the desired amount of plates and thus the detection limit can be varied depending on the sensitivity of the crop of interest without affecting the performance of a method. However, our suggestion is to use at least 5 Petri dishes, since Termorshuizen et al. (1998) observed that the greatest variation did not occur among soil samples, but among dishes within the same soil samples. Dry plating methods are generally less variable than wet plating methods (Table 1), but at low inoculum densities (approx. smaller than 10 CFU g^{-1} soil) dry plating methods are more variable than wet plating methods (Termorshuizen et al., 1998).

In summary, when deciding which method to use, focus should be placed on (1) the relative detection percentage and (2) the relative detection limit per Petri dish. By doing so (Table 1), it appears that none of the methods listed in Termorshuizen et al. (1998) combines a high detection percentage with a low relative detection limit per Petri dish, but dry plating methods generally combine the two characteristics better than wet plating methods. Whenever artificially infested soils are included, they have to be different origins, infested with microsclerotia from different sources. To compare variability, methods should use the same number of Petri dishes. To gain more insight in real detection limits, the inclusion of a number of soils with very low inoculum levels is necessary, as was done by Harris et al. (1993).

Conclusions

One may wonder why the impressive research efforts have not yet led to a robust and reliable detection technique. One reason may be that the semi-selective polypectate medium used for *V. dahliae* is not as selective as those developed for other soil-borne pathogens such as *Fusarium oxysporum* (Komada, 1975) and *Pythium* spp. (e.g., Stanghellini *et al.*, 1982). Another reason is that *V. dahliae* is able to incite damage to plants at relatively low inoculum densities. Inoculum densities as low as 1 microsclerotium g^{-1} (artificially infested) soil have resulted in symptom development during climate chamber experiments (Soesanto, 2000; unpublished data). Significant symptoms have occurred in the field at detected inoculum

levels around 1 CFU g^{-1} naturally infested soil in cotton (Paplomatas et al., 1992), strawberry (Harris and Yang, 1996), and Norway maple (Goud et al., 2000). However, actual densities were probably two to three times higher, because plating methods detect only part of the microsclerotia (Termorshuizen et al., 1998).

The observation that the largest variation in detection of *V. dahliae* occurs at the Petri dish level and not at the subsample level (Termorshuizen et al., 1998), may indicate that the mechanism that explains variability in detection of *V. dahliae* has a stochastic behaviour. For example, the absolute distance between the germinating microsclerotium and other organisms, capable to grow on the medium, could be the decisive factor whether or not *V. dahliae* will form a visible colony. Possibly the best approach to test whether colony formation is inhibited, is by concentrating microsclerotia onto agar media, and quantifying the microsclerotia using molecular methods. After incubation, molecular methods could target at the germinated microsclerotia, and results of molecular quantification and CFU counts could be compared. Insight into the mechanism could well yield information that can be extrapolated to the microsclerotium behaviour in rhizosphere soil.

Alternatives to plating methods include bioassays and molecular methods, but bioassays are very time-consuming and prone to variation according to growing conditions, and molecular methods focusing on direct DNA-isolation presume that a known, same amount of DNA is present in each microsclerotium and that a fixed fraction of the DNA is viable. Until molecular quantification methods have been tested for correlations with disease on a larger scale, plating methods will remain the most reliable means of quantification in soil. However, due to soil type × method interactions it is questionable whether a worldwide standardisation of methods is possible. Interlaboratory comparison of plating and molecular methods will remain important to assess relative performances.

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Chapter 3

Morphology of *Verticillium dahliae* and *V. tricorpus* on Semi-Selective Media used for the Detection of *V. dahliae* in Soil

J.C. Goud, A.J. Termorshuizen & W. Gams, 2003
Mycological Research 101: 822-830.

Abstract

The morphology of 2 soil-borne *Verticillium* species, *V. dahliae* and *V. tricorpus*, was studied on 2 semi-selective agar media, in the absence and presence of soil. Morphology of the fungi differed considerably between the media, with respect to presence and shape of microsclerotia, dark hyphae (*i.e.* short melanised hyphae attached to the microsclerotia) and dark mycelium (*i.e.* melanised mycelium throughout the colony). On modified soil extract agar (MSEA), a pectate based agar, *V. dahliae* always had globose to elongate microsclerotia, without dark hyphae or dark mycelium, whereas *V. tricorpus* always had dark hyphae or dark mycelium, and microsclerotia, whenever present, were globose to irregular in shape. On ethanol agar (EA), *V. dahliae* had large microsclerotia and abundant dark hyphae, whereas *V. tricorpus* did not form microsclerotia but always abundant dark mycelium. For the first time we observed formation of dark hyphae by *V. dahliae* to such a great extent. In the presence of soil, most characteristics were less pronounced, and *V. dahliae* microsclerotia were smaller, but *V. tricorpus* produced large microsclerotia, even when they were absent in pure culture. Morphological characteristics suitable for discrimination between the 2 species on MSEA plates in the presence of soil were selected and tested with fresh isolates from agricultural fields. The 2 fungi could be distinguished using qualitative characteristics and microsclerotial size. Molecular analysis and morphology on potato dextrose agar confirmed all identifications made on soil dilution plates.

Abbreviations: EA – ethanol agar; MSEA – modified soil extract agar; PDA – potato dextrose agar

Introduction

Representatives of the form-genus *Verticillium* Nees 1816 in the strict sense (anamorphs of the *Phyllachorales* (Messner *et al.* 1996), are commonly found in agricultural soils (Domsch, Gams & Anderson 1980). The genus includes the virulent plant pathogenic species *V. dahliae* Kleb. 1913, *V. longisporum* (Stark) Karapapa, Bainbridge & Heale 1997 and *V. albo-atrum* Reinke & Berth. 1879, which have low saprotrophic abilities, *V. nubilum* Pethybr. 1918 and *V. nigrescens* Pethybr. 1919 which are saprobes and weak pathogens, and *V. tricorpus* Isaac 1953 which has intermediate saprotrophic ability and is pathogenic to a limited number of crops. All these species can be associated with the same crop, e.g. potato or tomato (e.g. Domsch *et al.* 1980, Isaac 1967, Pegg & Brady 2002, Skotland 1971).

The 6 species can be differentiated morphologically by the types of resting structures they form in and on the surface of plant material, and on many artificial agar media, such as potato dextrose agar (PDA). *V. dahliae* and *V. longisporum* form microsclerotia, which are black melanised clumps, formed by budding of mycelial cells; *V. albo-atrum* forms melanised

resting mycelium; *V. nigrescens* and *V. nubilum* form chlamydospores, and *V. tricorpus* forms microsclerotia, resting mycelium and chlamydospores. The morphological differences of the microsclerotia of *V. dahliae* and *V. tricorpus* are pronounced on PDA, as described by Isaac (1949, 1953) and Smith (1965): *V. tricorpus* forms large and irregularly shaped microsclerotia, usually with melanised hyphae growing from them, and basally pigmented conidiophores, whereas *V. dahliae* forms smaller and oval to elongate microsclerotia which are sharply differentiated from the hyaline mycelium and hyaline conidiophores. Moreover, *V. tricorpus* often causes a yellow discoloration of the PDA medium upon first isolation. *V. longisporum* is known to be a heterodiploid between *V. dahliae* and *V. albo-atrum*. Morphologically *V. longisporum* is most similar to *V. dahliae* but it can be differentiated from *V. dahliae* in pure culture by the shape of the microsclerotia, the number of phialides per node and, particularly, its larger conidia, 7.9 × 2.5 µm. *V. longisporum* is mainly known from cruciferous hosts (Karapapa *et al.* 1997). Because of the resemblance of *V. dahliae* and *V. tricorpus* on poor isolation media, our study is mainly concerned with these 2 species.

In a number of field crops, but also many woody species, *V. dahliae* can cause serious wilt disease, whereas *V. tricorpus* is generally harmless (Hiemstra 1998). *V. tricorpus* has even been recommended for biological control against *Rhizoctonia solani* in cotton seedlings (DeVay *et al.* 1988, Paplomatas, Tzalavaras & DeVay 2000) and *V. dahliae* in potato (Davis *et al.* 2000). There is a great need for reliable assessment of soil population densities of *V. dahliae* in order to predict possible problems in tree nurseries and agricultural crops. Plating soil samples on semi-selective media is currently the most reliable way to assess population densities of *V. dahliae* in soil (Termorshuizen *et al.* 1998). Microsclerotia are detected indirectly by giving rise to characteristic new colonies with microsclerotia on a semi-selective agar medium. However, microsclerotia of *V. tricorpus,* when present in these soil samples, can also form similar colonies with microsclerotia. Rich media, like PDA, cannot be used during quantification of *V. dahliae* in soil, because rapidly growing and sporulating fungi, present in all soils, will overgrow *V. dahliae*, resulting in failure of quantification. While morphological differences are pronounced on rich media, on (poor) semi-selective media *V. dahliae* and *V. tricorpus* are easily confused, even by experienced researchers (Termorshuizen *et al.* 1998). Isolated colonies can be characterised using molecular techniques (Robb *et al.* 1994). However, the method of transferring colonies from semi-selective media to PDA, and purification for identification of species is too laborious to be employed as a standard procedure. Therefore it is important for disease prediction to compare the morphology of the 2 species on the original semi-selective media directly, and describe differential characteristics of the 2 species.

In this study we (1) compared the morphology of *V. dahliae* and *V. tricorpus* on semi-selective media in pure culture and in the presence of insterile soil, (2) distinguish characteristics that can be used to separate the 2 species on semi-selective media in the presence of soil, and (3) test these characteristics on colonies from soil samples of agricultural fields. The identity of isolates was always verified by molecular analysis.

Materials and methods

In Experiment 1 five single-spore isolates of both *V. dahliae* and *V. tricorpus* were studied. In Table 1 these isolates and their origin are listed. Morphology was first studied in pure culture

on PDA to determine the species: isolates with irregularly shaped microsclerotia, chlamydospores, melanised mycelium, and yellow discoloration of the medium were classified as *V. tricorpus*. Isolates with globose, oval to elongate microsclerotia and without chlamydospores or melanised mycelium were classified as *V. dahliae* (Isaac 1949, 1953, Smith 1965). All isolates fitted these descriptions, except for the *V. tricorpus* isolates 384.84 mc-1 and Vtx mc-1 (Table 1) which did not form microsclerotia on PDA, and did not show yellow discoloration of the medium.

Table 1. Origin of isolates of *V. dahliae* and *V. tricorpus* used in Experiment 1.

Species	Isolate[1]	Host of origin	Year of isolation	Location	Isolation by
V. dahliae	A59 mc-1	Blackberry (*Rubus fruticosus*)	1996	Spijk, The Netherlands	J.W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A45 mc-1	Strawberry (*Fragaria* sp.)	1996	Elst, The Netherlands	J.W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A60 mc-1	Maple (*Acer* sp.)	1993	Swolgen, The Netherlands	Auf 'm Keller, Plant Protection Service
V. dahliae	A56 mc-1	Rose (*Rosa* sp.)	1996	Elst, The Netherlands	J.W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A63 mc-1	*Forsythia* sp.	1997	Aalsmeer, The Netherlands	B. Wessels, Plant Protection Service
V. tricorpus	A36 mc-1	*Alstroemeria* sp.	1996	Rijnsburg, The Netherlands	J.W. Veenbaas-Rijks, Plant Protection Service
V. tricorpus	127.79A mc-1	Tomato (*Lycoper-sicon esculentum*)	1979	Roxborough, New Zealand	B. Taylor, -[2]
V. tricorpus	384.84 mc-1	Potato (*Solanum tuberosum*)	1984	Wageningen, The Netherlands	J. v. d. Spek, Inst. Plant Protection Research
V. tricorpus	227.84 mc-1	Potato (*Solanum tuberosum*)	1984	Dronten, The Netherlands	T. Hofman, Wageningen University
V. tricorpus	Vtx mc-1	-	-	-	-

[1] Voucher cultures are preserved in CBS (Utrecht)
[2] Unknown

As experimental treatments, 2 semi-selective media were compared: modified soil extract agar (MSEA) (Harris, Yang & Ridout 1993), which is a pectate-based medium often used for quantitative detection in soil, and ethanol agar medium (EA) (Nadakavukaren & Horner 1959), often used for isolating *V. dahliae* from plant material. Each semi-selective medium was amended with 50 mg l^{-1} oxytetracycline after autoclaving as the single bacteriostatic agent. Media for Experiment 1 were produced once, to avoid batch to batch variation. Ten microsclerotia per plate, produced on PDA covered with cellophane to stimulate formation of microsclerotia (DeVay *et al.* 1974), were placed individually on the 2 media.

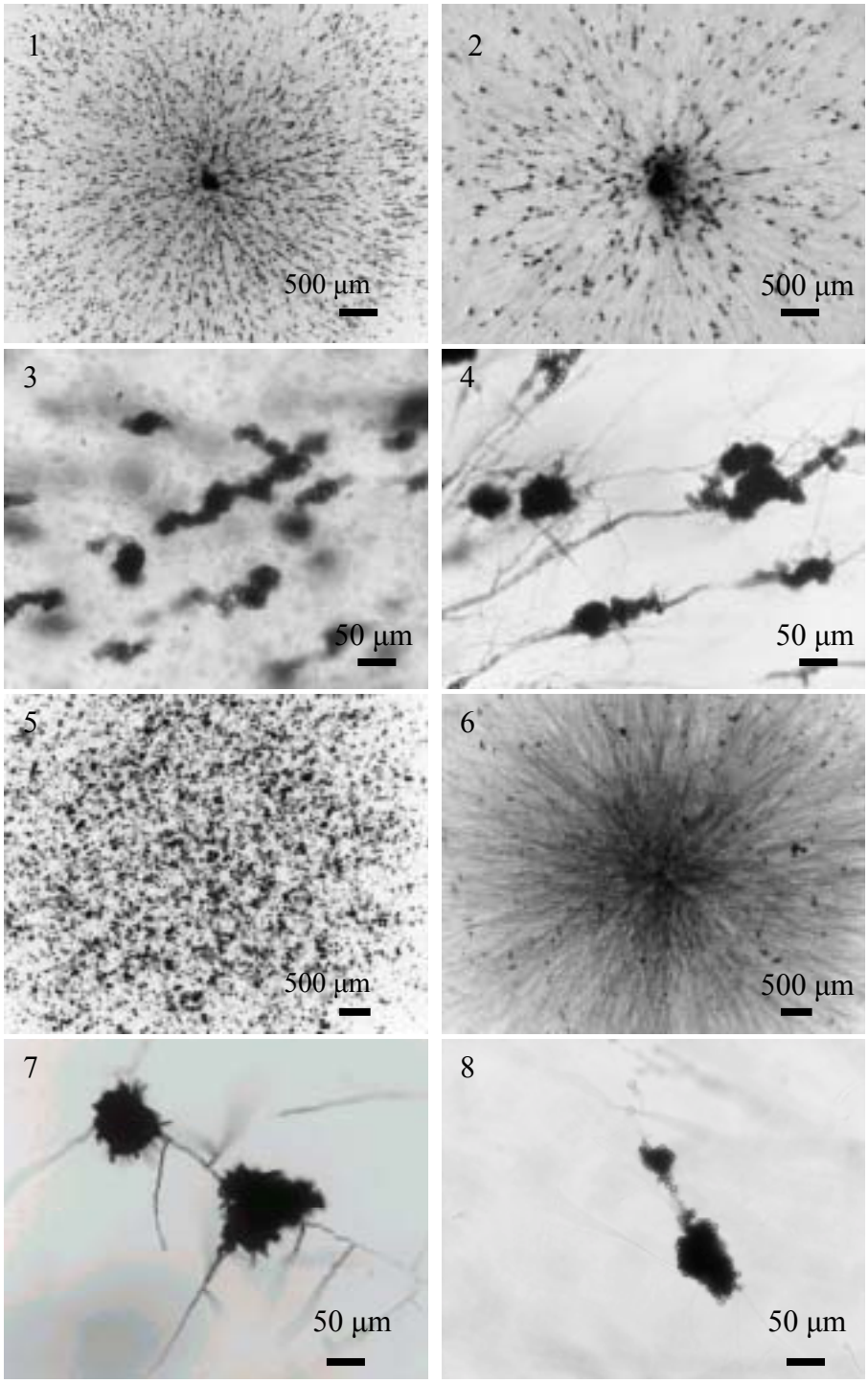
Colony growth of *Verticillium* species was studied with and without the presence of soil. For the latter treatment, 0.8 ml a suspension of non-sterile, *Verticillium*-free sandy soil was added to the surface of the inoculated agar plates. The soil had been steamed and cropped with wheat for 6 weeks before the start of the experiment. During the experiment, the soil was

treated in the same way as a soil sample is treated during the protocol for quantitative detection of *V. dahliae* (Harris *et al.* 1993): 12.5 g of dry soil was sieved, the 20-106 μm fraction was suspended in 0.08% water agar, and 0.8 ml of this suspension was spread over the agar surface in a 9.4 cm diam. Petri dish, after which the plates were air dried for 15 min. The inoculated Petri dishes, with or without soil, were incubated upside down in closed plastic bags for 6 weeks at 20 °C. Five Petri dishes were incubated per treatment per isolate with and without soil.

Numbers of microsclerotia and their shape were recorded for each colony. Length and width of 10 microsclerotia per colony were measured on 3 colonies of each combination. The microsclerotia to be measured were randomly chosen from the area halfway between the centre and the edge of the colony. Presence and intensity of dark melanised mycelium, growing radially from the centre throughout the colony was recorded (Figures 2 and 6). The intensity was estimated as the percentage of the colony covered by this type of mycelium. In our study, this type of mycelium will be referred to as 'dark mycelium'. The term 'dark hyphae' will be used for short portions (shorter than 120 μm) of melanised hyphae. Dark hyphae usually arise from microsclerotia (Figures 4 and 7), but they can also occur singly or several together without connection to a microsclerotium. Dark hyphae cannot be observed properly when dark mycelium is abundant. Colony characteristics like the pattern in which microsclerotia are formed (radial (Figure 1), scattered (Figures 5 and 6) or intermediate) and yellow discoloration of the agar medium were recorded. Chlamydospores were not scored because they were regarded unsuitable for discrimination, for two reasons: 1) chlamydospores are mostly hyaline and therefore difficult to spot under a dissecting microscope and 2) similar chlamydospores can be formed by various soil-borne fungi and therefore usually occur abundantly throughout semi-selective soil plates. Size of individual microsclerotial cells was not included in the study, because 1) individual cells cannot be observed properly through a dissecting microscope and 2) preliminary observations revealed overlap between cell sizes of *V. dahliae* and *V. tricorpus.*

In Experiment 2 ten individual colonies on MSEA soil plates surface-inoculated with a 0.8 ml soil suspension from agricultural fields were examined. Only colonies with microsclerotia were taken into account. Mainly small colonies were chosen, with fewer than 25 microsclerotia, as they are the most difficult to classify as either *V. dahliae* or *V. tricorpus.* Length and width of 10 microsclerotia were measured, the pattern in which the microsclerotia were formed, and the presence or absence of connected dark hyphae or dark mycelium was scored. Based on the morphological characteristics of Experiment 1, isolates of Experiment 2 were classified as *V. dahliae* or *V. tricorpus*. Then, pure isolates of these colonies were obtained by retrieving individual microsclerotia from these colonies, washing them several times in sterile demineralised water, and re-plating them on MSEA. Colonies were transferred to clean MSEA plates until they were pure, after which they were grown on PDA for species identification, using the morphological characteristics described above (Isaac 1949, 1953). Microsclerotia of some (later proven to be *V. tricorpus*) colonies were sporulating, and those isolates could be put onto PDA directly, by touching the conidiophore with a sterile needle.

The identity of the 10 soil isolates was checked with species-specific primers (Robb *et al.* 1994). In brief, DNA was extracted from conidia and mycelium and purified, followed by a PCR using a primer pair specific for amplification of either *V. dahliae* or *V. tricorpus* rDNA of the ITS region. When amplification of the DNA occurs, this is visible as a 334 bp band when the PCR product is run through a gel.



Figures 1-8. Morphology of *V. dahliae* and *V. tricorpus* on semi-selective media without soil. **Figure 1.** *V. dahliae* colony on MSEA. The microsclerotia are formed in a radial pattern, which is typical for *V. dahliae*, although a scattered pattern can also occur. **Figure 2.** *V. tricorpus* colony on MSEA, with dark mycelium in a radial pattern and microsclerotia in a scattered to intermediate between radial and scattered pattern. **Figure 3.** Globose to elongate *V. dahliae* microsclerotia on MSEA. **Figure 4.** Globose to irregularly shaped *V. tricorpus* microsclerotia on MSEA. **Figure 5.** *V. dahliae* colony on EA, with microsclerotia in a scattered pattern. **Figure 6.** *V. tricorpus* colony on EA, with microsclerotia in a scattered pattern and dark mycelium in a radial pattern. **Figure 7.** Large, globose to irregularly shaped *V. dahliae* microsclerotia with connected dark hyphae on EA. **Figure 8.** Globose to oval (to irregularly shaped, not shown) *V. tricorpus* microsclerotia on EA. Bars; Figures 1–2 and 5–6 = 500 µm; Figures 3–4 and 7–8 = 50 µm.

Data on microsclerotial size were analysed using the GLM procedure of SAS version 8.0 (SAS Institute Inc., Cary, NC, USA). Specific research questions were tested with t-tests (mentioned directly in the text) or using contrast analyses (presented in the tables).

Results

Experiment 1a. Morphology in the absence of soil

***V. dahliae* on MSEA.** Microsclerotia (N = 150) abundant (250–3000 per colony), shape usually elongate to oval (Figure 3), sometimes globose (A45 mc-1) (Table 1), (30–)32–113(–190) × (10–)15–45(–55) µm (the 5th and 95th percentiles between brackets) (average 62 × 29 µm; length-width ratio (l/w) (1.0–)1.1–4.9(–10.5), average 2.3), radially distributed through the colonies (Figure 1). Dark hyphae usually absent, though 1 isolate (A60 mc-1) exhibited dark hyphae connected to some (1%) of the microsclerotia or unconnected to microsclerotia in several (4%) of the colonies. Dark mycelium throughout the colony absent. Yellow discoloration of the medium absent.

***V. tricorpus* on MSEA.** Microsclerotia (N = 90), present only in isolates A36 mc-1 (1000–1500 per colony), 227.84 mc-1 (1000–2500 per colony), and in 24% of the colonies of isolate 127.79A mc-1 (0–50 per colony), irregularly shaped (Figure 4), (15–)25–85(–270) × (10–)20–50(–70) µm (average 50 × 31 µm, l/w = 1.0–2.1(–5.4), average 1.7), usually scattered through the colony, but sometimes intermediate between radial and scattered (Figure 2). Dark hyphae were present and connected to all microsclerotia in every colony of isolates A36 mc-1 and 227.84 mc-1 (Figure 4), but occurred mostly separate from the microsclerotia in 52% of the colonies in isolate 127.79A mc-1. Dark hyphae that were not connected to microsclerotia could not be scored in colonies with abundant presence of dark mycelium. Dark mycelium usually present and abundant (covering 80–100% of the colony area), but absent in 127.79A mc-1 and scarce in A36 mc-1 (present in 44% of the colonies and on average covering 5% of the colony area) (Figure 2). Yellow discoloration of the medium present in all colonies of isolate 227.84 mc-1 and several colonies of isolate A36 mc-1, and absent in all other colonies.

***V. dahliae* on EA.** Microsclerotia (N = 150) mostly abundant (1000–6000 per colony), but variable in A63 mc-1 (several–2000 per colony), globose to elongate or irregular in shape (Figure 7), (30–)40–156(–220) × (25–)30–90(–170) µm (average 86 × 56 µm, l/w = 1.0–2.3(–4.0), average 1.5), scattered through the medium (Figure 5), except for isolate A59 mc-1 which shows a radial distribution and isolate A63 mc-1 which shows a scattered distribution in colonies containing few (less than 50) and a random distribution in colonies containing more microsclerotia. Dark hyphae connected or close to most (80–95%) of the microsclerotia in most isolates (Figure 7), but less frequent in A63 mc-1 (50%) and A56 mc-1 (10%), some dark hyphae also occurring unconnected to microsclerotia. Dark mycelium throughout the colony mostly absent, but rare in A60 mc-1 and A63 mc-1, covering 5 percent of the colony diameter. Yellow discoloration of the medium absent.

***V. tricorpus* on EA.** Microsclerotia absent after 6 weeks of incubation. Dark mycelium abundant, covering 100% of the colony diameter (Figure 6). Dark hyphae that were unconnected to microsclerotia could not be scored properly, because of the dark mycelium. After scoring and leaving the Petri dishes at room temperature for another 6 weeks, microsclerotia with dark hyphae were formed in some colonies of isolate A36 mc-1 (0–200 per colony). Microsclerotia (N = 30) globose (Figure 8) to irregular in shape, (20–)30–141(–160) × 20–70(–120) (average 62 × 39 µm, l/w = 1.0–2.7(–3.2), average 1.6), formed in a radial to scattered pattern (Figure 6). Yellow discoloration of the medium absent, except for 1 colony of isolate 227.84 mc-1 and visible only during the second week of incubation.

Experiment 1b. Morphology in the presence of soil

***V. dahliae* on MSEA**. Microsclerotia (N = 150), usually present (around 200 per colony; less abundant or sometimes absent in A59 mc-1 (0–100 per colony)), globose to elongate, 20–80(–100) × 10–50(–60) µm (average 45 × 23 µm, l/w = 1.0–4.8(–10.0), average 2.3), radially distributed except for A59 mc-1 which showed a scattered distribution and A45 mc-1 which showed a scattered distribution in the colony centre and radial distribution near the colony edges. Dark hyphae or dark mycelium absent. No discoloration of the medium.

***V. tricorpus* on MSEA.** Microsclerotia (N = 90) present in about half of the colonies (isolates A36mc-1 and 127.79A mc-1 always showed microsclerotia (10–100 per colony)), usually (around 90%) with dark hyphae connected to them, except for isolates 384.84 mc-1 and Vtx mc-1 without, and 227.84 mc-1 with few microsclerotia (0–50 per colony), often irregularly shaped, sometimes globose to oval, (10–)20–116(–260) × (10–)15–50(–100) µm (average 48 × 28 µm, l/w = 1.0–2.9(–6.5), average 1.7), usually scattered through the colony, but sometimes intermediate between radial and scattered. Dark mycelium present in about half of the colonies, covering about 5% of the total colony area. Isolate 227.84 mc-1 showed numerous dark hyphae that were not connected to microsclerotia. No discoloration of the medium.

***V. dahliae* on EA.** Microsclerotia (N = 90) present in about half of the colonies, usually not abundant (0–100 per colony) except for isolate A45 mc-1 (around 400 per colony), globose to elongate, (20–)30–140(–700) × (10–)15–61(–120) µm (average 75 × 37 µm, l/w = (1.0–)1.2–4.2(–14.0), average 2.2), scattered through the colony. Dark hyphae or dark mycelium absent. No discoloration of the medium.

***V. tricorpus* on EA.** Microsclerotia (N = 90) present in about half of the colonies, but never abundant (0–30 per colony), irregular in shape, small and elongate in 127.79A mc-1 and large and globose in A36 mc-1, (20–)23–83(–170) × 10–50(–80) µm (average 47 × 28 µm, l/w = (1.0–)1.1–3.0(–5.3), average 1.8), usually scattered through the colony, but radially distributed in 227.84 mc-1. Dark hyphae and dark mycelium present in about half of the colonies, covering about 40% of the colony area. No discoloration of the medium.

Experiment 1. Overall effects on microsclerotial size

Microsclerotial size is generally different for the 2 species (larger for *V. dahliae*) and is severely affected by choice of the medium (larger on EA) and presence or absence of soil (larger if absent) (Table 2). The actual size, the surface that is visible through a microscope, is probably most clearly expressed in the factor length × width. The significant medium × soil interaction for the factors width and visible surface (Table 2) illustrates that microsclerotial size is more stable on MSEA. Significance levels of the species × soil interaction and the three-way interaction are misleading, because microsclerotia were often not formed.

Table 2. Significance levels [1] of Type III Sums of Squares of overall effects in Experiment 1.

Factor	Variable		
	Length of microsclerotia	Width of microsclerotia	Visible surface of microsclerotia
Species [2]	< 0.0001	< 0.0001	0.0001
Medium [3]	< 0.0001	< 0.0001	< 0.0001
Soil [4]	0.0006	< 0.0001	0.0001
Species × medium interaction	0.0006	< 0.0001	< 0.0001
Species × soil interaction	0.39	0.0048	0.26
Medium × soil interaction	0.57	< 0.0001	0.0096
Species × medium × soil interaction	0.16	0.22	0.86

[1] Calculated using the GLM procedure of SAS v. 8.0.
[2] *V. dahliae* or *V. tricorpus*.
[3] Modified soil extract agar or ethanol agar.
[4] Present or absent.

The effect of adding soil on morphology. Presence of a mixed soil microflora can alter the morphology of the 2 species considerably. On MSEA, the presence of soil resulted in shorter and narrower ($P < 0.001$) *V. dahliae* microsclerotia, but had no significant effect on the size of *V. tricorpus* microsclerotia. On EA, the presence of soil resulted in shorter ($P = 0.036$) and narrower ($P < 0.001$) *V. dahliae* microsclerotia, whereas in *V. tricorpus*, soil induced formation of microsclerotia in 2 isolates, and accelerated formation of microsclerotia in isolate A36 mc-1, without affecting the size.

On MSEA without soil, *V. dahliae* microsclerotia were longer ($P = 0.037$) but not wider than those of *V. tricorpus*, whereas in the presence of soil, *V. tricorpus* microsclerotia were wider ($P = 0.015$) and slightly, but not significantly, longer than those of *V. dahliae* (Figure 9). On EA, *V. dahliae* microsclerotia were longer and wider than those of *V. tricorpus*, both without and with soil (P values < 0.004). Despite these consistent differences, EA is less suited for quantification of *V. dahliae* in soil, because *V. dahliae* microsclerotia are not always formed (see above). Morphological characteristics suitable to separate *V. dahliae* and *V. tricorpus* on MSEA in the presence of soil are summarised in Table 3.

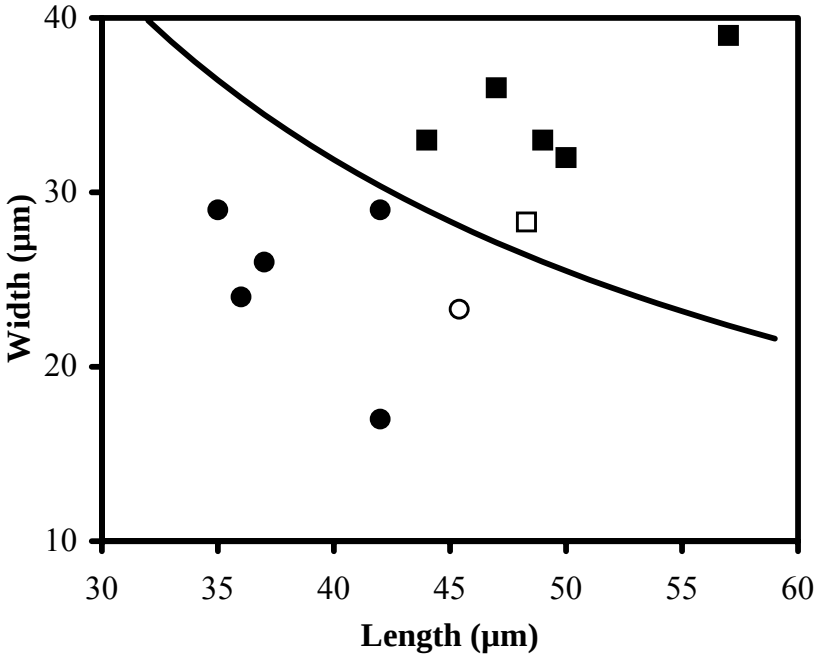


Figure 9. Average length and width of microsclerotia of the 10 colonies from MSEA soil plates from agricultural fields (Experiment 2), together with the average length and width of microsclerotia of *V. dahliae* and *V. tricorpus* reference isolates (Experiment 1). ●, *V. dahliae* field isolates; ■, *V. tricorpus* field isolates; ○, average of *V. dahliae* reference isolates; □, average of *V. tricorpus* reference isolates; the line indicates a length × width of microsclerotia, *i.e.* the visible microsclerotial surface, which was iteratively set at 1275 µm^2.

Experiment 2. Classification of colonies of soil samples from agricultural fields

On the basis of the differential features recognised on MSEA with added soil (Experiment 1b), 5 out of 10 field soil colonies were scored as *V. dahliae* and 5 as *V. tricorpus* (Table 3). In colony No. 2 microsclerotia were oval to elongate and arranged in a radial pattern, classifying it as *V. dahliae*. Colony No. 6 had globose to elongate microsclerotia in a more irregular pattern, and was also classified as *V. dahliae*, based on the elongate shape of the microsclerotia. Colonies 1, 3 and 7 had dark hyphae attached to the microsclerotia, classifying them as *V. tricorpus*. This identification was confirmed for colonies 1 and 3, which contained irregularly shaped microsclerotia. A comparison of the visible surface (length × width) of the microsclerotia of each field soil colony against the reference isolates of *V. dahliae* and *V. tricorpus* (Experiment 1) confirmed these identifications, and identified 3 more *V. dahliae* and 2 more *V. tricorpus* isolates (Table 3). Checking the identity of the purified isolate by PCR with specific primers and morphology on PDA confirmed the original identification based on morphological characteristics in the MSEA soil plates (Table 3).

Table 3. Morphological characteristics distinguishing *V. dahliae* and *V. tricorpus* on MSEA in the presence of soil.

Isolate(s) Reference isolates (Exp. 1b)	Colony pattern [1]	Microsclerotial shape	Approx. % of micro-sclerotia with connected dark hyphae	Dark mycelium	Average micro-sclerotial surface (length × width) (μm^2)	Probability [2] based on microsclerotial size of the field isolates being: *V. dahliae*	*V. tricorpus*	Species identification of isolates based on morphology on MSEA in the presence of soil	Check of identity of purified isolate by: PCR specific primers [3]	morphology on PDA [4]
V. dahliae	radial, scattered or intermediate	globose, oval to elongate	0	absent	1190	NA [5]	NA	NA	*V. dahliae*	*V. dahliae*
V. tricorpus	scattered or intermediate	globose, oval to irregular	90	absent or present	1725	NA	NA	NA	*V. tricorpus*	*V. tricorpus*
Field isolates (Exp. 2)								*V.*	*V.*	*V.*
1	intermediate	irregular	50	absent	1690	0.24 [6]	0.94 [6]	*tricorpus*	*tricorpus*	*tricorpus*
2	radial	oval-elongate	0	absent	720	0.26	0.02	*dahliae*	*dahliae*	*dahliae*
3	scattered	globose-irregular	80	absent	1520	0.43	0.66	*tricorpus*	*tricorpus*	*tricorpus* [7]
4	scattered	globose-oval	0	absent	2240	0.01	0.23	*tricorpus*	*tricorpus*	*tricorpus*
5	intermediate	globose-oval	0	absent	1020	0.69	0.10	*dahliae*	*dahliae*	*dahliae*
6	intermediate	globose-elongate	0	absent	880	0.46	0.05	*dahliae*	*dahliae*	*dahliae*
7	intermediate	globose-oval	70	absent	1710	0.22	0.97	*tricorpus*	? [8]	*tricorpus* [7]
8	scattered	globose-oval	0	absent	1810	0.14	0.84	*tricorpus*	*tricorpus*	*tricorpus*
9	intermediate	globose-oval	0	absent	1030	0.70	0.11	*dahliae*	*dahliae*	*dahliae*
10	scattered	globose-oval	0	absent	1260	0.87	0.28	*dahliae*	*dahliae*	*dahliae*

[1] Colony pattern is unclear when few microsclerotia are present.
[2] Contrast analysis of each isolate of Experiment 2 against the average of all isolates of *V. dahliae* and all microsclerotia forming isolates of *V. tricorpus*, respectively, of Experiment 1b on MSEA in the presence of soil (GLM procedure, SAS 8.0, SAS, Cary, NC).
[3] Robb *et al.* (1994).
[4] Isaac (1949, 1953).
[5] Not applicable.
[6] The higher probability value (either *V. dahliae* or *V. tricorpus*) indicates the most likely possibility.
[7] These isolates caused yellow coloration of the PDA medium.
[8] No signal with either *V. dahliae* or *V. tricorpus* primers.

Discussion

Morphology of *Verticillium* species can be highly variable on different semi-selective agar media. Shape of microsclerotia and abundance of dark hyphae of *V. dahliae* on EA resembles morphology of *V. tricorpus* on MSEA. Moreover, the distribution of microsclerotia through the colony on EA is scattered, whereas on MSEA it is usually (but not always) radial. This is the first report of the occurrence of dark hyphae of *V. dahliae in vitro. In vivo* production of dark hyphae has sometimes been reported in cotton (Garber & Houston 1966) and olive (Rodríguez-Jurado 1993). Researchers only familiar with *V. dahliae* on pectate-based agars can easily be confused when occasionally working with EA.

On MSEA, microsclerotia are the most important structures to recognise *Verticillium* colonies in the presence of soil, because many other fungi present in the soil are able to form melanised mycelium or chlamydospores (Domsch *et al.* 1980). Therefore, presence of dark hyphae is a useful characteristic only, when the dark hyphae are connected to the microsclerotia. Because this is the case with most of the *V. tricorpus* microsclerotia and never occurs in *V. dahliae*, this characteristic is very important for discrimination, and easy to observe. Shape of the microsclerotia is a useful characteristic, when the colony contains elongate (*V. dahliae*) or irregularly shaped (*V. tricorpus*) microsclerotia, which is also easy to observe. The pattern in which microsclerotia are formed discriminates only when it is clearly radial. When microsclerotia are scarce, the pattern is often unclear. Dark mycelium, when present, identifies a colony as *V. tricorpus*. However, dark mycelium did not occur in any of the *V. tricorpus* field colonies tested, so this characteristic does not seem to be useful for practice.

All previously mentioned qualitative characteristics fail to discriminate between *V. dahliae* and *V. tricorpus* when dark hyphae and dark mycelium are absent, and microsclerotia are globose to oval and arranged in an irregular pattern. Size of the microsclerotia is a useful character for discrimination, despite the overlap in length and width of the field isolates with the reference isolates (Figure 9). Size, however, is more clearly expressed as the average length × width of the microsclerotia, *i.e.* the visible surface. The visible surface differentiates clearly to the experienced observer, even without measuring the microsclerotia on a standard basis. Using both qualitative and quantitative characteristics, it is possible to discriminate *V. dahliae* and *V. tricorpus* on MSEA in the presence of soil.

These observations provide a more practical (yet reliable) method to characterise *Verticillium* colonies on MSEA soil dilution plates. The alternatives, to purify individual colonies and identify them using morphological or molecular characteristics, are too laborious and costly if large numbers of colonies need to be identified.

These observations in part confirm those of Davis & McDole (1979) and Davis *et al.* (2000) on the pectate based agar NPX (Butterfield & DeVay 1977) and of Huisman (1988) on a similar pectate medium. All authors mentioned the radially grouped *V. dahliae*, and scattered, dark hyphae bearing *V. tricorpus* microsclerotia, as well as the microsclerotial size difference. However, they did not mention any deviations from these typical characteristics.

MSEA is used for quantification of *V. dahliae* in research (Termorshuizen *et al.* 1998) and commercially (Anon. 1997) in soils with the 2 species present. Quantitative detection using MSEA has given a good prediction of disease for *V. dahliae*, e.g. in *Acer platanoides* (Goud *et al.*, 2001), but for *V. tricorpus* the method was not conclusive because some isolates did not form microsclerotia. EA cannot be used for quantification of *V. dahliae* in soil. EA is

suitable for working in the absence of soil, e.g. during plating of stem pieces of crops that can suffer from both species, like potato (e.g. Nagtzaam, Termorshuizen & Bollen 1997).

The addition of soil to MSEA Petri dishes affected microsclerotial size in *V. dahliae* more than in *V. tricorpus* isolates, which could be explained by the nature of the 2 species. *V. dahliae* is regarded as a plant pathogen, with low saprobic ability, whereas *V. tricorpus* is regarded as being more a soil inhabitant (Isaac 1967). The formation of microsclerotia by *V. tricorpus* on EA in the presence of soil but not in pure culture, or only after prolonged incubation, might be induced by competing microorganisms. Probably, microsclerotia are more durable resting structures than melanised mycelium, and are more frequently formed in the presence of other soil organisms. This hypothesis needs further testing.

In conclusion, the morphological characters used to identify pure cultures of dark *Verticillium* species cannot be used uncritically for soil dilution plates. For distinguishing *V. dahliae* and *V. tricorpus* on MSEA soil dilution plates we recommend the following key:

1	Dark hyphae or dark mycelium present	*V. tricorpus*
	Dark hyphae and dark mycelium absent	2
2(1)	Microsclerotia irregularly shaped	*V. tricorpus*
	Microsclerotia not irregularly shaped; globose to oval to elongate	3
3(2)	Microsclerotia elongate	*V. dahliae*
	Microsclerotia globose to oval	4
4(3)	Distribution of microsclerotia within medium clearly radial	*V. dahliae*
	Distribution of microsclerotia within medium not clearly radial	5
5(4)	Average surface (average length × average width) of microsclerotia (N = 10) < 1275 μm^2	*V. dahliae*
	Average surface (average length × average width) of microsclerotia (N = 10) > 1275 μm^2	*V. tricorpus*

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Chapter 4

Pathogenicity and virulence of the two Dutch VCGs of *Verticillium dahliae* to woody ornamentals

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Abstract

Two experiments were performed in two consecutive years to test whether isolates of different vegetative compatibility groups (VCGs) differ in their ability to cause disease in woody ornamentals, to study the host specificity of the isolates, and to get an insight into disease development in woody hosts. A range of woody ornamental plant species, including *Acer campestre*, *Acer platanoides*, *Acer pseudoplatanus*, *Catalpa bignonioides*, *Cotinus coggygria*, *Robinia pseudoacacia*, *Rosa canina*, *Syringa vulgaris* and *Tilia cordata*, were root-dip inoculated with six isolates of *Verticillium dahliae*, belonging to the two VCGs that occur in the Netherlands (VCG NL-1 and VCG NL-2). Isolates belonging to each vegetative compatibility group caused severe symptoms of verticillium wilt in most plant species tested. Disease progress differed between plant species, but was generally the same for the two VCGs. No overall differences in virulence were observed between the two VCGs for external wilt symptoms, number of dead plants, or shoot length. No significant VCG x plant species interactions were present for these characteristics. However, isolates of VCG NL-1 caused more vascular discolouration than did isolates of VCG NL-2 in the plant species tested. Isolates within VCGs often differed considerably in their virulence to certain hosts, as shown by highly significant isolate x plant species interactions. Isolates were more virulent on their original host. These findings imply that VCG identification does not contribute to disease prediction for a range of woody hosts.

Abbreviations: AUDPC – area under the disease progress curve; VCG – vegetative compatibility group.

Introduction

In tree nurseries all over the world considerable losses are reported from verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae* (Piearce and Gibbs, 1981; Sinclair *et al*., 1989). In the Netherlands, 30% of the area used for tree production is estimated to be infested (Anonymous, 1990). Growing susceptible tree species on infested soil can cause up to 50% loss (Anonymous, 1990; Goud *et al.*, 2000). Crop rotation is the only way to manage verticillium wilt, since chemicals for disinfestation of the soil become less and less available (Annis and Waterford, 1996; Anonymous, 1990), while non-chemical methods for the temperate zones are still in development (Blok *et al.*, 2000). Most of the current methods used to quantify the soil inoculum level do not provide information about the virulence of the soil population (Termorshuizen *et al.*, 1998), though it is known that isolates can differ significantly in virulence to woody hosts (Ashworth, 1983; Donohue and Morehart, 1978; Schnathorst and Sibbett, 1971; Tjamos, 1981).

Because the sexual cycle is absent in *Verticillium*, parasexuality is considered the single means of exchange of genetic material between individual mycelia (Heale, 1988). Vegetatively incompatible strains belong to different vegetative compatibility groups (VCGs) and therefore are genetically isolated from each other. This means that VCGs may vary in many characteristics, including those related to pathogenicity and virulence (Rowe, 1995). Work on pathogenic isolates of *Fusarium oxysporum* has shown that VCGs are subdivisions within formae speciales, i.e., the intra-specific pathogenicity grouping. Each forma specialis of *F. oxysporum* contains one to many VCGs, and each VCG occurs within one forma specialis (Katan and di Primo, 1999).

Vegetative compatibility grouping in *V. dahliae* is done with the use of nitrate non-utilising (*nit*) mutants, which express mutant growth on a minimal medium. Isolates are judged to be vegetatively compatible when complementing *nit*-mutants are able to mate and produce wild-type growth. This is the result of successful fusion of hyphae between strains and subsequent heterokaryon formation (Joaquim and Rowe, 1990; Puhalla, 1985). Following this approach, Joaquim and Rowe (1990) grouped isolates from *V. dahliae* into VCG 1 – 4. Later, isolates of VCG 3 were regrouped into VCG 4 (Bell, 1995; Katan, 2000; Strausbaugh *et al.*, 1992).

In the Netherlands, two VCGs of *V. dahliae* are present, provisionally called VCG NL-1 and VCG NL-2 (Hiemstra and Rataj-Guranowska, 2000, 2003). It is not exactly known how the isolates used in this study fit into the American vegetative compatibility grouping system, but tester strains of VCG NL-1 were compatible with both the American tester strains of VCGs 3 and 4, whereas the tester strains of VCG NL-2 were compatible with both the American tester strains of VCGs 1 and 2 (Hiemstra and Rataj-Guranowska, 2003; Rataj-Guranowska and Hiemstra, 2000).

Links of some American VCGs with pathogenicity have been reported, e.g., VCG 1 contains all cotton-defoliating strains and VCG 2 and VCG 4 contain all cotton non-defoliating strains (Daayf *et al.*, 1995). Grouping of isolates from certain hosts into specific VCGs has been observed in several inventories, e.g., isolates from watermelon in Greece were VCG 2 (Elena, 2000), most isolates from potato were VCG 4 (Rowe *et al.*, 2000), and most American isolates from woody hosts were VCG 1 (Chen, 1994). Such inventories can only be used for disease prediction when pathogenicity tests are performed. This has been done for single crops, like potato (Joaquim and Rowe, 1991; Strausbaugh, 1993; Strausbaugh *et al.*, 1992), or several (Gennari *et al.*, 2000) to many (Bhat and Subbarao, 1999) herbaceous crops, but never for a range of woody hosts. This is important because disease development in woody hosts can differ from that in herbaceous plants due to differences in anatomy and multi-year effects (Hiemstra, 1998).

All plants used in the present study were listed as susceptible to *V. dahliae* (Hiemstra, 1998) and often show wilt symptoms in nurseries in the Netherlands. The hypotheses were: (1) isolates of different VCGs in the Netherlands differ in virulence to woody hosts; (2) these isolates are host specific, and (3) disease development differs for the hosts studied. Preliminary results have been published in an abstract (Goud and Termorshuizen, 2000).

Material and methods

Experimental set-up

Two experiments were carried out in 1997 and 1998, with *Acer campestre* (field or hedge maple), *Acer platanoides* (Norway maple), *Acer pseudoplatanus* (sycamore maple), *Catalpa bignonioides* (southern catalpa), *Cotinus coggygria* (European smoketree) and *Robinia pseudoacacia* (black locust). The plant species *Syringa vulgaris* (common lilac) and *Tilia cordata* (littleleaf linden) were tested in Experiment 1, but because they did not show clear disease symptoms (no difference between the inoculated plants and the controls) they were not included in Experiment 2. *Rosa canina* cultivar 'Laxa' (a dog rose selection which is used as rootstock) was tested in Experiment 2 only. All plants were seedlings with 6 to 8 leaves, except for plants of *T. cordata* and *A. pseudoplatanus* in the first experiment and *R. canina* in the second, which were one year old.

Experiment 1 consisted of 25 blocks in a randomised complete block design, with each block including all plant species in combination with six isolates, representing the two VCGs, and three control plants, so 25 plants were used per species x isolate combination, together with 75 control plants per species (a total of 225 plants per species). Control plants were used to distinguish between disease symptoms and plant reactions caused by environmental factors. Plants were grown in a screenhouse with a plastic cover and open sides. Temperature generally fluctuated between 10 and 35 °C and relative humidity between 20 and 100%. Experiment 2 consisted of 10 blocks in a randomised complete block design, including two control plants, so 10 plants were used per species x isolate combination, together with 20 control plants per species (a total of 80 plants per species). Plants were grown in a ventilated greenhouse, with temperatures generally fluctuating between 15 and 25°C and a relative humidity between 40% and 80%, but temperatures in the greenhouse exceeded 30 °C during day time 3 – 5 weeks after inoculation.

Plant maintenance

Pots were placed in individual saucers to prevent cross contamination. Plants were watered two to four times per week, depending on their needs, by filling up the saucers. Each experiment was fertilised once with 100 ml solution of 2 g l^{-1} of Kristalon fertiliser 19-6-20-3+ microelements (Hydro Agri Rotterdam BV, Vlaardingen, The Netherlands), and in Experiment 2 Osmocote Plus controlled release fertiliser 15-11-13-2+ microelements (Scotts Europe BV, Heerlen, The Netherlands) was mixed through the potting soil at a density of 4 g l^{-1} soil.

In Experiment 1, all *Acer* species, *R. pseudoacacia,* and *C. coggygria* were sprayed against powdery mildew three times during summer with Curamil (Hoechst Holland N.V. Agro Chemie, Amsterdam, The Netherlands), Rubigan Flow (Dow-Elanco BV, Antwerp, Belgium), and Baytan Flow (Bayer BV, Mijdrecht, The Netherlands) respectively, all at a concentration of 0.5 ml l^{-1}. In Experiment 2, *R. canina* was sprayed once with Funginex (Shell Nederland Chemie BV, Rotterdam, The Netherlands) and all plants were sprayed against spider mites with Masaï (Bayer BV, Mijdrecht, The Netherlands) and against woolly aphids with Pirimor (ICI Agro, Ridderkerk, The Netherlands) all at a concentration of 0.5 ml l^{-1}. After the sprays spider mites were controlled biologically using Spidex (Koppert BV, Berkel and Rodenrijs,

The Netherlands) and woolly aphids with *Cryptolaemus* spp. (Entocare BV, Wageningen, The Netherlands). Though no negative effects of the applied fungicides and pesticides to *V. dahliae* were known from the literature, pots were covered during all sprays to prevent dripping of chemicals into the soil.

Inoculation

Six *Verticillium* isolates were used from different locations in the Netherlands (Table 1). Monoconidial cultures were made of the original isolates by the first author in 1997, except for G3 and S12,2 which were received as monoconidial cultures from the researchers who isolated them. Between 1997 (Experiment 1) and 1998 (Experiment 2) monoconidial cultures were maintained on potato dextrose agar at 4°C. Roots were washed free of soil and the plants were root-dip inoculated with a conidial suspension of *V. dahliae* at $1.0x10^6$ conidia ml^{-1} for 2 min. Inoculated seedlings were planted in 0.7 l-pots with potting soil (Nr. 4, Lentse Potgrond, Lent, The Netherlands) which had been steam-sterilised one month before. Control plants were dipped in autoclaved inoculum of the same conidial density.

Table 1. Collection and storage information for isolates of the two vegetative compatibility groups (VCGs) of *Verticillium dahliae* used in the experiments.

VCG	Isolate	Host of origin	Location	Date	Isolation by	Storage
NL-1[1]	G3	potato (*Solanum tuberosum* L.)	Wageningen	1-7-1994	A.J. Termorshuizen Wageningen University	agar plugs in water at 4°C
NL-1	es120 mc-1	ash (*Fraxinus excelsior* L.)	Lelystad	10-3-1988	J.A. Hiemstra, Plant Research International	agar plugs in water at 4°C
NL-1	AplatI mc-1	Norway maple (*Acer platanoides* L.)	Grubbenvorst	28-9-1993	J.A. Hiemstra, Plant Research International	agar plugs in water at 4°C
NL-2 [2]	A40 mc-2	red currant (*Ribes rubrum* L.)	Buurmalsen	26-1-1996	B. Wessels, Plant Protection Service	potato dextrose agar plates at 4°C
NL-2	A59 mc-1	blackberry (*Rubus fruticosus* L.)	Spijk	20-9-1996	W. Veenbaas, Plant Protection Service	potato dextrose agar plates at 4°C
NL-2	S12,2	lilac (*Syringa vulgaris* L.)	Aalsmeer	30-9-1996	J.A. Hiemstra, Plant Research International	agar plugs in water at 4°C

[1] Compatible with American VCGs 3 and 4.
[2] Compatible with American VCGs 1 and 2.

Disease observations

In the first experiment, disease scores were rated 75 days after inoculation on a 0 to 5 scale: 0= healthy plant, 1= one or two leaves affected (=wilted, dead or fallen off; two top leaves showing epinasty for *Acer* species), 2= three leaves or up to 30% of the leaves affected, 3= 30 to 60 % of the leaves affected, 4= 60 to 80% of the leaves affected, 5= more than 80% of the leaves affected or plant dead. In *A. pseudoplatanus* only the four top leaves could be taken into account in Experiment 1, due to a hypersensitivity reaction of the older leaves to the anti-mildew spray with Baytan Flow. Shoot length was measured at the end of the growing season. Vascular discolouration was observed for all plants from 15 blocks on cross sections of the stem base and rated on a 0 (absent) to 5 (xylem tissue of the cross section completely discoloured) scale. Stem pieces of all 72 plants in one block and random samples from the other blocks were plated on ethanol agar (Nadakavukaren and Horner, 1959) to check for presence of the fungus. The 10 remaining blocks were kept in a frost-free greenhouse during winter and transferred back in spring to score for either death or regrowth. In the second experiment, disease scores were taken every 2 or 3 weeks for 28 weeks and area-under-the-disease-progress curves (AUDPC) were calculated (Campbell and Madden, 1990). Death of plants was also recorded. For *C. bignonioides* and *R. pseudoacacia* rating of the disease scores was stopped 18 weeks after inoculation because all diseased plants were either dead or cured after initial symptom expression. Vascular discolouration was observed as in Experiment 1 after the last disease score was taken. Stem pieces of all plants in one block and random samples from the other blocks were plated on modified soil extract agar (Harris *et al.*, 1993) to check for presence or absence of the fungus.

Statistical analyses

Data presented in the second part of the Results section (concerning VCG main effects) were analysed using a mixed statistical model. This is a model in which fixed and random variables can be combined. In this analysis, plant species, VCG, and plant species x VCG interaction were treated as fixed variables, whereas block, isolate (within VCG), and plant species x isolate (within VCG) interaction were treated as random variables. This was done because the individual isolates were chosen randomly from each VCG, and no information was available about whether their variability represented adequately the variability within their VCG. This type of data analysis is conservative, in the sense that variation is first ascribed to the random effects and after that to themain effects (e.g., VCG). AUDPC and shoot length data were square-root transformed and analysed using the MIXED procedure of SAS version 8 (SAS Institute Inc., Cary, N.C., U.S.A.). Residuals of the transformed data were normally distributed. Ordinal data (classes) of disease scores of Experiment 1 and vascular discolouration of Experiments 1 and 2 can not be analysed adequately by parametric statistical models because class data are not normally distributed and step sizes between classes are not always similar for all classes (Oude Voshaar, 1994). Moreover, averages of ordinal scores may not always be meaningful. The threshold model (McCullagh, 1980) overcomes these problems by quantifying the ordinal data and constructing an underlying continuous scale. On this continuous scale thresholds are estimated between class 0 and 1, class 1 and 2, etc. Step sizes between these thresholds correspond with step sizes between classes. Next, data are fitted on this scale in the way that represents best the frequencies of plants scored in different

classes. Data were fitted best when a normal distribution was used to create this continuous scale (i.e., the 'link function'). The analysis of the ordinal data, using a mixed statistical model, was performed with the use of the CLASS procedure addition (Candy and Wilkinson, 1997; Keen, 1998) of GENSTAT version 5.4.1 (Genstat, Rothamsted Experimental Station, Harpenden, U.K.). Because vascular discolouration was absent in *S. vulgaris* and *R. canina*, these species were not included in the statistical analysis of the discolouration data. Plant death is a binomial phenomenon (either alive or dead) and is therefore not normally distributed. Plant death data for each experiment were analysed by means of the MIXED procedure of SAS version 8. Because this procedure assumes normality, the analysis was checked using a Chi-square test (Snedecor and Cochran, 1989), after pooling the data for all blocks and all isolates within VCGs. Data for *R. pseudoacacia* and *R. canina* had to be omitted during calculation of the Chi-square of Experiment 2, because none of the plants of those species died.

Data presented in the last part of the Results section (concerning individual isolates) were analysed using a statistical model with fixed effects only: isolate, plant species, isolate x plant species interaction, and block. AUDPC and shoot length data were square-root transformed and analysed using the GLM procedure of SAS version 8. Residuals of the transformed data were normally distributed. Ordinal data (classes) of disease scores of Experiment 1 and vascular discolouration of Experiments 1 and 2 were analysed using the threshold model GENMOD of SAS version 8, using the normal distribution as link function (see above). Specific questions, e.g., whether there were differences between VCGs, were estimated during the analysis using orthogonal contrasts (Snedecor and Cochran, 1989), assuming that the three isolates are correct representatives of each VCG. Analysis of the binomial plant death data by means of the GLM procedure of SAS version 8 was checked using a Chi-square test.

Results

Disease development

Isolates of each of the VCGs were capable of causing disease symptoms in most of the woody plant species. The most prevalent symptoms were acute wilt, necrosis and defoliation. *Acer* species and *C. bignonioides* showed all three symptoms, whereas *C. coggygria* mainly showed acute wilt, *R. canina,* a slowly developing necrosis, and *R. pseudoacacia* and *T. cordata* defoliation. Wilting plants of the *Acer* genus, especially *A. platanoides,* often showed epinasty of the two top leaves as a first symptom. In *S. vulgaris* curling of the leaves was observed and wilt or death of leaves was absent. Inoculated plants were generally different from the controls except for *S. vulgaris* and *T. cordata*. Plating of stem pieces revealed that control plants were not infected by *Verticillium*. Leaf curling of *S. vulgaris* and defoliation of *T. cordata* could have been a result of the mock inoculation procedure or natural senescence.

Disease development over time is described in Figure 1 for Experiment 2 for all isolates on *A. campestre*, *C. bignonioides* and *C. coggygria*. Disease developed slowly in *A. campestre* (Figure 1a) but eventually disease scores reached high values for the most virulent isolates. Disease progress of *A. platanoides* and *A. pseudoplatanus* was similar to that

in Figure 1a (data not shown). For *R. canina* the trend was similar, but disease scores were lower because of formation of new branches after onset of the disease (data not shown). *C. bignonioides* (Figure 1b) and *R. pseudoacacia* (data not shown) developed severe symptoms, including death of some *C. bignonioides* plants, during the first four weeks after inoculation. After that, all living diseased plants recovered. *C. coggygria* plants (Figure 1c) showed acute wilt within four weeks after inoculation. Seven weeks after inoculation most of the inoculated plants were killed and disease did not progress in the remainder of the inoculated plants. Disease progress curves within plant species were generally the same for each VCG.

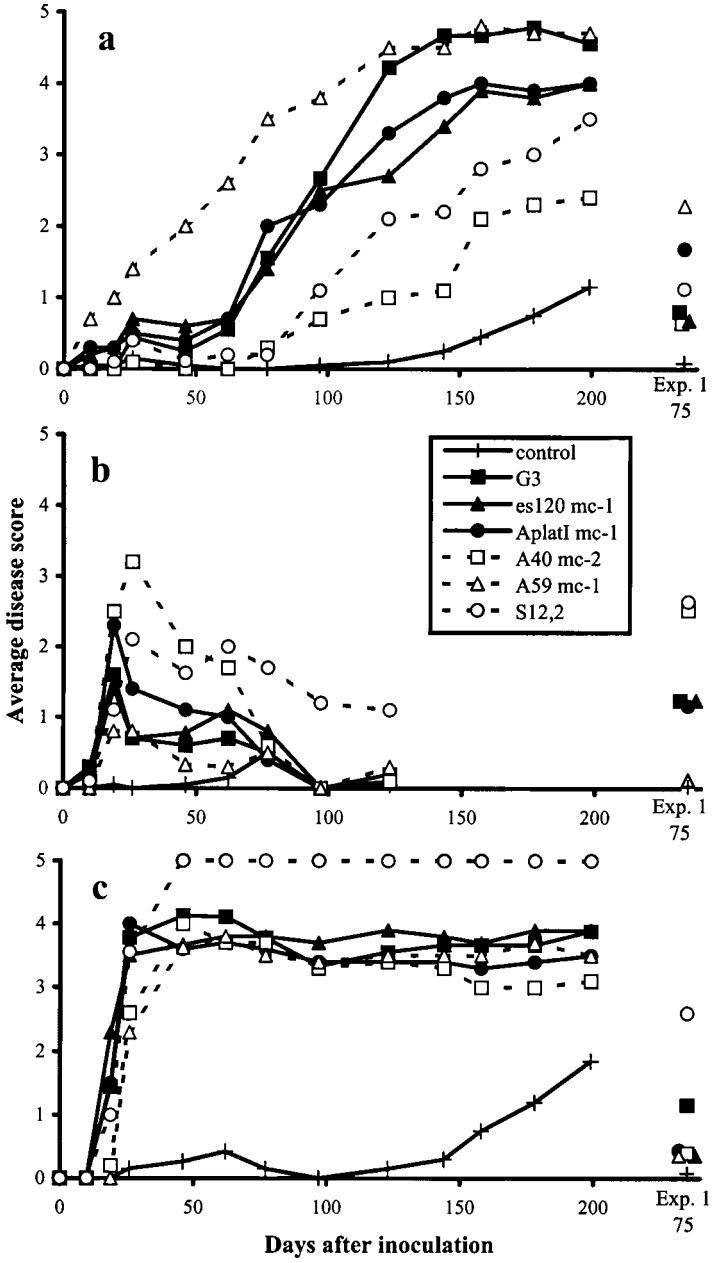

Figure 1. Disease progress curves of Experiment 2 and average scores from Experiment 1 (Exp. 1) 75 days after inoculation (data points on the right), for a: *Acer campestre*, b: *Catalpa bignonioides* and c: *Cotinus coggygria*. Solid lines with closed markers represent isolates of vegetative compatibility group (VCG) NL-1 of *Verticillium dahliae*, dashed lines with open markers represent isolates of VCG NL-2, and + indicates the control.

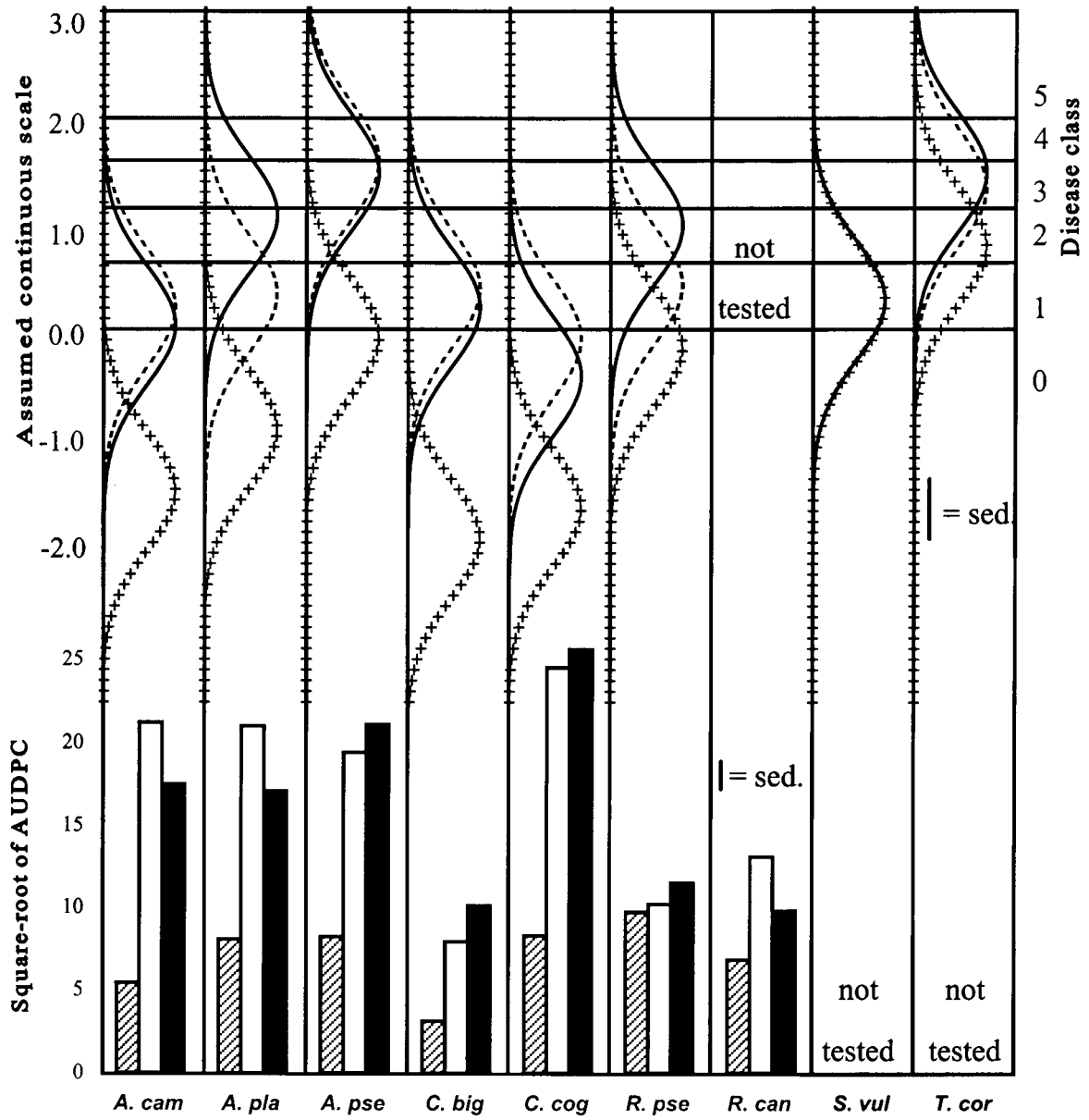

Figure 2. Estimated frequency distributions of the disease scores on an assumed underlying continuous scale (Experiment 1 – top) combined with average square-root transformed area-under-the-disease-progress curve (AUDPC) (Experiment 2 – bottom) for the two vegetative compatibility groups (VCGs) of *Verticillium dahliae* and the control. Experiment 1: ——— = VCG NL-1, ------ = VCG NL-2, +++++ = control. Experiment 2: White bars = VCG NL-1, black bars = VCG-NL-2, striped bars =control. *A. cam = Acer campestre, A. pla = Acer platanoides, A. pse = Acer pseudoplatanus, C. big = Catalpa bignonioides, C. cog = Cotinus coggygria, R. pse = Robinia pseudoacacia, R. can = Rosa canina, S. vul = Syringa vulgaris,* and *T. cor = Tilia cordata*. Horizontal lines indicate the thresholds between disease classes of Experiment 1. Individual graphs shifted towards higher classes (top) and taller bars (bottom) imply more disease. sed. = standard error of differences within plant species between vegetative compatibility groups. *S. vulgaris* and *T. cordata* were tested in Experiment 1 only and *R. canina* was tested in Experiment 2 only.

Vascular discolouration was present in most inoculated plants of all plant species except *S. vulgaris* (Experiment 1) and *R. canina* (Experiment 2), which rarely showed vascular discolouration symptoms. All other plant species showed significant differences

between inoculated plants and controls, in which vascular discolouration was absent. *T. cordata* (Experiment 1) showed clear vascular discolouration even though symptom expression was unclear. Vascular discolouration was grey/green to black in *Acer* species and *T. cordata*, light brown in *C. bignonioides,* and dark brown in *C. coggygria* and *R. pseudoacacia*.

VCG effects

Disease scores were not significantly different for the two VCGs (P=0.75), neither were AUDPC values (P=0.52) (Figure 2). Also for individual plant species significant differences between VCGs were not present in both experiments. Furthermore plant species x VCG interactions were neither significant in Experiment 1 (P=0.61) nor in Experiment 2 (P=0.22). Differences between plant species were highly significant (P<0.0001) in both experiments.

Numbers of dead plants were not significantly different for the two VCGs in Experiment 1 (mixed P=0.47; Chi-square P=0.18) and Experiment 2 (mixed P=0.85; Chi-square P=0.79) (Table 2). No consistent significant plant species x VCG interaction was present for number of dead plants in Experiment 1 (mixed P=0.62; Chi-square P=0.03) and Experiment 2 (mixed P=0.54; Chi-square P=0.23). Differences in numbers of dead plants between plant species were highly significant (mixed and Chi-square P<0.0001) in both experiments.

Table 2. Percentage of dead plants per plant species combined for Experiments 1 and 2 per individual isolate (n=20) of *Verticillium dahliae* and the controls (n=50).

HOST		ISOLATE					
	Control	VCG NL-1[1]			VCG NL-2		
		G3	es120 mc-1	AplatI mc-1	A40 mc-2	A59 mc-1	S12,2
A. campestre	4	42 [2]	30	30	5	75	25
A. platanoides	12	20	26 [2]	15	25	15	5
A. pseudoplatanus	6	20	15	30	10	60	55
C. bignonioides	2	0	0	0	5	5	25
C. coggygria	4	63 [2]	70	45	50	35	100 [2]
R. pseudoacacia	2	10	15	0	0	0	0
R. canina [3]	5	0 [4]	0	0	0	0	0
S. vulgaris [5]	0	0	0	0	0	0	30
T. cordata [5]	3	10	0	20	0	0 [4]	40

[1] VCG = vegetative compatibility group; [2] n=19; [3] tested in Experiment 2 only, so n=10 and control n=20; [4] n=9; [5] tested in Experiment 1 only, so n=10 and control n=30.

For vascular discolouration no significant differences were observed between the two VCGs in Experiment 1 (P=0.22) or Experiment 2 (P=0.37). There were no significant plant species x VCG interactions in Experiment 1 (P=0.49) or in Experiment 2 (P=0.17). For *A. platanoides* and *R. pseudoacacia* there was a repeatable trend of more vascular

discolouration after inoculation with isolates of VCG NL-1 compared with isolates of VCG NL-2. However, this trend was significant only for *A. platanoides* in Experiment 2 ($P<0.05$). For other plant species there were no trends. Differences in vascular discolouration between plant species were highly significant ($P<0.0001$) in both experiments.

Inoculated plants were generally 11% shorter than control plants ($P<0.05$). However, no significant difference was observed between the two VCGs ($P=0.63$) and there was no plant species x VCG interaction ($P=0.36$).

Isolate effects

Data analysis with fixed factors only showed that for disease symptoms, plant species and plant species x isolate interaction were highly significant ($P<0.0001$) in both experiments. The interaction reveals specificity of certain isolates to cause symptoms on certain plant species. The overall isolate effect was significant as well ($P<0.01$). Similar P values are observed for data on number of dead plants, shoot length, and vascular discolouration (Table 3).

Figures 3 and 4 illustrate the isolate x plant species interaction, e.g., for *A. campestre* and *C. bignonioides* inoculated with isolates belonging to VCG NL-2: on *A. campestre* isolate A59mc-1 was most virulent and A40mc-2 and S12,2 were among the least virulent, whereas on *C. bignonioides* the effect was opposite.

Contrasts for isolates belonging to VCG NL-1 versus VCG NL-2 were significant only for vascular discolouration (Table 3). Isolates of VCG NL-1 caused more vascular discolouration than did isolates of VCG NL-2 in Experiment 1 ($P=0.0031$) and Experiment 2 ($P=0.041$). All cross sections of two plant species x isolate combinations in Experiment 2, viz., isolate G3 (VCG NL-1) on *A. campestre* and S12,2 (VCG NL-2) on *C. coggygria*, were entirely discoloured and were given the maximum score of 5. Consequently, no variation in the data for these combinations was present, which caused problems during the statistical analysis. Therefore, data for these two combinations had to be omitted during calculation of the contrast, to allow convergence of the statistical procedure.

Table 3 also shows contrasts for *S. vulgaris* inoculated with isolate S12,2, originally isolated from *S. vulgaris*, compared with other isolates. No clear symptoms (different from the control plants) were observed in *S. vulgaris* during the growing season, but during winter three out of ten plants that had been inoculated with this isolate died (Table 2), while none of the *S. vulgaris* plants inoculated with other isolates died (glm $P=0.014$; Chi-square $P=0.0001$). This isolate also caused the largest reduction in shoot length (25%) on *S. vulgaris* ($P=0.0005$). The same isolate was the most virulent on *C. coggygria* causing the severest symptoms and 100% mortality in both experiments (Table 2).

Contrasts for isolate AplatI mc-1, originally isolated from *A. platanoides*, versus other isolates showed that this isolate caused more disease symptoms in Experiment 1 and more vascular discolouration in Experiment 2 than other isolates tested on *A. platanoides*. Tested against all other isolate x plant species combinations, AplatI mc-1 on *A. platanoides* caused above average severe wilt symptoms in Experiment 1 ($P<0.0001$) and Experiment 2 ($P=0.0013$). The same isolate tested on all three representatives of the *Acer* genus caused significantly more disease symptoms in Experiment 1 ($P<0.0001$) and Experiment 2 ($P=0.035$) than other isolates on these hosts (Table 3).

Table 3. Significance levels of fixed effect factors and contrasts for all tested variables in Experiments 1 and 2. Data for control plants are excluded.

Tested variable	disease score	AUDPC [1]	plant death	plant death	plant death	plant death	shoot length	vascular discolouration	vascular discolouration
Experiment	1	2	1	1	2	2	1	1	2
Statistical procedure (SAS v. 8)	genmod	glm	glm	chi-square [2]	glm	chi-square	glm	genmod	genmod [3]
FACTORS									
Plant species	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Isolate	0.0032	0.0017	0.0005	0.021	0.012	0.22	0.084	0.0012	<0.0001
Plant species x isolate interaction	<0.0001	<0.0001	<0.0001	0.018	<0.0001	0.11 [4]	<0.0001	<0.0001	<0.0001
Block	0.10	0.22	0.012	- [5]	0.27	- [5]	0.72	0.0011	0.27
CONTRASTS									
VCG NL-1 vs. VCG NL-2 [6]	0.42	0.15	0.08	0.18	0.69	0.79	0.48	0.0031	0.041
S. vulgaris isolate vs. other isolates on *S. vulgaris*	0.18	- [7]	0.014	0.0001	- [7]	- [7]	0.0005	- [8]	- [7]
A. platanoides isolate vs. other isolates on *A. platanoides*	0.0002	0.14	0.66	0.49	0.86	0.87	0.27	0.54	0.0016
A. platanoides isolate vs. other isolates on all *Acer* species	<0.0001	0.035	0.27	0.46	0.97	0.95	0.73	0.88	0.0021

[1] AUDPC = area-under-the-disease-progress curve. [2] Chi-square test was performed to check binominal plant death data analysis. [3] To facilitate convergence of the statistical procedure for the contrast statements, two plant species x isolate combinations were excluded, viz., isolate G3 (VCG NL-1) on *A. campestre* and S12,2 (VCG NL-2) on *C. coggygria*. All plants of these two combinations were scored in the highest vascular discolouration class. [4] Data for *R. pseudoacacia* and *R. canina* were omitted during calculation of the chi-square, because none of the plants of those species died. [5] Blocks were pooled during calculation of the chi-square. [6] VCG = vegetative compatibility group. [7] *S. vulgaris* was tested in Experiment 1 only. [8] *S. vulgaris* did not show vascular discolouration.

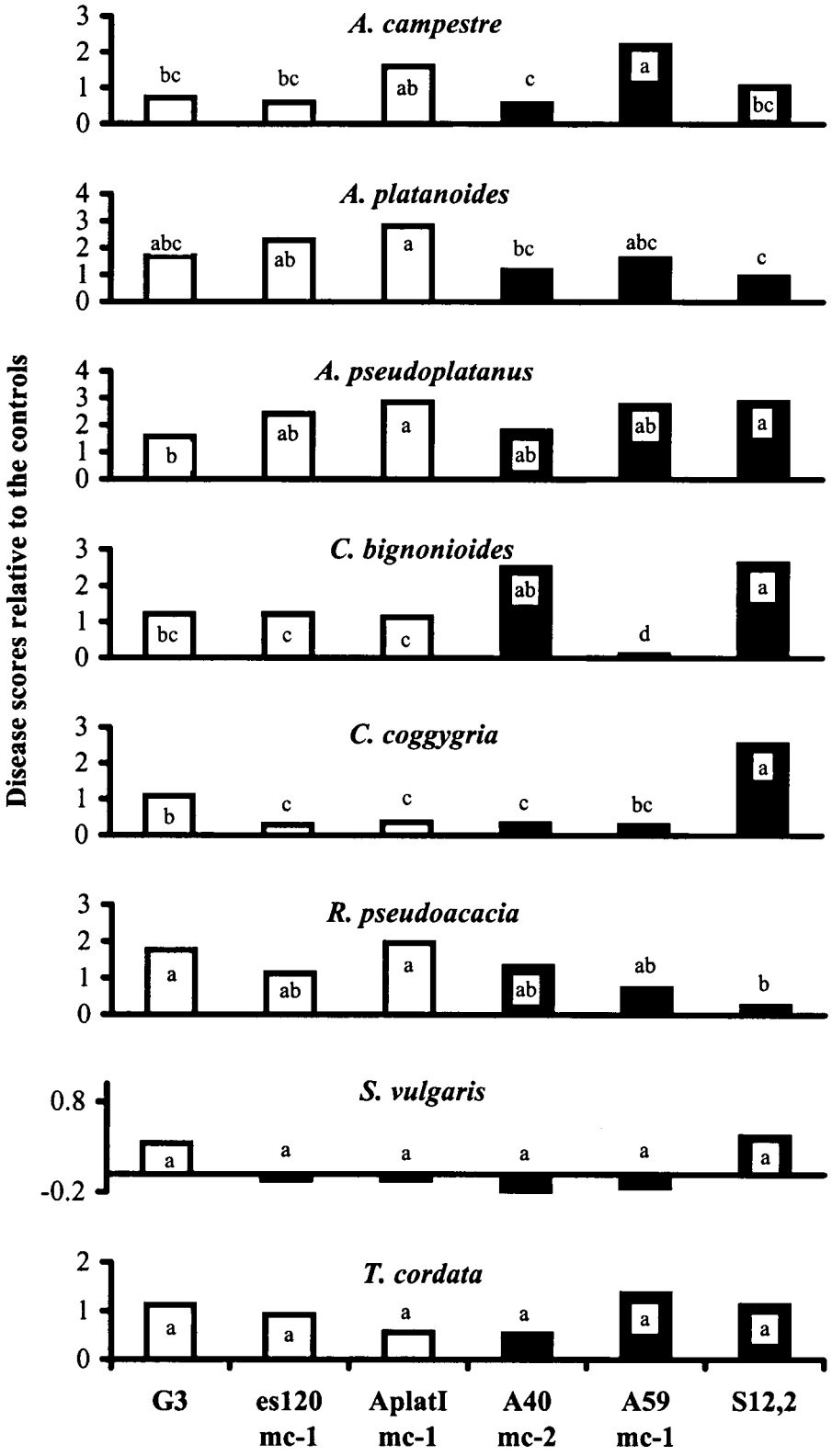

Figure 3. Disease scores of Experiment 1 for the different isolates of *Verticillium dahliae*, presented as averages after subtraction of the average control values per plant species. The first three isolates represent vegetative compatibility group (VCG) NL-1 (white bars), the last three isolates represent VCG NL-2 (black bars). Different characters within plant species indicate significant differences (overall $P<0.05$; $P<0.0033$ per comparison). Statistics were performed on the original data as described in the Material and Methods section.

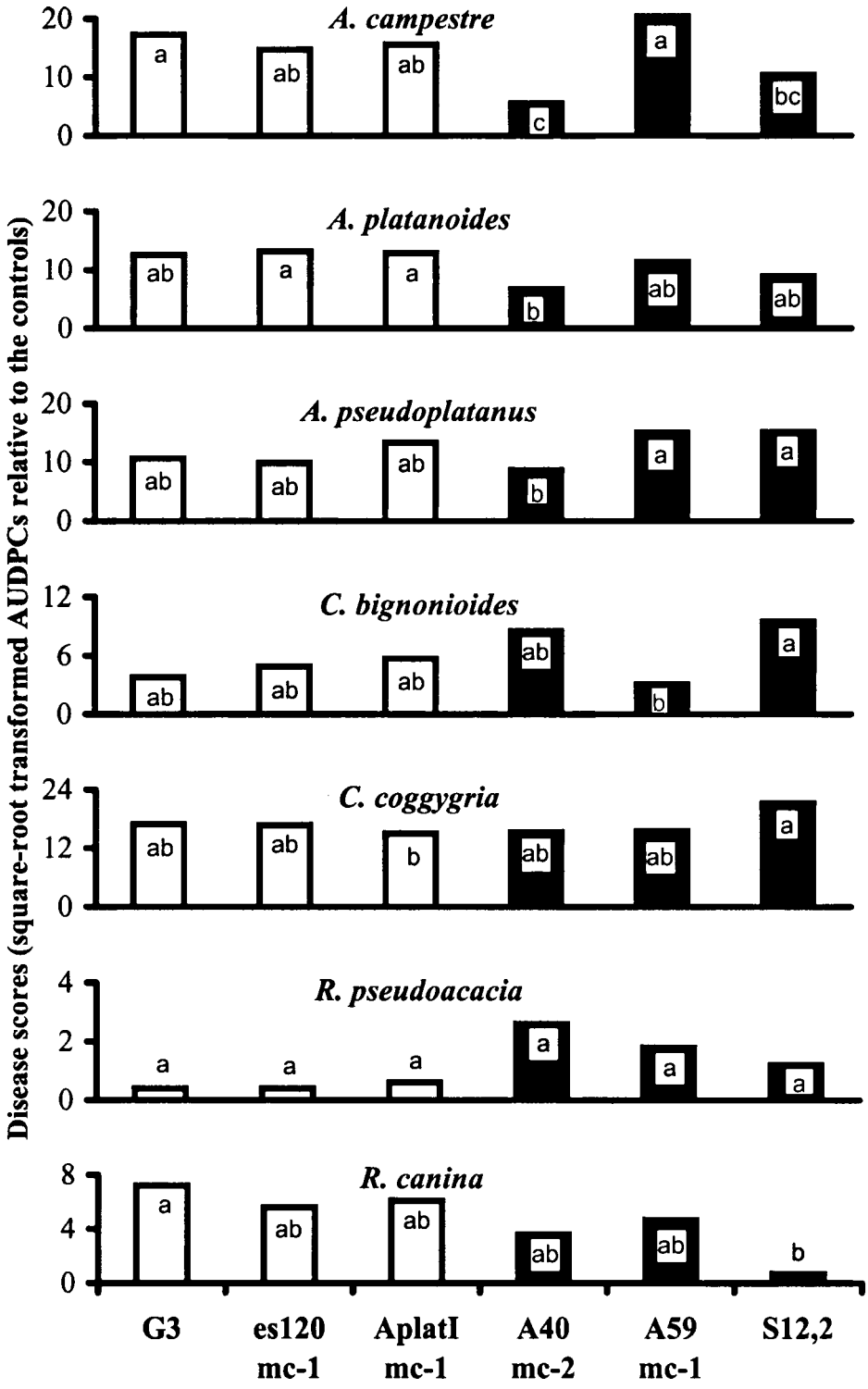

Figure 4. Square-root transformed area-under-the-disease-progress curves (AUDPCs) of Experiment 2 for the different isolates of *Verticillium dahliae*, presented as averages after subtraction of the average control values per plant species. The first three isolates represent vegetative compatibility group (VCG) NL-1 (white bars), the last three isolates represent VCG NL-2 (black bars). Different characters within plant species indicate significant differences (overall $P<0.05$; $P<0.0033$ per comparison). Statistics were performed as described in the Material and Methods section.

Discussion

The observation that isolates from each of the VCGs can cause verticillium wilt in woody hosts, and that no difference in virulence between VCGs was found has major consequences for disease prediction in woody hosts. Absence of a link between VCG and virulence implies that VCG determination of the population of *V. dahliae* present in infested soil does not contribute to disease forecasting in woody hosts in general. This was expressed clearly in all external disease symptoms and was repeated in both experiments. Other unknown characteristics at the individual isolate level were found to be of higher importance than VCGs.

No reports exist on virulence testing of VCGs on a range of woody hosts. Bhat and Subbarao (1999) tested 14 herbaceous crops and observed no correlation among VCG and reduction of plant length and root and shoot dry weight. Unfortunately, data for external wilt symptoms are not presented in their paper. Gennari and co-workers (2000) found no differences in virulence between three Italian VCGs tested on tomato and melon. On the other hand, virulence differences between VCGs were reported frequently for individual host species, e.g., for potato (Joaquim and Rowe, 1991; Strausbaugh, 1993; Strausbaugh *et al.*, 1992) and cotton (Daayf *et al.*, 1995; Korolev *et al.*, 2000), though sometimes different results were obtained when isolates from different countries were used.

We repeatedly observed more severe vascular discolouration caused by isolates of VCG NL-1 than that caused by isolates of VCG NL-2. This is an indication that different VCGs can cause differential reactions in plants. Apparently, these reactions do not affect wilt symptoms and plant length. Our findings are not in agreement with those of Bhat and Subbarao (1999), who observed no difference in vascular discolouration between VCGs. Vascular discolouration differs from other symptoms because it can extend beyond infected tissue (Mace, 1989) and it is permanent: if the plant recovers from the disease by formation of new vascular tissue, external symptoms can disappear, but vascular discolouration of the older xylem tissue remains visible. Vascular discolouration is generally absent in *S. vulgaris* (Van der Meer, 1925) and can be present (Nienhaus *et al.*, 1992) or absent (McCain, 1976) in *R. canina*.

Isolates within VCGs can vary considerably with respect to virulence (Bao *et al.*, 1998; Elena, 1999; Gennari *et al.*, 2000). All isolates used in the present study were not absolutely host specific, despite the highly significant plant species x isolate interaction. Some degree of specificity was visible in most of the isolates, e.g., in the two isolates that were more virulent on their original host. This latter phenomenon has been reported before (Douhan and Johnson, 2001; Mol, 1995; Resende *et al.*, 1994; Sinclair *et al.*, 1989), and also in *Acer* (Adams and Tattar, 1976; Hiemstra, 1995), though host specificity appears not to be a rigid character (Jeger *et al.*, 1996): Fordyce and Green (1963) have shown, through repeated inoculation and isolation, that isolates from peppermint that were not pathogenic to tomato became pathogenic after one or more passages through tomato plants. Quantitatively, potato is the most important host in the Netherlands, because *V. dahliae* can increase well on this host. Van der Meer (1925) already observed that woody and herbaceous plants on former potato fields were often infected. The potato isolate used in this study had an average virulence, compared with the other isolates. Adams and Tattar (1976) observed that isolates from potato, tomato, and chrysanthemum were less pathogenic on *A. saccharum* than isolates from woody hosts. More potato isolates should be tested to discover trends.

Recovery of diseased *C. bignonioides* and *R. pseudoacacia* plants in Experiment 2 is most probably caused by the high air temperatures in the greenhouse. *V. dahliae* hardly grows at temperatures above 30°C (Schnathorst, 1981), whereas *C. bignonioides* and *R. pseudoacacia*, the two most thermophilic plant species tested, reacted with rapid growth resulting in recovery from the disease. Other plant species tested did not grow faster at high temperatures. Isolation of *V. dahliae* from recovered *C. bignonioides* and *R. pseudoacacia* plants at the end of the experiment showed that the fungus was still present. Recovery of infected trees during hot summer periods has been reported for apricot (Taylor and Flentje, 1968), avocado (Latorre and Allende, 1983; Zentmyer, 1949), and olive (Wilhelm and Taylor, 1965). Recovery of diseased trees should be further examined, focusing on unravelling the mechanisms involved and gathering quantitative information to get insight about the relative importance of recovery.

The highly significant differences between plant species indicate the importance to test a range of species. VCG determination may be useful only for disease prediction in specific crops. More isolates need to be tested, because it is unclear whether three isolates is a sufficiently large number to represent an entire VCG. Future research also needs to investigate whether most isolates of VCG NL-2, like the tester isolates, are compatible with American VCG-1 and VCG-2 tester isolates, thus being an entire bridging population. A situation like that exists in *Armillaria* where three North American intersterility groups are partially (two groups) or fully (one group) interfertile with one European intersterility group (Anderson *et al*., 1980; Guillaumin *et al*., 1991). Research on disease prediction for a range of woody hosts should focus on molecular characterisation of isolates to discover virulence genes. For example, differentiation between the defoliating and non-defoliating strains of *V. dahliae* has been shown for cotton (Pérez-Artéz *et al.*, 2000) using random amplified polymorphic DNA analysis. This type of approach could help to discover genes that contribute to virulence. Because of significant effects of both isolate and isolate x plant species observed in our study, one should search for genes involved with virulence to woody hosts in general, and virulence to specific (woody) plant species.

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Chapter 5

Long-term effect of biological soil disinfestation on verticillium wilt

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Plant Pathology: *submitted*

Abstract

Biological soil disinfestation (BSD), involving incorporation of grass combined with plastic mulching, eliminates many soil-borne pests and diseases through the creation of anaerobic conditions. BSD was compared with a control treatment, a grass alone treatment and a plastic mulching alone treatment, at two locations. After BSD, plots were cropped with *Acer platanoides* and *Catalpa bignonioides*, for four years. Soil inoculum levels of *Verticillium dahliae* were reduced by 85% after BSD, relative to the control, and did not increase during the four-year period. Stem infection with *V. dahliae* was reduced by 80-90%. *Pratylenchus fallax* populations in the soil and in roots were reduced by 95 – 99%. Verticillium wilt was significantly reduced in *A. platanoides* in most years, and significantly fewer plants died. Plant height and width were larger after BSD, compared with the control, at the location with the wider plant stand. Value of the marketable plants, including only those that were never diseased, was up to € 140,000 ha^{-1} higher for *A. platanoides* and up to € 190,000 ha^{-1} higher for *C. bignonioides*, compared with the control. BSD is an effective, economically profitable and environmentally friendly control method for tree nurseries.

Abbreviations: BSD – biological soil disinfestation, i.e. soil disinfestation through anaerobic conditions, caused by incorporation of fresh organic matter and plastic mulching; CFU – colony forming units; SEM – standard error of the mean; AUDPC – area under the disease progress curve

Introduction

Verticillium wilt is caused by the soil-borne fungus *Verticillium dahliae* Kleb. and occurs in many crops all over the world. The disease can be a severe problem in potato, strawberry and olive production, and in nurseries for strawberries and ornamental trees (Pegg & Brady, 2002; Sinclair et al., 1989). In tree nurseries, losses up to 50% have been reported (Anonymous, 1990). Interactions of *V. dahliae* with plant pathogenic nematodes have been observed, e.g., in potato (Martin et al., 1982; Wheeler et al., 1994), and sugar maple (*A. saccharum*) (Dwinell & Sinclair, 1967).

Until recently, effective control of *V. dahliae* and plant pathogenic nematodes was achieved by fumigating the soil with chemicals. However, restrictions for soil fumigants point towards the need for non-chemical alternatives. In developed countries, the application of methyl bromide will be forbidden by 2005 and in developing countries in 2015 (Duniway, 2002). In the Netherlands, the application of methyl bromide has already been banned since 1992, after a 10-year phase-out period (Mus & Huygen, 1992). Currently, metham sodium is the only liquid soil fumigant allowed in the Netherlands, and its use is restricted to a maximum of once every five years. Moreover, 1,3-dichloropropene and metham sodium are

effective against nematodes, but they have only a slight effect on plant pathogenic fungi. Effective non-chemical methods exist, but their application is often limited to certain cropping systems. Steaming the soil requires high investments and is therefore only applied in high value crops (Corsten et al., 2002). Soil solarization (Katan et al., 1976) and flooding (Stover, 1979) reduce inoculum levels of soil-borne pathogens (Ashworth et al., 1982; Pullman & DeVay, 1982), but solarization is restricted to warmer areas, and flooding is not feasible on many locations.

Recently, a new approach was reported by Blok et al. (2000), referred to as Biological Soil Disinfestation (BSD). The method offers a promising alternative to reduce soil inoculum levels of a wide variety of plant pathogenic soil-borne fungi and nematodes (Blok et al., 2000; unpublished). BSD combines the incorporation of fresh organic amendments in soil and mulching with airtight plastic. This combination creates an anaerobic condition in the soil, resulting in pathogen elimination. BSD is essentially different from solarization, since death of the pathogens is not caused by high temperatures (Blok et al., 2000). The method has been proven effective in several large scale field experiments, and is advocated for use in high value crops. It has broad spectrum activity against many soil-borne fungi and plant-pathogenic nematodes with an effectiveness similar to that of methyl bromide (Blok et al., 2000, unpublished).

Every type of soil disinfestation, either chemical or non-chemical, introduces the potential risk of lowering the general soil suppressiveness towards pathogens by disturbing the soil microflora (Stover, 1979). *V. dahliae* is unable to grow through non-rhizosphere soil, because of inhibition by the general microbial soil community. It can, however, actively colonise sterilised soil. Active growth of *V. dahliae* through the soil could cause enhancement of the pathozone, and therefore, an increased disease conduciveness. Otherwise, inactivation of specific antagonists of *V. dahliae* could lead to the situation where inoculum levels are much reduced initially, but build up to high levels rapidly, resulting in high disease levels. In theory, recolonization of the soil by *V. dahliae* can occur directly after BSD or gradually during the years after BSD by formation of microsclerotia in senescing and dying plant tissue (annual plants) or petioles (perennial plants) (Hiemstra, 1998; Rijkers et al., 1992).

The objectives of the present study were to investigate: (1) the effectiveness of BSD in reducing soil inoculum levels of *V. dahliae*; (2) whether there was increased disease conduciveness for verticillium wilt after BSD, and (3) the occurrence of enhanced recolonization of the soil by *V. dahliae*.

Materials and methods

General layout

To test the efficacy and persistence of biological soil disinfestation (Blok et al., 2000) against *V. dahliae*, a full factorial two-factor field experiment in a randomised complete block design with 5 repetitions was started at two locations: at Wageningen in 1997 and at Meterik in 1998. Experimental factors included (1) the incorporation of freshly mown grass (0 or 40-54 ton ha^{-1}) and (2) covering the field with nearly-airtight plastic (yes or no). A description of BSD is given below. After this treatment the soil was left fallow till the next spring, when seedlings of *Acer platanoides* L. (Norway maple) and *Catalpa bignonioides* Walt. (southern catalpa)

were planted. Verticillium wilt disease severity and soil inoculum densities of *V. dahliae* were followed during the subsequent four years. Plots measured 8 × 8 m (Wageningen) or 7.5 × 10.5 m (Meterik). To avoid side-effects, measurements were performed only in the inner area, measuring 5 × 5 m (Wageningen) or 4.5 × 7.5 m (Meterik).

Field characteristics and infestation

The soil at Wageningen (experimental farm of Wageningen University) was a loamy sand (0-20 cm soil layer: pH-$CaCl_2$ = 5.8, organic matter (OM) = 3.3%; 30-60 cm: pH-$CaCl_2$ = 5.7, OM = 1.9%) that was cropped with potato in the year prior to BSD and had an inoculum level of 3.0 colony forming units (CFU) g^{-1} dry soil weight. An increase in inoculum level was expected, because of decomposition of potato debris leading to release of microsclerotia into the soil (Mol et al., 1996). At the experimental farm 'Meterikse Veld' (Meterik, province of Limburg, the Netherlands) a field (loamy sand, 0-20 cm soil layer: pH-$CaCl_2$ = 4.9, OM = 2.8%; 30-60 cm: pH-$CaCl_2$ = 5.0, OM = 2.2%) that was cropped with *Tagetes* sp. during two years prior to BSD, was artificially infested with *V. dahliae* from potato stems. Potato stems, covered with microsclerotia at a density of approx. 1 million microsclerotia g^{-1} potato stem, were collected in October 1997 from various agricultural fields in the provinces of Drenthe and Gelderland (the Netherlands), air-dried and stored during winter at approx. 5 - 10 °C, milled shortly before application, mixed 1 : 1 (v/v) with sand, spread by hand and mixed through the top 20-25 cm soil layer using a rototiller at a rate of approx. 15 g of potato stems m^{-2}.

Biological soil disinfestation

Biological soil disinfestation (BSD) was applied in Wageningen in the summer of 1997, and in Meterik in the summer of 1998, as described by Blok et al. (2000). The organic amendment treatments were (i) non-amended (control), and (ii) Italian ryegrass (*Lolium multiflorum* Lam.) mixed through the soil using a rototiller. In Wageningen, freshly mown young grass was applied from a production pasture (C/N ratio = 13.9) and mixed through the top 25 cm soil layer at the rate of 40 tons fresh weight ha^{-1} (= 4.56 tons oven dry weight ha^{-1}) on July 8 1997. In Meterik, grass was sown on the experimental plots on May 11, 1998 and incorporated through the top 35 cm soil layer 12 wk later on August 3 (C/N ratio = 21.4). At the time of incorporation, the biomass (shoots + roots) was estimated to be 54 tons fresh weight ha^{-1} (= 15.4 tons oven dry weight ha^{-1}). Soil in all amended and control plots was rototilled, soil was compacted by riding over it with a tractor, and irrigated with approx. 50-70 mm water. The tarping treatments were (i) non-covered, and (ii) covered with three-layered ensilage plastic with low oxygen permeability. In Wageningen the plastic was Hytileen (Klerks Plastic Industrie, Noordwijkerhout, the Netherlands), 0.135 mm thick, with a black (upper) and a white (under) side. In Meterik the plastic was Hermetix (Klerks Plastic Industrie, Noordwijkerhout, the Netherlands), 0.115 mm thick, with a green upper and a white under side. The edges of the cover were buried approx. 10 cm deep. The plastic cover was present from July 11- October 10 1997 (13 wk) at Wageningen and from August 6 – October 13 1998 (10 wk) at Meterik. After removal of the plastic cover, the soil was left fallow for six months, until the next spring.

Assessment of soil inoculum levels of V. dahliae and plant pathogenic nematodes

To determine pre-planting inoculum levels of *V. dahliae* (Wageningen and Meterik) and free-living plant pathogenic nematodes (Wageningen), 50 soil cores (0-25 cm deep) were collected per plot, in a V- and inverse-V-pattern. Soil samples were thoroughly mixed, and stored at 4°C until processed. For *V. dahliae*, the soil sample was air-dried at room temperature, dry-sieved through a 2-mm sieve, and 12.5 g of the air-dry soil was subsequently wet-sieved through 106- and 20-µm nested sieves. The fraction retained on the 20-µm sieve was suspended in 50 ml 0.08 % water agar, and 0.8 ml of this suspension was plated on modified soil extract agar (MSEA), a semi-selective medium (Harris *et al.*, 1993) containing soil extract prepared with a sandy garden soil and 50 ppm oxytetracycline as the single antibiotic. After four weeks of incubation at 20 °C, colonies were counted and colony forming units g^{-1} dry soil were calculated. Post-planting soil inoculum levels of *V. dahliae* were assessed similarly once each following spring, but soil sampling occurred along the plant rows, at approx. 15 cm distance from the stem bases. To follow the soil inoculum development in association with the two plant species, soil samples were taken separately for *A. platanoides* and *C. bignonioides*, except for the second year in Wageningen. To determine soil inoculum densities of free-living plant-pathogenic nematodes, a 200-g subsample was washed in an Oostenbrink elutriator (Oostenbrink, 1960).

Plant origin and maintenance

Seeds of *A. platanoides* were obtained from Boevé BV (Boskoop, the Netherlands) for the Wageningen experiment and for the Meterik experiment germinated seeds were obtained from the municipal plant nursery of Wageningen (Wageningen, the Netherlands). Seeds of *C. bignonioides* were obtained from Boevé BV for both locations. Plants were raised in the greenhouse for 10 wk and planted in the field 7 months after BSD. In Wageningen, each plot was planted with three rows of 20 seedlings (8-10 leaves) of *A. platanoides* and three rows of 20 seedlings (6-8 leaves) of *C. bignonioides*, maintaining a within-row planting distance of 25 cm. Each set of three rows was surrounded by a guard row of the same species. After the first growing season the middle row and after the second growing season another row of 20 plants was harvested to provide growing space for the remaining plants, and for assessment of infection. Row distance was 33 cm in the first year and 100 cm in the 3^{rd} and 4^{th} years. In Meterik, 7 months after BSD, 60 seedling plants of *A. platanoides* and *C. bignonioides* per plot were planted in two rows of 30 plants. One row was harvested after the first growing season, and plants were thinned alternately within the row after the second growing season. Row distance was 50 cm in the first year and 100 cm in the 2^{nd}, 3^{rd}, and 4^{th} year. Distance within the rows was 25 cm during the first two years, and 50 cm during the 3^{rd} and 4^{th} year. Plants were cut at 1.80 m height to arrive at a better height/width ratio after year 2 (Meterik) and year 3 (Wageningen and Meterik). Plants were fertilised two times with 75 kg of N ha^{-1} (50% NO_3-N / 50% NH_4) and one time 100 kg of Mg ha^{-1} during each growing season. Weeding was done mechanically or by hand at regular intervals. *A. platanoides* plants were sprayed periodically with anti-mildew sprays (3 times Rubigan, 20 ml 100 l^{-1} water, Dow Elanco, Wilrijk, Belgium, 1 time Meltatox, 250 ml 100 l^{-1}, BASF, Arnhem, The Netherlands, 3 times Baycor Flow, 60 ml 100 l^{-1}, Bayer, Mijdrecht, The Netherlands, and 1 time again Meltatox).

Disease measurements

Wilt symptoms. Disease scores were taken regularly during 4 consecutive years, for each individual plant on a 0 - 5 scale (0 = healthy, 1 = wilt of several leaves (up to 5%); epinasty in *A. platanoides*, 2 = 5 - 40% wilt or defoliation, 3 = 40 - 60% wilt or defoliation, 4 = 60 - 80% wilt or defoliation, and 5 = more than 80% wilt or defoliation or plant dead). Area-under-the-disease-progress-curve (AUDPC) values (Campbell & Madden, 1990) were calculated per plot for each plant species. Dead plants or dead plant parts (in those that had recovered) of the previous year(s) were not included in the disease assessment of the following year(s).

Infection with *V. dahliae*. After the first growing season in Wageningen, stem infection with *V. dahliae* was assessed for all 20 plants that were removed of each plant species. Plants were harvested per block. Blocks 1 to 5 were harvested on September 9, September 25, October 19, October 29, and November 10, 1998 respectively. Infection was assessed at the stem base, by sterilizing the stem pieces in 1% sodium hypochlorite for 1 min., followed by washing in sterile water, aseptically removing the bark, cutting discs with a pruning knife, and placing 5-7 discs onto MSEA. After incubation for 4 weeks at 20°C, presence of *V. dahliae* was scored. In Meterik, confirmation of infection by plating was sought only incidentally, when symptoms were unclear.

Shoot length and width. At each location, shoot length was measured at the end of each growing season. Stem width was measured at the stem base after the first (Wageningen and Meterik) and second (Wageningen) growing season, and at 1 m height after the following growing seasons. Dead plants or dead plant parts of the previous year(s) were not measured in the following year(s).

Root infection with *Pratylenchus fallax*. In Wageningen, the number of *Pratylenchus fallax* Seinhorst nematodes g^{-1} root was determined during the first growing season on a sample of pooled roots of 5 random plants of the row that was removed. A 50-g (fresh weight) subsample was used by cutting the clean roots in approx. 1 cm pieces, placing them in a mist incubator, and collecting the nematodes in the run-off water during a seven-day period (Oostenbrink, 1960).

During the second season, *P. fallax* was assessed in roots of 4 random plants, which were harvested on the same day, and analysed separately, with the procedure described above, using 20-30 g (fresh weight) of roots per plant.

In Meterik nematodes were not present, as was revealed by incidental measurements, because of two consecutive croppings of *Tagetes* sp. in the years prior to BSD.

Data analysis

Data were analysed with SAS version 8.0 (SAS Institute Inc., Cary, NC). The GLM procedure was used for analysis of *V. dahliae* and nematode inoculum levels in the soil, AUDPC-values, numbers of *P. fallax* per plant root, and stem length and width. Soil inoculum levels of *V. dahliae* and nematodes, and numbers of *P. fallax* per plant root were log-transformed and percentages of diseased and dead plants were arcsine-transformed to obtain normal distribution of residuals. Contrast analyses were performed between the combined

grass + plastic treatment and the control, to obtain insight in the effectiveness of the BSD treatment. Enhanced disease conduciveness was tested by covariance analysis of the data of the grass + plastic treatment and the control. By treating *V. dahliae* soil inoculum levels as a covariate, effects of BSD on disease were corrected for different *V. dahliae* soil inoculum levels (Cody & Smith, 1997). Marketable yield was calculated per plot, based on stem width of plants that were healthy during all growing seasons. Plot values were divided by the maximum numbers of plants (20 in Wageningen and 15 in Meterik) per plot to arrive at averages per plant.

Results

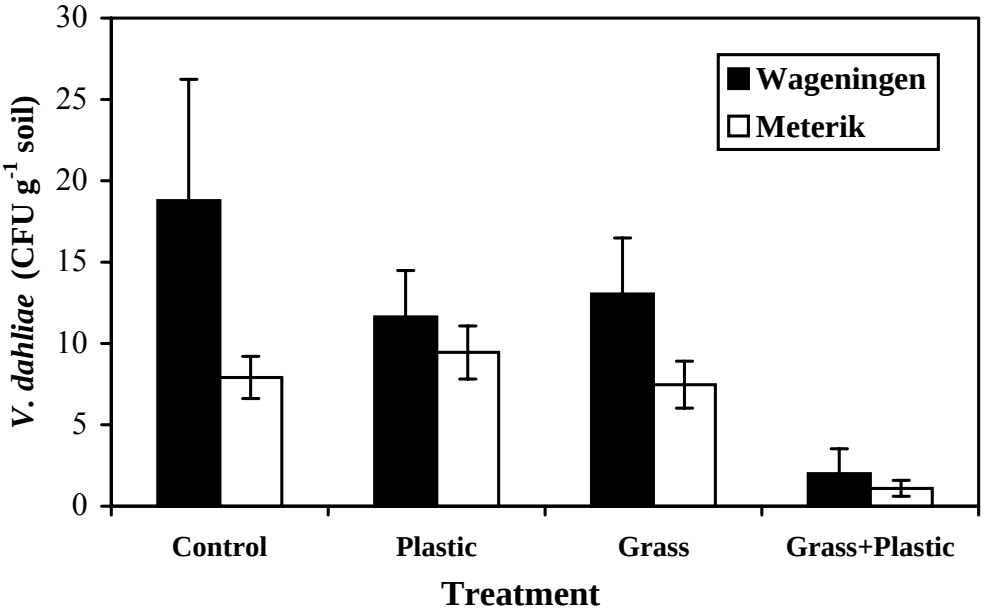

Figure 1. Effect of four treatments (control, plastic mulching, grass amendment, and BSD (grass amendment combined with plastic mulching)) on detected soil inoculum level (colony forming units (CFU)) of *Verticillium dahliae* in the next spring in the tree nursery plots at two locations. Error bars indicate the standard error of the mean (SEM).

Effect of BSD on inoculum densities. The combination of grass amendment with plastic tarping had a greater than additive effect on the reduction in soil inoculum levels of *V. dahliae*, as is indicated by the (near) significance of the grass x plastic interaction (Wageningen P=0.06; Meterik P<0.01). The contrast of the grass + plastic combination with the control was significant in each experiment (P<0.01), while the grass or plastic treatments by themselves did not differ significantly from the control treatment. Compared with the control, *V. dahliae* levels were reduced by 85% in plots of the grass + plastic combination, in each field experiment (Figure 1). Likewise, the numbers of *Pratylenchus* sp. were significantly reduced (P<0.01) in the combined grass + plastic treatment, compared with the control (Figure 2). Also the contrast of plastic alone compared with the control was significant (P=0.05) with respect to soil inoculum levels of *Pratylenchus.* Other naturally occurring plant

pathogenic nematodes were present in low numbers and contrasts between grass + plastic and the control were significant for *Trichodorus* spp. only (P=0.04) (Figure 2).

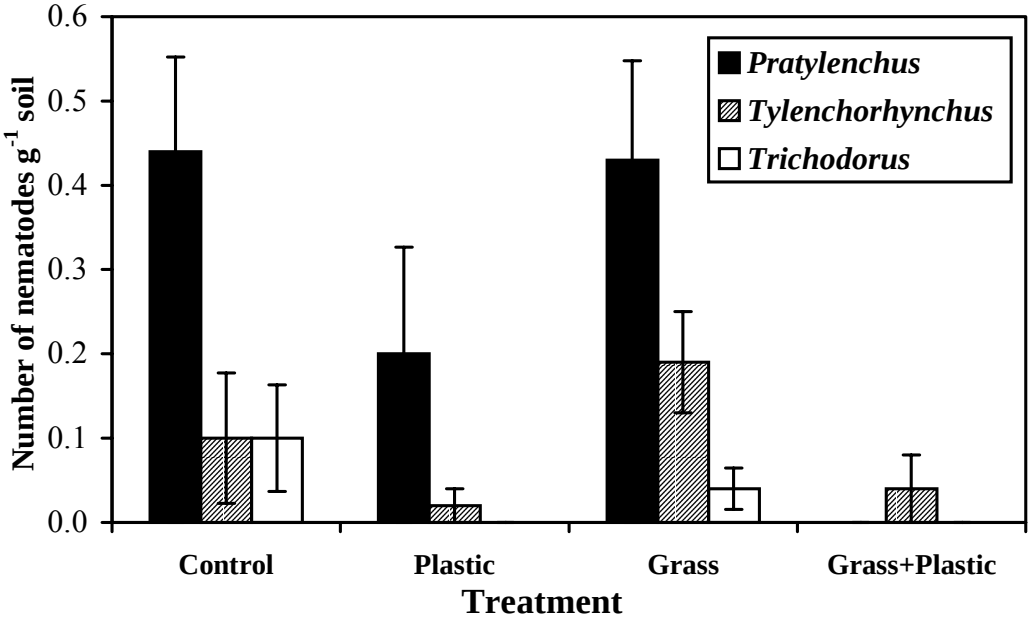

Figure 2. Effect of four treatments (control, plastic mulching, grass amendment, and BSD (grass amendment combined with plastic mulching)) on number of free living plant pathogenic nematodes in the next spring in the tree nursery plots (Wageningen location only). Error bars indicate SEM.

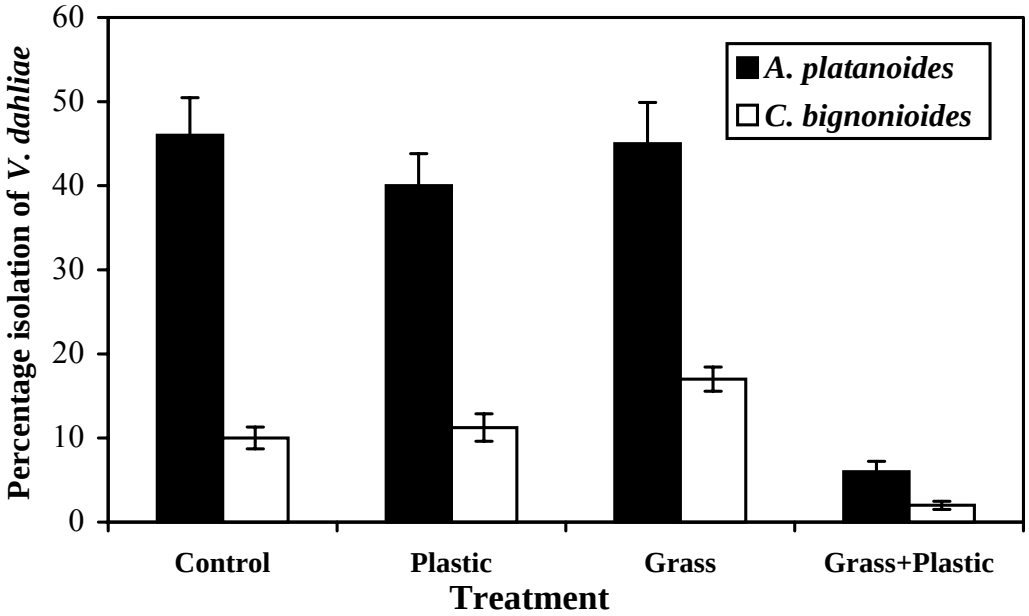

Figure 3. Effect of four treatments (control, plastic mulching, grass amendment, and BSD (grass amendment combined with plastic mulching)) on percentage of isolation of *Verticillium dahliae* from the stem base of *Acer platanoides* and *Catalpa bignonioides* at the end of the growing season (Wageningen location only). Error bars indicate SEM.

Effect of BSD on plant infection. In Wageningen, more than 40% of the *A. platanoides* plants and 10-17% of the *C. bignonioides* plants became infected with *V. dahliae* in fields of the control, grass alone and plastic alone treatments (Figure 3). These percentages were reduced by 80-90% in the grass + plastic combination (Figure 3) (contrast *A. platanoides* $P<0.01$; *C. bignonioides* $P=0.10$). Likewise, the numbers of *P. fallax* nematodes were 230 g^{-1} root in *A. platanoides* and 32 g^{-1} root in *C. bignonioides* in the control treatment, and were reduced by 95-99% in the combined grass + plastic treatment (Figure 4) (contrast *A. platanoides* $P<0.01$; *C. bignonioides* $P=0.01$). This effect was still present in the second year ($P<0.01$), though numbers extracted from *A. platanoides* were smaller than during the first year (Figure 4). The nematode was identified as the species *Pratylenchus fallax* Seinhorst (Seinhorst, 1977). Though nematodes were present in the roots in large numbers, no lesions were observed on the roots.

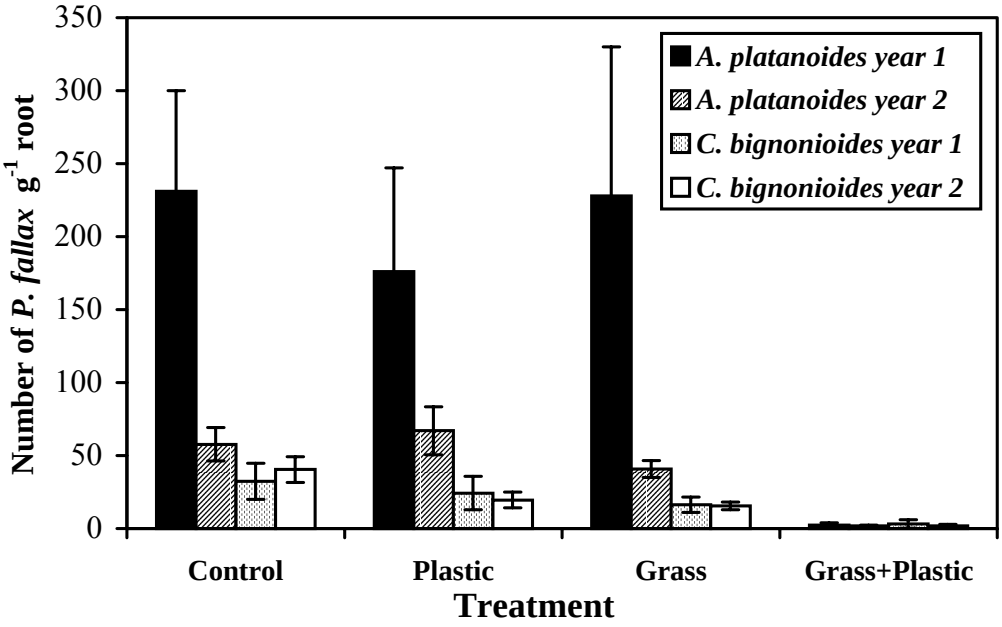

Figure 4. Effect of four treatments (control, plastic mulching, grass amendment, and BSD (grass amendment combined with plastic mulching)) number of *Pratylenchus fallax* nematodes isolated g^{-1} of fresh root of *Acer platanoides* and *Catalpa bignonioides* plants at the end of the growing season in Wageningen. Error bars indicate SEM .

Effect of BSD on verticillium wilt. The combined grass + plastic treatment resulted in significantly lower AUDPC values compared with the control, for *A. platanoides* in Meterik during all experimental years, and in Wageningen during the first two years (Figure 5). For *C. bignonioides,* the contrast between the combined grass + plastic treatment and the control was significant in the first year in Meterik and the second year in Wageningen only (Figure 5). The most important reason for the absence of a difference in later years was the low level of disease in *C. bignonioides*. Few plants of *C. bignonioides* died (Table 1), because of the species' strong capacity to recover from verticillium wilt (Goud & Termorshuizen, 2002). Grass + plastic resulted in lower percentages of dead *A. platanoides* plants during most of the years compared with the control (Table 1). After the second growing season, *A. platanoides* plants were higher and thicker in the grass + plastic combination, compared with the control

(Table 1) though significant in Meterik only. In Wageningen, plant stands appeared to be too dense for *A. platanoides* to detect differences in growth (Table 1). Moreover, death of many plants in the control plots provided growing space for their surviving neighbours. *C. bignonioides* is known to need more growing space than *A. platanoides*. Probably, that is the reason for the absence of significant differences in shoot length and stem width of *C. bignonioides* in Wageningen. In Meterik, where the plant stands were wider, differences in shoot length were significant in each year and differences in stem width were significant in the first two years (Table 1).

Table 1. Significance levels of the contrast analyses between the combined grass + plastic treatment and the control for living shoot length, stem width of the living plants and percentages of dead plants, at the end of the four consecutive growing seasons after BSD, for the two plant species at the two locations.

	Wageningen		Meterik	
	A. platanoides	*C. bignonioides*	*A. platanoides*	*C. bignonioides*
shoot length [1]				
year 1	0.03 [2]	0.13	0.26	<0.01
year 2	0.06	0.55 [2]	<0.01	<0.01
year 3	0.15	0.27	<0.01	0.01
year 4	0.22	0.39 [2]	<0.01	0.04
stem width [1,3]				
year 1	0.14 [2]	0.95 [2]	0.04	<0.01
year 2	0.20	0.76 [2]	<0.01	0.01
year 3	0.11	0.43	<0.01	0.37
year 4	0.11	0.06 [2]	0.01	0.18
% dead plants [4]				
year 1	0.12	0.31	0.11	0.73
year 2	<0.01	1.00	<0.01	0.50
year 3	<0.01	0.42 [5]	0.02	0.98
year 4	<0.01	0.42 [5]	0.06	0.98

[1] Shoot length and stem width of the living part; i.e. dead stems of recovered plants not included.
[2] Shoot lengths and stem widths of control plot plants larger than grass + plastic plot plants.
[3] Stem width was measured at the stem base after the first (Wageningen and Meterik) and second (Wageningen) growing season, and at 1m height after the following growing seasons.
[4] Recorded during spring following the year BSD was applied.
[5] Percentages of dead plants in control plots lower than in grass + plastic plots.

In tree nurseries, recovery of diseased plants does not occur, because they are removed immediately, and consequently are a complete loss to the grower. Therefore, the benefit of the grass + plastic combination compared with the control is most clearly expressed in percentages of plants that do not show disease symptoms during any of the four growing seasons. These percentages were significantly higher in grass + plastic plots than in the control in Meterik (Table 2). In Wageningen, there was a similar trend for *A. platanoides*, but not for *C. bignonioides*. Differences in marketable yields expressed similar results (Table 2).

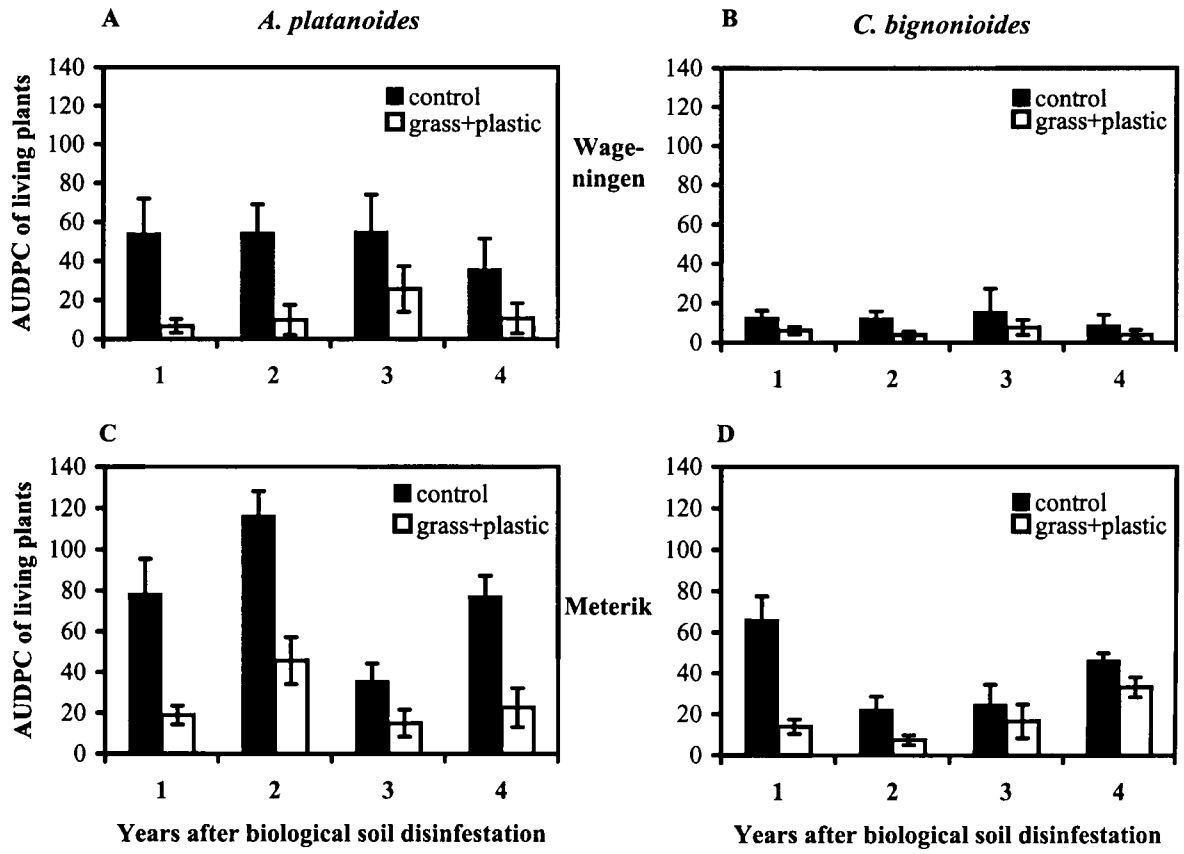

Figure 5. Area under the disease progress curves (AUDPCs) of BSD (grass amendment combined with plastic mulching) and the control during the four growing seasons following BSD. A. *Acer platanoides* in Wageningen, B. *Catalpa bignonioides* in Wageningen, C. *A. platanoides* in Meterik, D. *C. bignonioides* in Meterik. Error bars indicate SEM.

Effects of BSD on conduciveness to verticillium wilt. Covariance analysis indicated that, after correction for the *V. dahliae* soil inoculum level, the grass + plastic treatment did not have higher AUDPCs than the control in any of the experimental years at each location. In other words, the BSD treatment did not significantly contribute to the model when *V. dahliae* inoculum level was already in the model, except for *A. platanoides* in Meterik during the first year, where the grass + plastic treatment had a lower AUDPC than the control (P=0.02). This suggests that there are no indications for changes in conduciveness to verticillium wilt after BSD.

Long-term effects of BSD on development of *V. dahliae* inoculum levels in the soil. Figure 6 shows the development of the detected *V. dahliae* inoculum densities (initial and after one, two, and three growing seasons) in the two field experiments, for the four treatments and the two plant species. In Wageningen, inoculum levels did not significantly change over the years. In Meterik, there was a trend of decrease in inoculum level, which was significant in the plastic plots only. Most importantly, no enhanced recolonization occurred in the grass + plastic plots. There were no significant differences between soils cropped with *A. platanoides* and *C. bignonioides*.

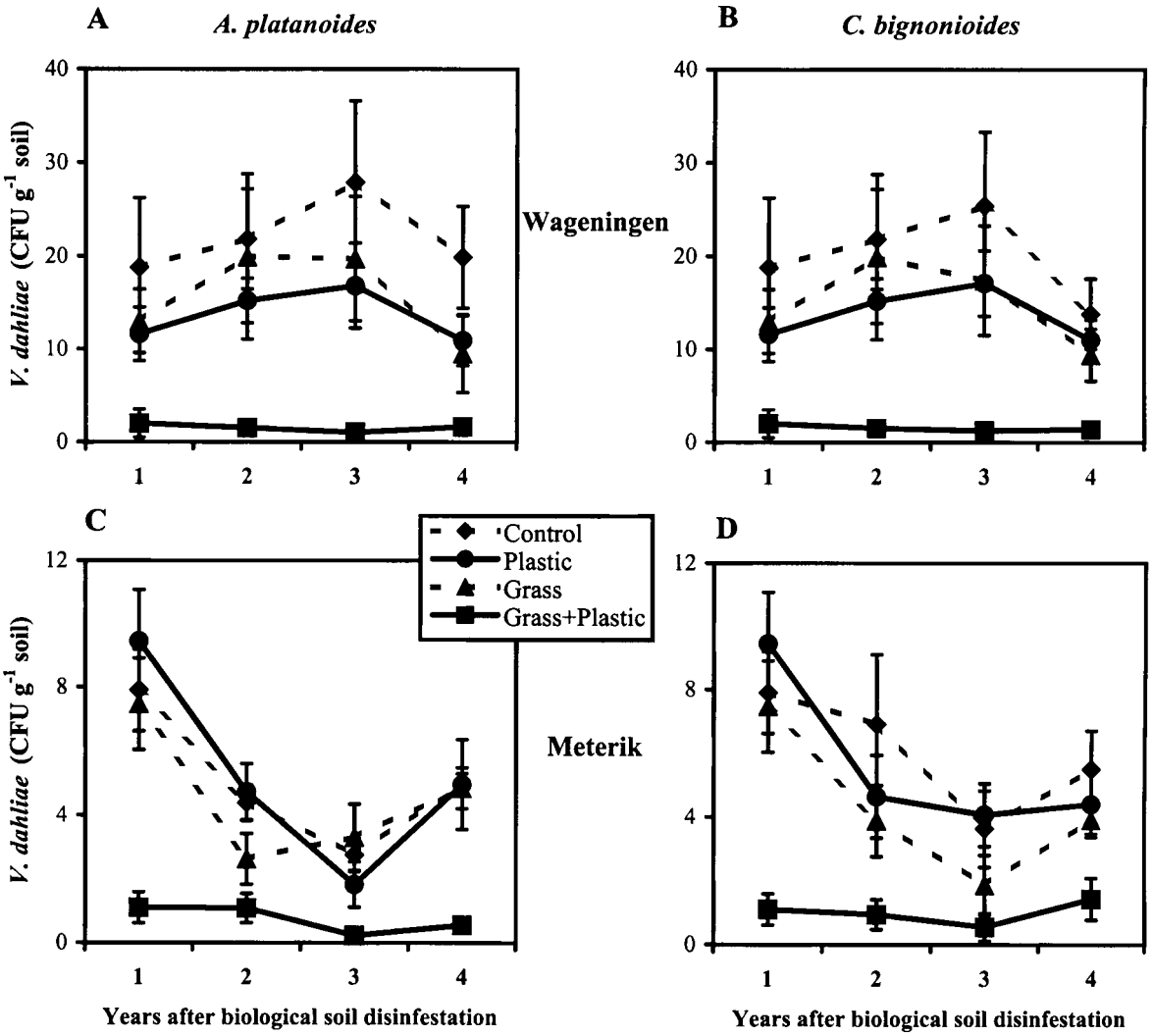

Figure 6. Detected inoculum densities of *Verticillium dahliae* of four treatments (control, plastic mulching, grass amendment, and BSD (grass amendment combined with plastic mulching)) during the four consecutive years after BSD (pre-planting and after one, two and three years). A. *Acer platanoides* in Wageningen, B. *Catalpa bignonioides* in Wageningen, C. *A. platanoides* in Meterik, D. *C. bignonioides* in Meterik. Soil samples were analysed separately for *A. platanoides* and *C. bignonioides* from the second (Meterik) or third (Wageningen) year onwards. Error bars indicate SEM.

Discussion

The obtained results are highly promising, and indicate that BSD (the grass + plastic combination) is a good, environmentally friendly alternative to chemical treatment of the soil with metham sodium or methyl bromide. Though *V. dahliae* populations are not completely reduced to zero, grass + plastic significantly reduces verticillium wilt problems. The results are better than those obtained by flooding (Pullman & DeVay, 1982) and comparable to those of soil solarization (Ashworth et al. 1982), incorporation of broccoli residues (Subbarao et al., 1999) and application of liquid swine manure (Conn & Lazarovits, 2000). Application of grass or plastic alone is not effective against *V. dahliae*, but plastic alone seems effective in reducing soil inoculum levels of *Pratylenchus*.

Table 2. Percentages of plants that remained healthy during all growing seasons, and their average financial value, for the control and the grass+plastic combination.

	Wageningen				Meterik			
	A. platanoides		*C. bignonioides*		*A. platanoides*		*C. bignonioides*	
	Control	Grass+ Plastic	Control	Grass+ Plastic	Control	Grass+ Plastic	Control	Grass+ Plastic
Percentages of plants healthy [1] during all 4 growing seasons	33	69	73	80	20	65	27	63
P-value [2]		0.06		0.78		<0.01		<0.01
Marketable yield (€) of plants that were healthy during all 4 growing seasons [3]	2.31	4.28	5.95	5.73	2.24	6.96	5.65	11.92
P-value		0.09		0.75		<0.01		<0.01

[1] Living plants that never received a disease score of 2 or higher.
[2] Significance level of the difference between Grass + Plastic and the Control. Percentages were arcsine transformed before calculation of the *P*-value.
[3] Total economical yield of plots was divided by the maximum number of plants per plot (20 in Wageningen and 15 in Meterik) to obtain averages per plant. Economical value is based on stem diameter at 1 m height of healthy plants and current advisory prices of the Dutch working group of road side, forest and park trees (H. Meijer, pers. comm.).

The absence of treatment effects on disease development during the first year, corrected for *V. dahliae* soil inoculum densities, implies that BSD did not increase conduciveness to verticillium wilt. In other words, the net multiplication rate of *V. dahliae*, including germination of the microsclerotia, infection of the plant root, colonisation of the plant, and formation of new microsclerotia, was not increased. Apparently, the time between the end of BSD and planting of a susceptible crop (half a year during winter) was sufficient to restore the most important part of the soil biota. However, it is known that specific antagonists return less quickly to soil (Cook & Baker, 1983). The absence of increased conduciveness to verticillium wilt during following years is an indication that specific antagonists (whenever present) are not killed by BSD. This needs to be further examined. The absence of enhanced recolonization during four years after BSD implies that plants are not more prone to infection, colonisation and microsclerotia formation. Both plant species seem to maintain *V. dahliae* at pre-planting levels.

Reduction of *V. dahliae* soil inoculum levels in the spring after BSD might be caused by direct killing during the BSD treatment, and a weakening effect on the microsclerotia. The percentages of direct killing of *V. dahliae* microsclerotia were 59% in Wageningen and 68% in Meterik (Blok *et al.*, unpublished). The strong reduction in verticillium wilt symptoms are in agreement with the 85% reduction in inoculum density observed during the next spring. We suggest that, on top of direct killing, BSD causes weakening of the microsclerotia, by

killing some of the cells that are clumped together into a microsclerotium, capable of germinating separately.

The *P. fallax* densities in the roots of *A. platanoides* and *C. bignonioides* were high in the control plots in year 1. This implies that the nematode can multiply on tree species. *P. fallax* is mainly pathogenic on grass species, but it has also been reported on rose and strawberry (Coolen & Hendrickx, 1972; McKinley & Talboys, 1979; Seinhorst 1977).

Differences in marketable yields between the grass + plastic combination and the control indicate that BSD is profitable in high value crops. Absence of verticillium wilt and stem width at 1m height are important quality measures for plant value. Though other quality criteria were not taken into account, these data provide a strong indication that BSD can serve as a method to avoid risk of yield loss in practice. Based on a commercial planting density of 30,000 plants ha^{-1}, the benefits of the grass + plastic combination compared with the control range from € 60,000 – 140,000 ha^{-1} for *A. platanoides* and from € 0 (or a € 13,000 disadvantage) – 190,000 ha^{-1} for *C. bignonioides*. These benefits are high compared with the direct costs of BSD (approx. € 2,000 ha^{-1}).

Our results clearly show that BSD can help to decrease soil inoculum levels of *V. dahliae* and some plant-pathogenic nematodes significantly. Moreover, BSD is active against a broad range of soil-borne plant pathogens (Blok et al., unpublished). The effectiveness of the method, in combination with the absence of increased conduciveness to verticillium wilt and the absence of enhanced recolonization of the soil with microsclerotia, makes it practically feasible and environmentally friendly. Thus, BSD can be recommended as a viable alternative to fumigation with methyl bromide.

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Chapter 6

Verticillium wilt in trees: Damage thresholds, spatial and temporal aspects.

J.C. Goud, A.J. Termorshuizen & A.H.C. van Bruggen

Abstract

Verticillium wilt is the cause of high losses in tree nurseries, because many tree species are susceptible and a symptomatic plant is a complete loss to the grower. It is important to be able to predict potential losses caused by pre-planting inoculum densities of *Verticillium dahliae*. Therefore, the relationship between verticillium wilt and soil inoculum densities of *V. dahliae* and *Pratylenchus fallax* was studied in two 4-year field experiments, in *Acer platanoides* and *Catalpa bignonioides*. Best fit regression equations showed that pre-planting inoculum densities of *V. dahliae* can be used to predict verticillium wilt in future years and that disease can occur at low inoculum densities. However, in some years, disease prediction was not significant, because disease levels were too low. The relationships between percentages of diseased plants (%) and detected pre-planting *V. dahliae* soil inoculum densities (colony forming units (CFU) g^{-1} soil), in years with high disease levels, were for *A. platanoides*: % = –0.1 + 2.85 (CFU g^{-1} soil), (R^2=0.92); and for *C. bignonioides*: % = 4.3 + 0.58 (CFU g^{-1} soil), (R^2=0.65). This implies that the 5% damage thresholds were at 1 (*C. bignonioides*) – 2 (*A. platanoides*) CFU g^{-1} soil. *P. fallax* contributed significantly to disease severity in *A. platanoides* in most experimental years, but usually only at low *V. dahliae* inoculum densities. Analysis of spatial relationships indicated that diseased plants sometimes had a higher range of influence (causing disease in neighbour plants) at low *V. dahliae* inoculum densities (<5 CFU g^{-1} soil) than at high densities (>5 CFU g^{-1} soil). This is an indication that *V. dahliae* microsclerotia occur clustered at low inoculum densities and scattered at high inoculum densities. Diseased plants often recovered from disease during the following year. After that year, recovered plants of each species at both locations, had a higher chance of becoming diseased again than plants that were healthy during the two previous years. At high inoculum densities (Wageningen location only), the higher disease chances of recovered *A. platanoides* and *C. bignonioides* plants were related to soil inoculum density of *V. dahliae*. This is an indication that inoculum density in the soil, rather than incomplete recovery, was the most important factor for disease chance.

Abbreviations: CFU – colony forming units; SEM – standard error of the mean; AUDPC – Area under the disease progress curve

Introduction

Verticillium wilt is caused by the soil-borne fungus *Verticillium dahliae* Kleb. and occurs in many crops all over the world. The disease can be a severe problem in many crops. Besides herbaceous plants, a large number of economically important woody species are susceptible, such as olive, avocado, stone fruits, pistachio, cocoa, and many ornamental tree species (Cirulli et al., 1998; Emechebe et al., 1971; Latorre & Allende, 1983; Paplomatas & Elena, 1998; Pegg & Brady, 2002, Sinclair et al., 1989). In herbaceous plants like potato, verticillium wilt may result in a relatively small amount of yield loss. In tree nurseries

however, wilt of trees implies a complete loss of value. Therefore, damage thresholds are low compared to those in other crops. Losses up to 50% have been reported (Anonymous, 1990).

Interactions of *V. dahliae* with plant pathogenic nematodes have been observed, e.g., in potato (Martin et al., 1982; Wheeler et al., 1994), and sugar maple (*A. saccharum*) (Dwinell & Sinclair, 1967). These nematodes often lower the damage threshold, and interactions have to be taken into account for reliable disease prediction.

Control of *V. dahliae* by chemicals is possible, but their use is restricted (Duniway, 2002) or forbidden (Mus & Huygen, 1992), because of potential environmental pollution and health risks. Alternative control methods to reduce the initial inoculum densities are available for application in greenhouses (Corsten et al., 2002), areas with a Mediterranean climate (Katan et al., 1976), and temperate zones (Blok et al., 2000), but they are costly. Therefore, reliable disease prediction is urgently needed to decide if these expensive management practices are warranted. This is especially true for high value plants like nursery trees. Disease prediction is possible by assessment of pre-planting inoculum densities of *V. dahliae* (Anonymous, 1997, Locke & Buck, 1997). Good relationships have been observed between soil inoculum densities and disease incidence in cauliflower (Xiao & Subbarao, 1998), cotton (Paplomatas et al., 1992; Pullman & DeVay, 1982), horseradish (Khan et al., 2000), tomato (Grogan et al., 1979), potato (Nicot & Rouse, 1987), and strawberry (Harris & Yang, 1990, 1996), also in naturally infested fields (e.g., Harris & Yang, 1990, 1996; Nicot & Rouse, 1987; Paplomatas et al., 1992). Inoculum levels of less than 1 colony forming units (CFU) g^{-1} soil can cause 5% (strawberry) or 3 – 10 % (cotton) disease incidence (Harris & Yang, 1990, 1996; Paplomatas et al., 1992; Pullman & DeVay, 1982) At inoculum levels of 5 CFU g^{-1} soil, disease incidence in cotton and potato can be 60 – 100% (Nicot & Rouse, 1987; Paplomatas et al., 1992) and in cauliflower 16 – 20% (Xiao & Subbarao, 1998). However, the harvested yield might be relatively unaffected (Paplomatas et al., 1992; Xiao & Subbarao, 1998). Until now there is a lack of quantitative information about inoculum density – disease incidence relationships and damage thresholds in trees.

Woody plant species differ from non-woody hosts in that they have the ability to recover from disease over time. Disease is overcome by confinement of the fungus to infected xylem vessels, and the formation of new, uninfected xylem tissue (Shigo, 1984). Even when severe die-back occurs, surviving plants may re-grow from the stem base. Especially ring-porous tree species (i.e., tree species with very large xylem vessels) like *Catalpa bignonioides*, are known to be able to recover from disease without severe die-back, because they depend mainly on newly formed xylem tissue for water and nutrient transport (Grosser, 1977; Hiemstra, 1998b; Shigo, 1984). However, recovery is not important for tree nurseries, since diseased trees are always removed. Nevertheless, it is an important aspect of verticillium wilt in established trees in gardens and orchards.

In practice, *V. dahliae* populations frequently occur aggregated through a field (Xiao et al., 1997) and this may affect distribution of disease incidence. Assessed inoculum densities are high when the inoculum is aggregated in several highly infested spots. In that case however, only a limited number of plants may be exposed to *V. dahliae*. Aggregation of nematode populations may contribute to the clumped distribution of verticillium wilt.

The objectives of this present study were: (1) to establish inoculum density – disease severity relationships and damage thresholds for young trees, taking nematode populations into account; (2) to establish the spatial pattern of verticillium wilt, and (3) to investigate the chance that recovered trees become diseased again during following years. Therefore we

followed disease development in 20 plots each at two locations for *Acer platanoides* and *Catalpa bignonioides* for a period of 4 years.

Materials and methods

Layout of field experiments

Two field experiments were performed, in Wageningen and in Meterik, the Netherlands. In Wageningen, the soil was naturally infested and in Meterik, the soil was artificially infested with *V. dahliae* (Chapter 5). Each field experiment consisted of 20 plots with different levels of *V. dahliae*. The 20 plots originated from a biological soil disinfestation experiment (Blok et al., 2000), performed in the summers of 1997 (Wageningen) and 1998 (Meterik). The plots with different biological soil disinfestation treatments did not differ in conduciveness to verticillium wilt (Chapter 5). In Wageningen, the root lesion nematode *Pratylenchus fallax* was present at various inoculum densities in the 20 plots. The nematode was identified after isolation from plant roots (Chapter 5; Seinhorst, 1977).

During the next spring, each plot was planted with 60 seedlings of *Acer platanoides* L. (Norway maple) and 60 seedlings of *Catalpa bignonioides* Walt. (southern catalpa). Soil inoculum densities of *V. dahliae* were assessed by wet plating (Harris et al., 1993) during the spring of each growing season (Chapter 5). In Wageningen, inoculum densities ranged from 0 – 50 CFU g^{-1} soil during the first year, and remained at a more or less constant level. In Meterik, inoculum densities ranged form 0 – 15 CFU g^{-1} soil, during the first year, dropped to about 0 – 10 CFU g^{-1} soil during the second year, and remained at a more or less constant level during the following years (Chapter 5). In Wageningen, pre-planting soil inoculum densities of *P. fallax* were measured (Oostenbrink, 1960). In Meterik, no nematodes were present. After the first two growing seasons, part of the plants was removed. In Wageningen, 20 plants and in Meterik 15 plants remained during the third and fourth year (Chapter 5). In Wageningen, the density of *P. fallax* nematodes in the roots was measured (Oostenbrink, 1960) during the first two years, in plants that were removed (Chapter 5). This was done because pre-planting soil inoculum densities of *P. fallax* were low and nematode populations are known to be able to increase rapidly to significant numbers in susceptible plant roots (Chapter 5).

Disease measurements

Wilt symptoms. Verticillium wilt severity was followed during four years, for each individual plant on a 0 - 5 scale (0 = healthy, 1 = wilt of several leaves (up to 5%); epinasty in *A. platanoides*, 2 = 5 - 40% wilt or defoliation, 3 = 40 - 60% wilt or defoliation, 4 = 60 - 80% wilt or defoliation, and 5 = more than 80% wilt or defoliation or plant dead). Area-under-the-disease-progress-curve (AUDPC) values (Campbell & Madden, 1990) were calculated per plot for each plant species. Dead plants or dead plant parts (in those that had recovered) of the previous year(s) were not included in the disease assessment of the following year(s). Recovery was scored, based on the presence or absence of disease. A plant was considered diseased during a particular year, when it received a maximum disease score of 2 or higher.

In Wageningen, after the first growing season, stem infection with *V. dahliae* was assessed for all 20 plants that were removed for each plant species and each plot, by plating stem base parts on modified soil extract agar medium (Chapter 5; Harris et al., 1993).

Data analysis

Inoculum density – disease relationships. Data were analysed with SAS version 8.0 (SAS Institute Inc., Cary, NC). The GLM procedure was used to calculate regression equations for AUDPC values or percentages of diseased or infected plants, in relation to *V. dahliae* inoculum densities in the soil and *P. fallax* inoculum densities in the soil or the roots. Soil inoculum densities of *V. dahliae* and *P. fallax* and numbers of *P. fallax* per plant root, were log-transformed and percentages of infected, diseased and healthy plants were arcsine-transformed, where appropriate.

Regression equations presented directly in the text, summarise the relations between the untransformed percentages of diseased plants (disease score 2 or higher) and the untransformed *V. dahliae* inoculum densities in the soil. For regression equations mentioned directly in the text, only the data of the first year in Wageningen were used, because disease levels and correlation coefficients were highest, and only the linear part of the curve was taken into account (including inoculum densities lower than 25 CFU g^{-1} soil). In this way it was possible to arrive at a worst-case prediction of damage thresholds.

Recovery. The FREQ procedure was used to perform Chi-square tests of numbers of diseased and healthy plants with different disease histories. Numbers of plants from years 3 and 4 with the same disease history were pooled, where appropriate. The REPEATED statement in the GENMOD procedure of SAS was used to perform a weighted regression of disease chance on pre-planting inoculum density of V. dahliae. During this analysis, the regression of numbers of diseased and healthy plants after recovery (diseased–healthy–diseased or diseased–healthy–healthy) on V. dahliae inoculum density was compared with the regression of numbers of diseased and healthy plants that were healthy during the two preceding years (healthy–healthy–diseased or healthy–healthy–healthy) on V. dahliae inoculum density.

Spatial aspects. Spatial aspects concerning diseased trees were analysed by calculating uni-directional semivariograms from the AUDPC values of year 1 of one row of 20 (Wageningen) and 30 (Meterik) plants per plant species. The semivariogram formula is: $\gamma(h) = c \times [(3h/2a) – (h^3/2a^3)]$ when $h<a$ and $\gamma(h) = a$ when $h>a$, where c is the maximum level (the horizontal part, or 'sill' of the curve), h is the distance (cm) between two plants, and a is the range of influence of a diseased plant (Clark, 1979). Semivariograms were calculated and plotted in Microsoft Excel version 97 SR-1 (Microsoft, Chicago, USA) and ranges and sills were determined graphically per plot for each plant species. The sills were log-transformed. The ranges and sills were analysed in two ways using the GLM procedure in SAS: they were related directly to *V. dahliae* inoculum densities and they were analysed for different *V. dahliae* inoculum density classes, viz. low and high inoculum densities (<5 and >5 CFU g^{-1} soil, respectively).

Table 1. Best fit regression models of annual AUDPC related to soil inoculum densities of *V. dahliae* (Wageningen and Meterik) and densities of *P. fallax* per g soil or root (Wageningen), as determined at the beginning of the experiment and annually in the spring.

ageningen						eterik		
platanoides								
Year	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil of year 1 (Vd) [1]	*P. fallax* g^{-1} soil (Pf) [2]	Interaction (int)	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil of year 1 (Vd) [1, 3]
1	0.95	-0.58	3.86 * Vd	39.3 * Pf	-2.67 * int	0.55	-9.45	48.7 * log (Vd + 1)
		0.93 [4]	<0.0001 [4]	0.0045	0.0068		0.51	0.0032
2	0.76	13.4	2.31 * Vd	67.2 * Pf	-2.49 *int	0.76	31.2	114.5 * log (Vd +1)
		0.33	0.032	0.0127	0.16		0.12	<0.0001
3	0.51	24.6	4.10 * Vd	35.2 * Pf	-3.75 * int	0.53	5.05	13.6 * log (Vd + 1)
		0.36	0.049	0.45	0.27		0.66	0.23
4	0.63	9.93	1.35 * Vd	3.22 * Pf	-	0.43	3.54	2.78 * Vd
		0.43	0.0067	0.86	ns [5]		0.85	0.12
Year	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil (Vd) [6]	*P. fallax* g^{-1} root (Pf) [7]	Interaction (int)	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil (Vd) [6, 3]
1	0.94	0.03	42.8 * log (Vd+1)	0.19 * Pf	0.19 * int	[8]		
		0.99	<0.0001	0.0070	0.0016			
2	0.62	21.4	36.2 * log (Vd+1)	-	-	0.50	24.8	132.6 * log (Vd + 1)
		0.17	0.0025	ns	ns		0.46	0.0040
3	0.50	33.8	1.64 * log (Vd+1)			0.51	10.43	12.0 * log (Vd + 1)
		0.12	0.0061	na [9]	na		0.28	0.36
4	0.72	15.2	1.61 * Vd			0.44	-0.66	4.49 * Vd
		0.077	0.0002	na	na		0.97	0.099
. *bignonioides*								
Year	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil of year 1 (Vd) [1]	*P. fallax* g^{-1} soil (Pf) [2]	Interaction (int)	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil of year 1 (Vd) [1, 3]
1	0.87	8.58	1.02 * Vd	15.5 * Pf	-3.50 * int	0.84	24.5	69.7 * log (Vd + 1)
		0.0017	<0.0001	0.18	0.0015		0.35	<0.0001
2	0.81	7.70	0.938 * Vd	10.1 * Pf	-2.46 * int	0.36	6.36	18.8 * log (Vd + 1)
		0.020	0.0011	0.51	0.053		0.58	0.11
3	0.53	18.5	0.807 * Vd	-	-	0.31	0.86	1.85 * Vd
		0.055	0.020	ns	ns		0.95	0.17
4	0.64	3.61	-0.344 * Vd	-8.17 * Pf	1.39	0.40	22.5	2.68 * Vd
		0.38	0.25	0.26	0.017		0.089	0.035
Year	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil (Vd) [6]	*P. fallax* g^{-1} root (Pf) [7]	Interaction (int)	R^2	Inter-cept	*V. dahliae* CFU g^{-1} soil (Vd) [6, 3]
1	0.84	7.47	0.88 * Vd	0.048 * Pf	.0096 * int	[8]		
		0.0049	0.0003	0.46	0.0073			
2	0.70	11.0	-0.60 * Vd	-3.96 * Pf	8.14 * int	0.34	5.14	19.6 * log (Vd + 1)
		0.042	0.0025	0.42	0.15		0.69	0.13
3	0.50	18.2	0.597 * Vd			0.24	9.17	-1.38 * Vd
		0.074	0.0331	na	na		0.28	0.35
4	0.25	5.80	0.348 * Vd			0.17	41.0	1.02 * log (Vd + 1)
		0.22	0.16	na	na		0.034	0.96

[1] Pre-planting *V. dahliae* soil inoculum density, assessed before year 1. [2] Pre-planting *Pratylenchus* soil inoculum density, assessed before year 1. [3] No nematodes present in Meterik; [4] *P*-value for significance of F-test. [5] not significant (model with highest R^2 did not contain this factor). [6] *V. dahliae* soil inoculum density assessed annually in spring each year. [7] *P. fallax* density g^{-1} root assessed annually in autumn each year. [8] Year 1 assessment is identical to pre-planting assessment. [9] not assessed.

Results

Inoculum density – disease severity relationships. Significant relationships between detected inoculum densities of *V. dahliae* in the soil and AUDPC values were observed in both plant species in most years (Table 1). In *A. platanoides* in Wageningen, numbers of *P. fallax* nematodes g^{-1} root made a significant ($P<0.05$) contribution to AUDPC in 1998. Both the additive term and the interaction term were positive and significant. However, in 1999, numbers of *P. fallax* nematodes g^{-1} root were low compared to 1998, and contributions were not significant (Table 1). In *C. bignonioides* in Wageningen in each year, the number of *P. fallax* nematodes g^{-1} root did not cause a significant increase of AUDPC. Fits of the regressions were best when nematode factors were included in the model, also when they were not significant (Table 1). In addition, fits were improved when AUDPC values of years 2 - 4 were related to pre-planting (year 1) soil inoculum densities of *V. dahliae* (Wageningen and Meterik) and *P. fallax* (Wageningen only), compared with annual inoculum densities (Table 1). In year 1 and 2, pre-planting soil inoculum densities of *P. fallax* contributed significantly to disease in *A. platanoides* (Table 1). Fits were independent of the weather during summer, with one exception for *A. platanoides* where *V. dahliae* severity was related to a low amount of global radiation (data not shown).

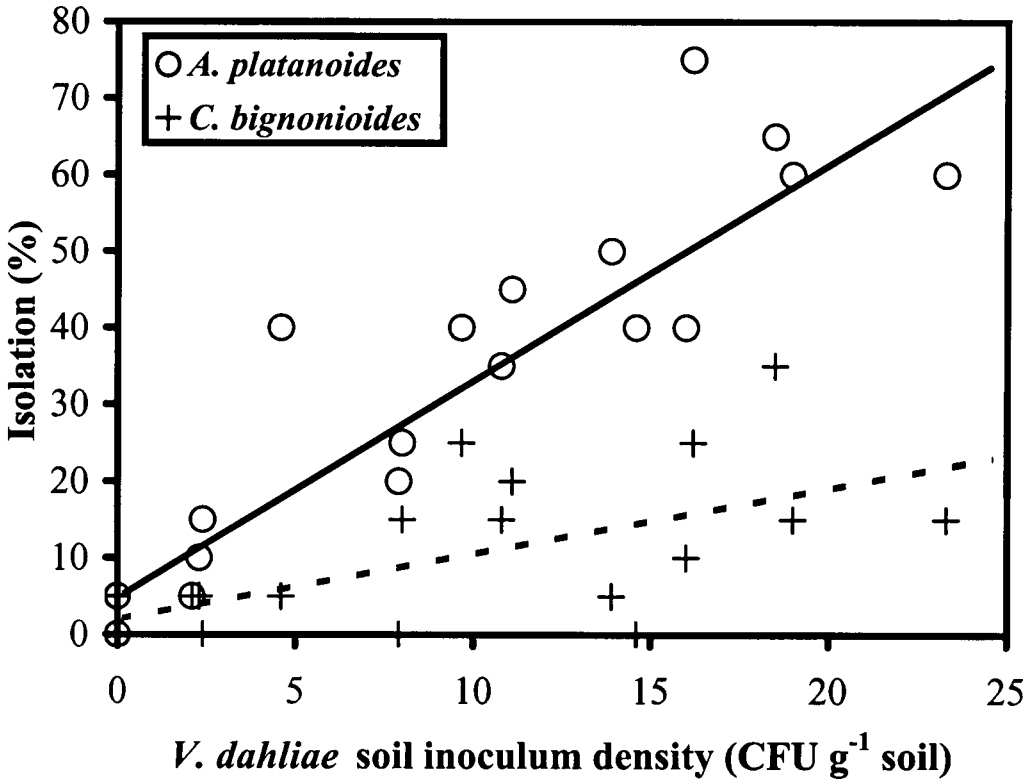

Figure 1. Percentages of isolation of *Verticillium dahliae* from the stem base of *Acer platanoides* and *Catalpa bignonioides* at Wageningen, year 1. The solid line is the regression line for *A. platanoides* (% = 5.6 + 2.9 * *V. dahliae* inoculum density; R^2= 0.83). The striped line is the regression line for *C. bignonioides* (%= 2.5 + 0.85 * *V. dahliae* inoculum density; R^2=0.36)

The worst-case regression equations based solely on pre-planting densities of *V. dahliae* (CFU g^{-1} soil) were: percentage of diseased *A. platanoides* plants = –0.1 + 2.85 (CFU g^{-1} soil) (R^2=0.92) and percentage of diseased *C. bignonioides* plants = 4.3 + 0.58 (CFU g^{-1} soil) (R^2=0.65).

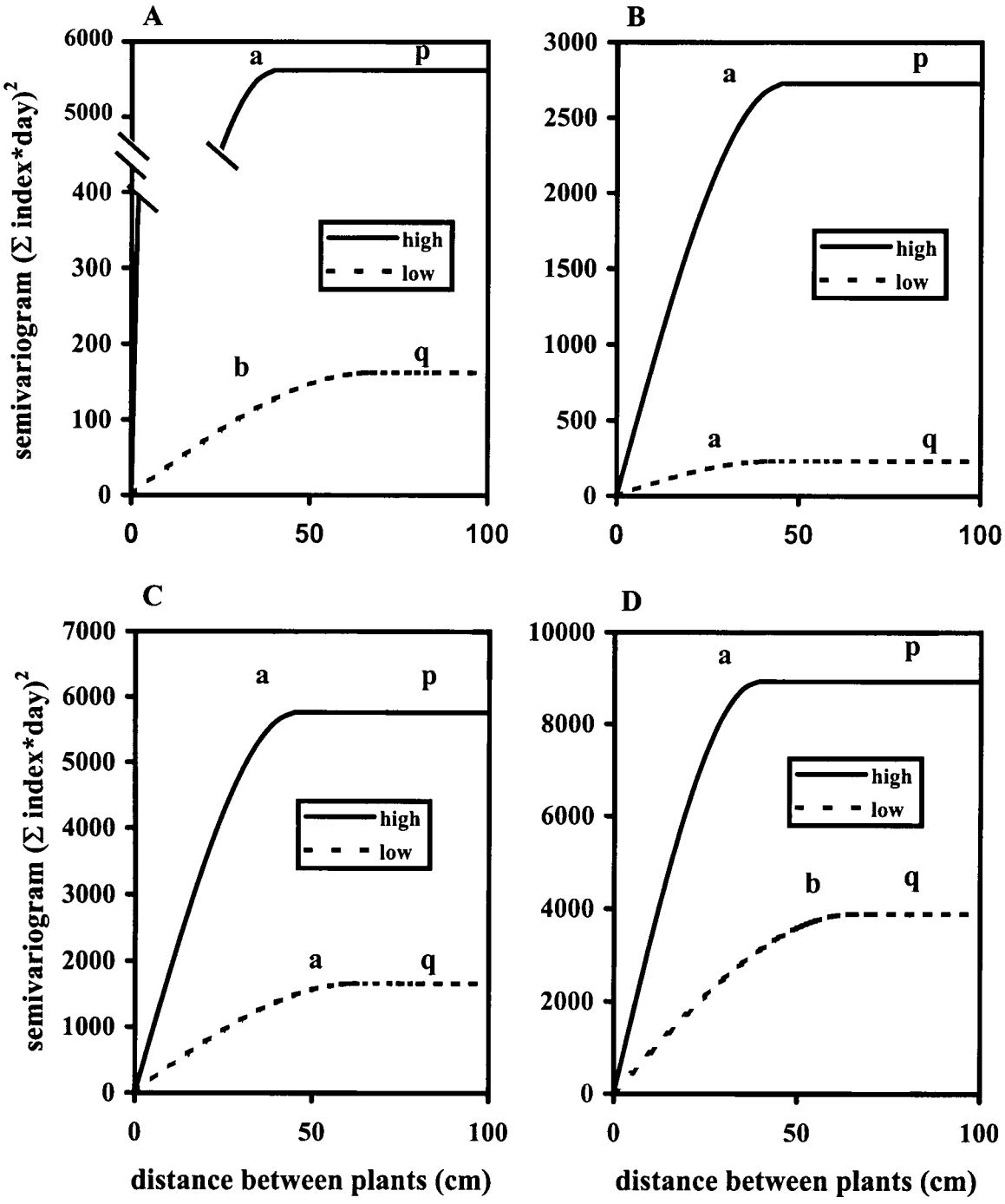

Figure 2. Pooled uni-directional semivariograms. A: *Acer platanoides* at Wageningen; B: *Catalpa bignonioides* at Wageningen; C: *A. platanoides* at Meterik; D: *C. bignonioides* at Meterik. Different letters indicate significant differences (P<0.05) for ranges (a or b) and significant differences (P<0.01) for sills (p or q).

Isolation and damage thresholds. Percentage of isolation of *V. dahliae* from the stem base of *A. platanoides* and *C. bignonioides* was dependent on pre-planting inoculum density of *V. dahliae* in the soil in each plant species ($P<0.01$) (Wageningen year 1 only) (Figure 1). Regression equations for isolation in relation to *V. dahliae* soil inoculum densities (Figure 1) were similar to the worst-case damage threshold given above. Numbers of *P. fallax* g^{-1} root of *A. platanoides* and *C. bignonioides* were dependent on pre-planting inoculum density of *P. fallax* in each year ($P<0.05$) (Wageningen only) (data not shown).

Spatial relations. Regressions of sills of semi-variograms (representing variability in disease) on pre-planting soil inoculum densities of *V. dahliae* were significant in both plant species at each location ($P<0.01$). Sills were significantly higher at high than at low inoculum densities of *V. dahliae*. Regressions of ranges of influence of diseased plants on pre-planting soil inoculum densities of *V. dahliae* were significant for *A. platanoides* in Wageningen and *C. bignonioides* in Meterik ($P<0.05$). Ranges were larger at low inoculum densities of *V. dahliae*. However, they were not significant for *C. bignonioides* in Wageningen and *A. platanoides* in Meterik (data not shown).

Analyses were confirmed by division of data into two *V. dahliae* infestation classes, viz. low (< 5 CFU g^{-1} soil) and high (>5 CFU g^{-1} soil). Sills were significantly higher at high than at low *V. dahliae* inoculum densities ($P<0.01$) (Figure 2). Ranges of influence of diseased plants were significantly higher at low than at high *V. dahliae* inoculum densities for *A. platanoides* in Wageningen and for *C. bignonioides* in Meterik ($P<0.05$). However, for *C. bignonioides* in Wageningen and *A. platanoides* in Meterik ranges were not significantly different (Figure 2).

Table 2. Numbers of plants that were healthy and diseased after being healthy during two consecutive years and after being recovered (diseased in one year and healthy in the following year).

Two years before One year before Assessment year [1]	healthy healthy healthy	healthy healthy diseased	diseased healthy healthy	diseased healthy diseased	Chi-square probability level [2]
Wageningen					
A. platanoides	361	38	70	39	<0.0001
C. bignonioides	623	39	49	12	<0.0001
Meterik					
A. platanoides	259	48	62	11	0.90
C. bignonioides	333	72	70	27	0.025

[1] Numbers of plants with different disease history were different.
[2] Significance of difference between the ratio of healthy–healthy–diseased / healthy–healthy–healthy and the ratio of diseased–healthy–diseased / diseased–healthy–healthy.

Recovery. Of each plant species, many trees recovered from disease during the following season. *A. platanoides* mainly re-grew from the stem base, whilst *C. bignonioides* mainly re-grew from the crown of the tree. A relatively larger number of diseased plants was observed in recovered plants (diseased–healthy) than in plants that were healthy during the two

previous years, except for *A. platanoides* in Meterik (Table 2). The number of times (one or two) that a tree was diseased before recovery occurred did not have a significant effect on disease chance in the fourth year for any of the plant species (data not shown). *A. platanoides* plants that remained healthy for one extra year after recovery (diseased– healthy– healthy in years 1, 2, and 3, respectively) had a significantly different disease chance than plants directly after recovery (diseased–healthy): in Wageningen the chance of becoming diseased was smaller and in Meterik it was larger (P<0.05) (data not shown).

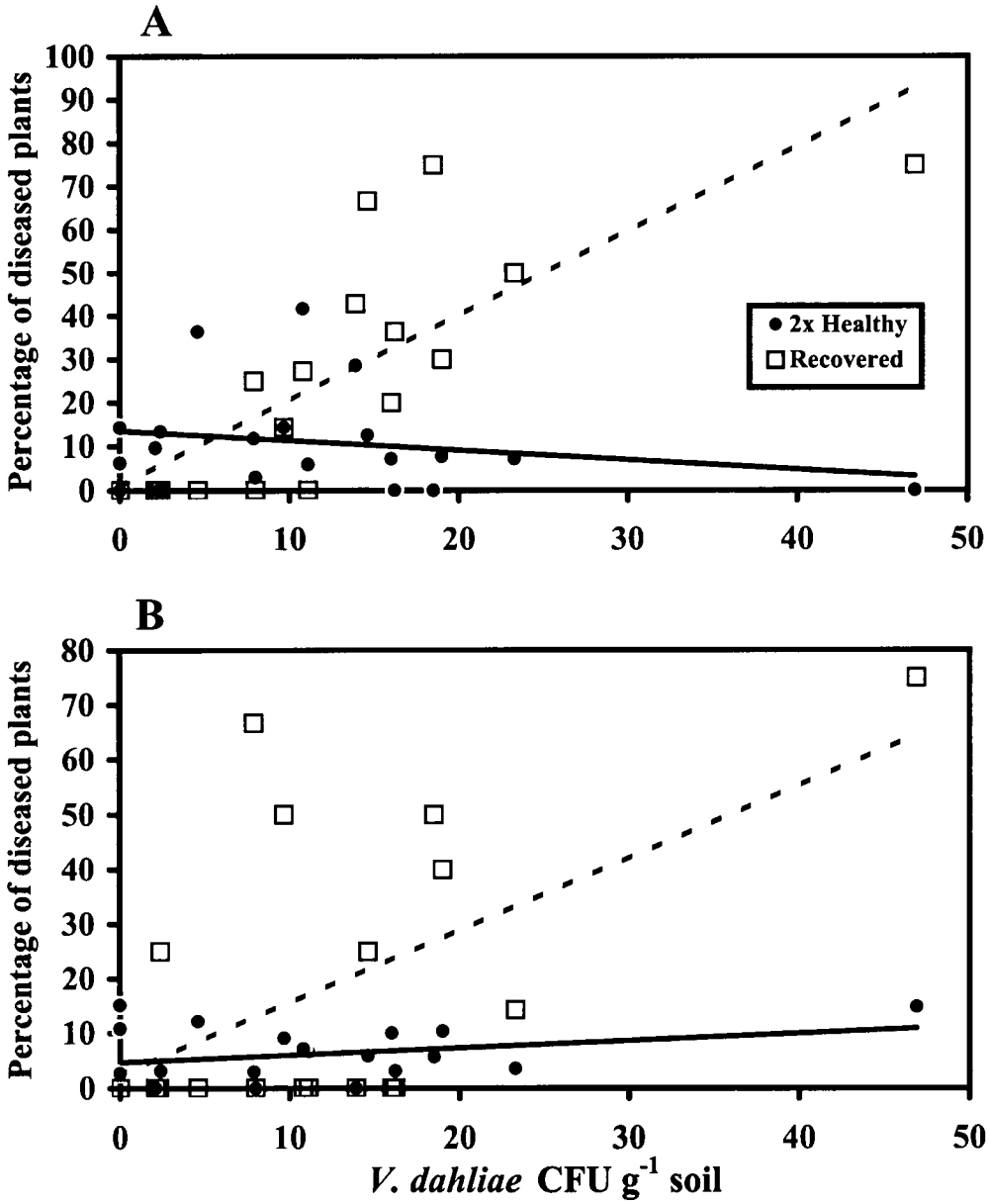

Figure 3. Percentages of diseased plants in the year after recovery (diseased–healthy–diseased) or after two consecutive years without disease (healthy–healthy–diseased), plotted against pre-planting *Verticillium dahliae* inoculum densities, at Wageningen. A: *Acer platanoides*; B: *Catalpa bignonioides*. □, percentage of disease in recovered plants; ●, percentage of disease after two consecutive years without disease; striped line, weighted regression line of disease in recovered plants on *V. dahliae* inoculum densities; solid line, weighted regression line of disease in plants that were healthy during the two previous years on *V. dahliae* inoculum densities. Regression lines were significantly different (P<0.05) for *A. platanoides* and *C. bignonioides*.

Percentages of disease in recovered plants were significantly correlated with pre-planting soil inoculum densities of *V. dahliae* for each plant species at Wageningen (P<0.05) (Figure 3). Disease percentages of plants that were healthy during the 2 previous years were used for comparison (Figure 3). The relationship between disease of recovered plants and *V. dahliae* soil inoculum density was significantly different from the relationship between disease in plants that were healthy during the 2 previous years (P<0.05) (Figure 3). At Meterik there were no significant relationships between disease chance and *V. dahliae* inoculum density (data not shown).

Discussion

Significant relationships were observed between disease severity (AUDPC) and soil inoculum densities of *V. dahliae* during most years. In years with low levels of disease, relationships were sometimes not significant. *V. dahliae* inoculum density usually was the most significant term in the best fit regression equation. *P. fallax* inoculum densities in the soil and in the roots were significant in year 1 and 2 for *A. platanoides*, but effects were not always clear. Most of the times, the additive term in the equation was positive, while the interaction term was negative. This indicates that *P. fallax* contributes to disease at low *V. dahliae* inoculum densities, but not at high *V. dahliae* inoculum densities. A lower damage threshold was also observed for other *Pratylenchus* species by Martin et al. (1982) in potato and Dwinell & Sinclair (1967) in sugar maple. Our results are in contradiction of those of McKinley & Talboys (1979), who found a higher effect of *P. penetrans* on verticillium wilt severity in strawberry at high *V. dahliae* densities, but not at low inoculum densities.

Damage thresholds based on disease (AUDPC) indicate that a visible amount of symptoms already occurred around detected inoculum levels of 1 or 0 CFU g^{-1} soil, thus, around the detection limit of the detection method. However, damage in trees is not directly related to disease severity (expressed in AUDPC), because the entire tree is lost commercially when disease symptoms are clear (disease score 2 or higher). Disease incidence is more suitable for assessment of damage thresholds. The observed damage thresholds for *A. platanoides* and *C. bignonioides* were below 2 CFU g^{-1} soil. This is low compared to cauliflower, cotton and potato, where disease incidence can be significant at such levels, however, without causing financial damage (Nicot & Rouse, 1987; Paplomatas et al., 1992; Pullman & DeVay, 1982; Xiao & Subbarao, 1998). It is comparable with the damage threshold of strawberry where 5% infection and wilt can occur below 2 CFU g^{-1} soil, causing significant yield losses (Harris & Yang, 1990, 1996). Verticillium wilt in commercial fields can vary to a large extent, because disease development is highly dependent on management practices (Davis & Everson, 1981). However, calculated damage thresholds can serve as worst-case scenarios. The observed 5% infection thresholds were 0 CFU g^{-1} soil (below the detection limit) for *A. platanoides* and 3.3 CFU g^{-1} soil for *C. bignonioides*, which are well in agreement with the damage thresholds.

Disease prediction was better at high inoculum densities (Wageningen) than at low inoculum densities (Meterik). At high inoculum densities, there usually were high disease levels. Multi-year effects, like recovery and especially death of plants, negatively affected fit of regression models. Better fits were obtained using pre-planting inoculum densities, also for

disease in years 2 – 4, compared with the densities of the yearly assessments. This indicates that disease prediction in trees remains valid for several years.

Many methods to quantify inoculum densities of *V. dahliae* exist (Termorshuizen et al., 1998). The wet plating method used in these current studies is a method with a low detection limit compared with most other methods (Chapter 2). However, each plating method for quantification of *V. dahliae* has the drawback of being soil-type dependent (Termorshuizen et al., 1998). This limits comparison of results with those for other soils.

The range of influence of a tree suffering from verticillium wilt was small (0 – 100 cm). This can be explained by the life cycle of *V. dahliae* (Chapter 1). After infection, the fungus mainly resides in the vascular tissue. Only when the infected plant tissue becomes moribund, microsclerotia are formed in the dying plant tissue, such as roots and petioles (Hiemstra, 1998a, 2000; Rijkers et al., 1992). Growth of *V. dahliae* through the soil is limited to the rhizosphere. *V. dahliae* spreads in soil mainly by colonising roots (Huisman, 1988). Microsclerotia in the soil are attached to soil particles and are mainly static. However, they can spread through the soil to some extent by earthworms (Hiemstra, 1998a; Rijkers et al., 1992), superficially by blowing dust, and by moving equipment and machinery.

Higher ranges of influence of a diseased plant at low *V. dahliae* soil inoculum densities were observed several times. This suggests that verticillium wilt occurred clustered when inoculum densities were low. In plots with low inoculum densities of *V. dahliae*, a local higher density is likely to have a relatively larger effect than in plots with high inoculum densities. Firstly, because at high inoculum densities, new inoculum production occurs throughout the field, and might not rise above the high background inoculum density. At low inoculum densities, infestation and new inoculum production are rare, and background densities are low. Secondly, a wilted plant does not compete well with surrounding plants with respect to nutrient uptake and light absorption. Thus, growth of surrounding plants is promoted, and directed towards the infected spot. This implies that it might be sensible for farmers to not only remove a wilted tree, but also to remove surrounding trees. However, this is a drastic and costly measure to prevent spread of *V. dahliae*. Alternatively, surrounding trees should be closely monitored for wilt symptoms. Root growth of surrounding trees into the infested spot should be minimised, e.g., by digging in a root barrier cloth and/or planting a non-host tree species on the infested spot.

The predominant observation was that recovered trees had a higher chance of becoming diseased again. This could be caused by the presence of original inoculum close to the tree (causing initial disease). The initial inoculum density seems to be the most important factor, since there was no difference between one or two times disease before recovery (and thus one or two times a possible inoculum build-up). Expansion of the root systems into previously unexplored infested areas is likely to occur both in recovered trees and in trees that were healthy all along.

Recovery from disease is known to occur in trees, as a result of confinement of *V. dahliae* to the infected xylem and the formation of new, uninfected, xylem vessels (Hiemstra, 1998b, Shigo, 1984). It usually occurs between growing seasons, when a new ring of vessels is produced also without disease (Grosser, 1977) but it is also observed within a growing season, e.g. as a result of high temperatures (Goud & Termorshuizen, 2002). However, the relationship with soil inoculum density was not investigated before. In Wageningen, high percentages of recovered plants became diseased during the next year, especially at high inoculum densities (above 15 CFU g^{-1} soil). In Meterik, disease in

recovered plants was not significantly related to inoculum density. The reason for this could be that inoculum densities above 15 CFU g^{-1} soil did not occur. This is an indication that disease after recovery is mainly caused by new infections of *V. dahliae* from the soil, and not by incomplete recovery.

Summarising, our results clearly demonstrate that verticillium wilt damage in tree nurseries can already occur at *V. dahliae* inoculum densities around the detection limit. Pre-planting soil inoculum densities of *V. dahliae* are highly indicative for disease during the following 1 – 4 years. Plant-pathogenic nematodes, like *P. fallax*, significantly contribute to disease severity at low *V. dahliae* inoculum densities. At low inoculum densities, diseased trees should be removed as quickly as possible, and growth of the surrounding trees into the infested spot should be avoided. Recovered trees have a higher chance of becoming diseased again, which is caused by build-up of the inoculum in the soil. Improvement of detection methods (especially molecular methods) is urgently needed. Relationships between inoculum densities and verticillium wilt in trees in commercial tree nurseries under different types of management and located on different soil types need to be further examined.

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Chapter 7

General discussion

The overall aim of this thesis was the prediction of verticillium wilt in tree hosts based on the detected inoculum level of microsclerotia of *V. dahliae* in the soil. Reliable insight in this relation is urgently needed by growers, to decide on the crops to be grown and on crop rotations. The relevance of this information is clearly related to the value of the crop involved, and thus highly significant for tree nursery growers. However, disease prediction is difficult for many reasons.

First of all, some problems are associated with the quality of the detection method itself, as described in Chapter 2. Using classical plating techniques, detection is known to depend on soil type (Termorshuizen et al., 1998). The reason for this general phenomenon may be that microsclerotia are quantified in the presence of many microbiota, because the media used are only semi-selective for *V. dahliae*. Since the soil microbial community varies in time and space and among soils, so is quantification of microsclerotia likely to vary. The influence of the microbial community in the semi-selective quantification plates probably is the reason that detection improves when the amount of soil added to the agar dish is reduced (Chapter 2). However, if the amount of soil per plate becomes too small, the detection limit is affected negatively. In woody hosts, *V. dahliae* can cause damage at, or below, the detection limit of 0.5 microsclerotia g^{-1} soil (Chapter 6), so there is a clear desire to reduce the detection limit. In Chapter 2, suggestions are presented for a better comparison of plating methods. Molecular quantification methods (Mahuku and Platt, 2002; Krishnamurthy et al., 2001) are more specific and much less dependent on the soil microbiota than plating methods. However, problems include the epidemiological interpretation of molecular data (which do not necessarily refer well to number of infectious propagules) and the detection of dead DNA. Nevertheless, molecular methods are highly promising alternatives to plating methods for the near future. The most important quality aspect of quantification methods is their disease predictive ability, but currently data are not available. The standard plating method used in this thesis resulted in good relationships between detected soil inoculum levels and verticillium wilt, in years conducive to verticillium wilt (Chapter 6). Therefore, the disease predictive ability of the traditional method is fairly good for woody hosts.

The second reason that disease prediction is difficult is that differentiation between *V. dahliae* and *V. tricorpus* on quantification media is cumbersome. In an interlaboratory study, Termorshuizen et al. (1998) concluded that part of the variation was due to improper recognition of these two species on semi-selective media. *V. tricorpus* is not known to cause disease in trees. Although not much is known about the dispersal of *V.* tricorpus, it has apparently a wide distribution since papers on this species appeared from China (Shang et al., 1998), South Africa (Uys et al., 1997), North and South America (e.g., Huisman, 1988), and Europe (Lamers and Termorshuizen, 1993, pers. obs.). Although *V. dahliae* and *V. tricorpus* are easy to differentiate when grown in pure culture condition, their morphology is different when grown on other media and in the presence of other organisms (Chapter 3). It is well known that the morphology of fungal cultures depends on growing conditions (Domsch et al., 1980), but it is peculiar that *V. dahliae* forms dark hyphae when grown on the ethanol semi-selective medium of Nadakavukaren and Horner (1959).

A third aspect of disease prediction deals with the variation within *V. dahliae*. In the Netherlands, two vegetative compatibility groups (VCGs) occur (Hiemstra and Rataj-Guranowska, 2000). Isolates of different VCGs are genetically separated from each other. In

Chapter 4, virulence of isolates belonging to each of the two VCGs was tested on a range of woody hosts. No overall differences were observed between the two VCGs. Therefore, determination of VCG does not contribute to disease prediction in woody hosts. However, considerable differences in virulence existed among isolates within VCGs: some isolates were highly virulent on some hosts and not on others, whereas other isolates were moderately virulent on all hosts tested. If differences in virulence are high, like is the case with the defoliating and non-defoliating pathotypes in cotton and olive (Jiménez-Díaz et al., 1998; Schnathorst and Sibbett, 1971), disease prediction becomes unreliable. Virulence of isolates to specific plant species can also occur in soils with a history of continuous monocropping: *V. dahliae* has been observed to be more virulent on its original host than on other hosts (e.g., Resende et al., 1994). Moreover, *V. dahliae* can become pathogenic to non-hosts after several passages through that non-host by forced inoculation (Fordyce and Green, 1963). For this reason, the cropping history of the soil must be known to improve disease prediction. If virulence differences between isolates are relatively small, disease prediction is reliable. In tree nurseries, usually a large number of susceptible and non-susceptible species are grown on each field, so specific isolate-to-species virulences are of minor importance. If disease prediction is needed for specific crops, e.g., continuous cropping of lilac (Hiemstra and Mouris, 2001), virulence of individual isolates becomes very important. In that case it would be relevant to study the genetic population structure of isolates of *V. dahliae*.

A general difficulty in predicting verticillium wilt in trees is that disease symptoms are more qualitative (presence or absence of wilt) than quantitative, although partial wilt can occur. Expression of symptoms seems best under circumstances that also favour plant growth, like a wide plant density (Chapter 5). Probably, more infections occur when growth is promoted, due to increased numbers of encounters between the root and microsclerotia of *V. dahliae*. Presence of plant pathogenic nematodes can increase verticillium wilt through additive and/or interactive effects (Chapter 6) (Kotcon et al., 1985; Martin et al., 1982), though *V. dahliae* inoculum densities remain the most important factor.

Finally, there are spatial and temporal aspects that influence disease. Chapter 6 describes that plants next to diseased plants have a higher chance of becoming diseased, indicating a clustered distribution of microsclerotia through the soil. Diseased woody plants may recover over time. For growers this is not relevant, because they would have removed the diseased tree. Nevertheless, if the tree is not removed, a recovered tree has a higher chance of becoming diseased again.

Despite the many difficulties and uncertainties, it was possible to describe inoculum density- disease severity relationships for Norway maple and southern catalpa in the presence and the absence of nematodes. *A. platanoides* showed 1% diseased plants at, or below the detection limit of 0.5 CFU g^{-1} soil, in the absence of nematodes. At low detected inoculum densities (1 CFU g^{-1} soil), 3% diseased plants can be expected. At higher inoculum densities, (5 CFU g^{-1} soil), 14% diseased plants can be expected. *C. bignonioides* showed 4% diseased plants at or below the detection limit of the method. At low *V. dahliae* inoculum densities, nematodes lowered the damage threshold for *A. platanoides*. These percentages are a rough, worst-case indication for weather conditions conducive to verticillium wilt.

Whenever much verticillium wilt is expected on the basis of high detected soil inoculum levels, growers have several alternatives: (1) go ahead as usual and take the risk of encountering high losses; (2) leave the soil, in the case that it is not owned by the grower; (3) grow non-susceptible crops only; (4) use resistant cultivars or rootstocks (Ruesink and Hiemstra, 2000; Hiemstra, 2001), or (5) apply biological soil disinfestation (Blok et al., 2000; Chapter 5). Most alternatives have the drawback of being costly or risky. Resistant rootstocks are likely to be available soon for *A. platanoides* (Hiemstra, 2001; Ruesink and Hiemstra, 2000). However, resistance is not absolute (Hiemstra, 2001) and might be ineffective in

highly infested soil. A solution could be to combine resistant rootstocks with biological soil disinfestation.

Selection for resistance in other tree species than *A. platanoides* is urgently needed. Selected clones should be tested under field circumstances for at least several years, to avoid plants escaping from infection (Hiemstra, 2001). Setting up breeding schemes is extremely time consuming in trees, but is needed to some extent. Disease prediction in woody hosts will probably always remain difficult, with respect to (unpredictable) weather conditions and the many other aspects that influence infection and disease development. Disease management by means of improvement of soil health needs to be further examined. Soil microbiota, physical soil characteristics, nematode densities and clustering of inoculum can, in principle, be measured, but often remain unknown. The influence of the soil microbiota on soil health and the relation of soil health with verticillium wilt need to be investigated. However, if the *V. dahliae* inoculum density is known, it is possible to arrive at a reliable worst-case disease prediction. This leaves quantification of *V. dahliae* in soil samples as the most important factor to improve in future research.

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Summary

The soil-borne fungus *Verticillium dahliae* is the causal agent of verticillium wilt in a large number of crops. In the Netherlands, susceptible species are e.g., potato, strawberry, chrysanthemum, rose, tomato and eggplant, and in other countries e.g., olive and cotton. Susceptible tree species are e.g., maple species, ash, catalpa, and linden. In tree nurseries, the financial damage can be considerable, because of the high value of the crop. Moreover, when soil is infested, a grower cannot grow susceptible plant species anymore, or has to find an alternative location.

Microsclerotia (diam. 0.05 mm), the surviving structures of *V. dahliae*, play an important role in its life cycle. Microsclerotia can survive in the soil for up to 10 years. Germination of the microsclerotia is induced when the root of a host plant passes, and *V. dahliae* can infect the root. After infection, the fungus colonises the vascular tissue and grows through the xylem vessels into the stem. There, the xylem is blocked, resulting in water stress, wilt, and plant death. Formation of toxins by the fungus can contribute to the development of disease symptoms. In dying tissue, new microsclerotia are formed.

Currently, no efficient methods to control *V. dahliae* are available for tree nurseries. Crop rotation with non-host cereals is successfully used to manage the disease. However, in tree nurseries, this is not economically feasible. Therefore, it is important to growers to get insight in the presence of *V. dahliae* in the soil, and the level of infestation, before the soil is rented or bought. Detection and quantification of microsclerotia of *V. dahliae* is usually done by plating soil on a semi-selective nutrient medium. This can be done using water (wet plating) or air (dry plating). Microsclerotia present in the soil sample, germinate and form colonies, which can be counted under a dissecting microscope. A large number of quantification methods exist, based on this principle. However, methods can differ significantly in many aspects (e.g., composition of the nutrient medium). Consequently, large differences exist in the detected amounts of microsclerotia. For this research, existing methods for quantification of *V. dahliae* were compared. The cause of the differences in performance among wet plating methods remained unclear. However, in this thesis it was demonstrated that the amount of soil plated per Petri dish was the most important factor among dry plating methods. Various modifications of the wet and dry plating methods were investigated in an attempt to improve performance of quantification methods. However, we were unsuccessful in developing a quantification method that was significantly better than existing methods.

Recognition of the *V. dahliae* colonies in the semi-selective detection medium can be hampered by the presence of another *Verticillium* species, viz., *V. tricorpus*. This species is harmless to trees. Misidentification could lead to erroneous conclusions about the infestation level of a field. Therefore, the morphology of both fungi was investigated on two semi-selective detection media. On the most widely used type of medium (pectate agar), *V. tricorpus* formed larger microsclerotia than *V. dahliae*, sometimes with dark hyphae attached to them. On the other semi-selective detection medium (ethanol agar), these characteristics were largely reversed. The abundance of dark hyphae of *V. dahliae* on ethanol agar was not described before. Discriminating morphological characteristics were successfully used to identify field isolates as *V. dahliae* or *V. tricorpus*. Species identification was confirmed by PCR with specific primers.

Although a sexual stage is absent in *V. dahliae*, and the fungus propagates by means of hyphal fusion, different isolates are not genetically uniform. With respect to this hyphal fusion, groups exist. These groups are referred to as vegetative compatibility groups (VCGs). Hyphal fusion can only occur between *V. dahliae* isolates within a VCG and not between

isolates of different VCGs. In literature, differences in virulence to certain crops are described between VCGs. In this thesis, these virulence differences were investigated for a range of woody ornamentals. No virulence differences existed between the two VCGs that occur in the Netherlands. Therefore, assessment of VCG is not needed during assessment of the soil inoculum density. However, there were large differences in virulence between individual isolates to certain plant species.

V. dahliae occurs throughout the Netherlands, and therefore, growers are often unable to find fields that are not infested. The question arises, as to which inoculum densities are still acceptable to the grower. In two field experiments, the relationships between soil inoculum densities and verticillium wilt in Norway maple and southern catalpa were investigated. The damage threshold (less than 5% diseased plants) was approx. 2 detected microsclerotia g^{-1} soil for Norway maple and 1 microsclerotium g^{-1} soil for southern catalpa. At inoculum densities of approximately 5 microsclerotia g^{-1} soil 7 (southern catalpa) to 14% (Norway maple) diseased plants can be expected. These percentages are indicative for years conducive to verticillium wilt. In years less conducive to verticillium wilt, damage in tree nurseries may be less. We discovered that presence of plant pathogenic nematodes lowered the damage threshold in Norway maple.

Wilted trees can recover from the disease during the next growing season. We observed that recovery occurred frequently, especially in southern catalpa. However, during following years, they had a higher chance of becoming diseased again. Moreover, neighbouring plants of diseased plants sometimes had a higher chance of becoming diseased. Thus, diseased trees need to be removed as quickly as possible to avoid build-up of the soil inoculum.

We investigated the effectiveness of biological soil disinfestation as a control method for *V. dahliae* in tree nurseries. Biological soil disinfestation involves the incorporation of fresh organic material (e.g., grass) through the soil combined with plastic mulching with airtight ensilage plastic, during 6-8 weeks in summer. As a result, soil inoculum densities of *V. dahliae* were reduced by 85%. Moreover, 99% of the plant pathogenic nematodes were killed. In high value crops, with a low damage threshold, such as nursery trees, biological soil disinfestation is a good method to prevent damage by *V. dahliae*.

Despite the advances made in this thesis, there still remain some problems to be solved. Research should focus on selection of resistant rootstocks, analysis of factors influencing crop health, the relationship between soil health and the occurrence of verticillium wilt, and the improvement of quantification of *V. dahliae* in the soil with molecular methods.

Samenvatting

De bodemschimmel *Verticillium dahliae* is de oorzaak van verticillium-verwelkingsziekte in een groot aantal gewassen, zoals in Nederland aardappel, aardbei, chrysant, roos, tomaat en aubergine en in het buitenland o.a. olijf en katoen. In de boomteelt treedt de ziekte op in o.a. esdoorn, es, trompetboom en linde. In deze kapitaalintensieve teelt kan de financiële schade erg groot zijn. Dit komt doordat, bij geconstateerde besmetting, een teler òf de teelt van zijn vatbare bomen moet staken, òf een nieuwe locatie moet zoeken.

De levenscyclus van *V. dahliae* wordt vooral gekenmerkt door microscopisch kleine structuren (diam. 0,05 mm), de microsclerotiën. Deze kunnen zo'n 10 jaar in de bodem overleven. Wanneer er een wortel van een waardplant langs een microsclerotium groeit, dan kiemt deze en kan de schimmel de plant infecteren. In de plant koloniseert de schimmel snel de houtvaten, waardoorheen zij zich naar de stengel verplaatst. Eenmaal in de stengel, verstopt de schimmel de houtvaten, waardoor de plant watergebrek krijgt, verwelkt en tenslotte sterft. De vorming van toxinen door de schimmel kan verder bijdragen aan de ziektesymptomen. In afstervend weefsel vormt de schimmel weer nieuwe microsclerotiën.

Momenteel bestaan er geen efficiënte bestrijdingsmethoden voor *V. dahliae* in de boomteelt. In de praktijk is vruchtwisseling met o.a. onvatbare graangewassen het meest gebruikelijk, maar voor boomtelers bedrijfseconomisch onhaalbaar. Voor de boomteelt is het belangrijk om vóór de verwerving van de grond te weten of, en in welke dichtheden, er een besmetting met microsclerotiën van *V. dahliae* aanwezig is. Het aantonen en kwantificeren van deze microsclerotiën geschiedt doorgaans door de grond nat (met behulp van water) of droog (met behulp van lucht) op een semi-selectief voedingsmedium te brengen. Microsclerotiën in het grondmonster groeien dan uit tot een kolonie en kunnen onder een prepareermicroscoop worden geteld. Er bestaan veel kwantificeringsmethoden die gebaseerd zijn op dit werkingsmechanisme, maar die in de uitwerking (bijv. samenstelling van het voedingsmedium) sterk kunnen verschillen. De hoeveelheid *V. dahliae* die wordt aangetoond met deze verschillende methoden loopt dan ook sterk uiteen. In dit proefschrift werden verschillende kwantificeringsmethoden vergeleken. De reden van de verschillen in uitkomst bij natte uitplaatmethoden is niet duidelijk geworden. De meest bepalende factor bij droge uitplaatmethoden bleek de hoeveelheid grond per petrischaal te zijn. Voorts werden er verscheidene aanpassingen aan de natte en droge uitplaatmethoden onderzocht. Het is vooralsnog niet gelukt om een betere dan de bestaande methode te ontwikkelen.

Het herkennen van kolonies van *V. dahliae* in het semi-selectieve uitplaatmedium kan sterk worden bemoeilijkt door de aanwezigheid van een andere *Verticillium*-soort, *V. tricorpus*. Deze soort is echter onschadelijk voor bomen. Bij verkeerde identificatie zouden verkeerde conclusies kunnen worden getrokken omtrent de besmetting van een veld. De morfologie van beide schimmels werd daarom vergeleken op twee semi-selectieve uitplaatmedia. Op het meest gangbare medium (pectaat-agar) vormde *V. tricorpus* microsclerotiën die iets groter zijn dan die van *V. dahliae*, en waaraan bovendien soms donkere hyfen (schimmeldraden) zaten. Op het andere semi-selectieve uitplaatmedium (ethanol-agar) waren deze kenmerken grotendeels omgekeerd. De overvloedige aanwezigheid van de donkere hyfen bij *V. dahliae*, zoals die op ethanol-agar aanwezig bleken te zijn, is niet eerder beschreven. De morfologische kenmerken werden met succes toegepast voor de determinatie van praktijkisolaten als *V. dahliae,* dan wel *V. tricorpus*. Determinaties werden bevestigd m.b.v. moleculaire methoden.

De genetische samenstelling van isolaten van *V. dahliae* is niet uniform, hoewel *V. dahliae* zich uitsluitend ongeslachtelijk voortplant door middel van versmelting van hyfen. Er bestaan met betrekking tot deze versmelting verschillende groepen. Deze groepen worden

vegetatief-compatibele groepen (VCG's) genoemd. Tussen isolaten van *V. dahliae* behorende tot dezelfde VCG kan versmelting plaatsvinden en tussen isolaten van verschillende VCG's niet. In de literatuur komen soms verschillen voor tussen VCG met betrekking tot virulentie (ziekteverwekkend vermogen) op bepaalde gewassen. In dit proefschrift werd bekeken of dit ook gold voor een reeks van boomkwekerijgewassen. Er bleken geen verschillen te bestaan tussen de twee in Nederland voorkomende VCG's: er hoeft bij de bepaling of een grond wel of niet besmet is met *V. dahliae* dus geen rekening gehouden te worden met welke VCG men te maken heeft. Wel bleken er grote virulentie-verschillen te zijn van individuele isolaten op bepaalde plantensoorten.

V. dahliae komt in Nederland zeer algemeen voor en het is dus voor een boomteler moeilijk om grond te vinden die geheel vrij is van deze schimmel. Als dit een gegeven is, wat is dan de maximale besmetting waar een boomteler genoegen mee mag nemen? Dit werd onderzocht in twee veldproeven waarbij de relatie werd vastgesteld tussen verwelkingsziekte in Noorse esdoorn en trompetboom en besmettingsgraad van de bodem. De schadedrempel (minder dan 5% zieke planten) bleek voor Noorse esdoorn ca. 2 gedetecteerde microsclerotiën per gram grond te zijn, en bij trompetboom ca. 1 microsclerotium per gram grond. Bij besmettingen van ca. 5 microsclerotiën per gram grond kan al 7 (trompetboom) tot 14% (Noorse esdoorn) zieke planten worden verwacht. De percentages zieke planten gelden echter alleen voor jaren waarin veel verticillium-verwelkingsziekte optreedt. In jaren met minder verwelkingsziekte kan de schade in de praktijk meevallen. Een complicerende factor is verder dat de aanwezigheid van plantenparasitaire nematoden (aaltjes) de schadedrempel verder kan verlagen.

Zieke bomen kunnen in het volgende seizoen van de ziekte herstellen. In de veldproeven werd herstel vaak waargenomen, vooral bij trompetboom. Echter, in jaren daarna hadden herstelde bomen een grotere kans om weer ziek te worden. Bovendien hadden buurplanten van zieke planten soms een verhoogd risico om ziek te worden. Zieke bomen dienen dus direct te worden verwijderd om opbouw van de besmetting in de grond tegen te gaan.

Voorts werd de effectiviteit van biologische grondontsmetting onderzocht als bestrijdingsmethode voor *V. dahliae* in de boomkwekerij. Bij biologische grondontsmetting wordt vers organisch materiaal (bijvoorbeeld gras) door de grond gemengd en wordt grond afgedekt met luchtdicht kuilplastic, gedurende zes tot acht weken in de zomer. Het resultaat was dat de besmettingsgraad van de bodem met *V. dahliae* met 85% werd verlaagd. Bovendien werd 99% van de plantenparasitaire nematoden gedood. In hoogrenderende gewassen met een lage schadedrempel, zoals boomkwekerijgewassen, is biologische grondontsmetting een goede methode om schade door *V. dahliae* te voorkomen.

Ondanks de voortgang die is geboekt in dit proefschrift, zal een aantal problemen m.b.t. verticillium verwelkingsziekte in de boomteelt in de nabije toekomst blijven bestaan. Vervolgonderzoek dient zich te richten op het selecteren van resistente onderstammen, analyse van factoren die van invloed zijn op gewasgezondheid, de relatie tussen bodemgezondheid en het optreden van verticillium verwelkingsziekte, en het verbeteren van het aantonen van *V. dahliae* in de bodem met moleculaire kwantificeringsmethoden.

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Curriculum vitae

Jan Cornelis Goud werd op 1 oktober 1968 geboren te Schore. Na het behalen van zijn VWO-diploma aan het Buys Ballot College te Goes in 1987, begon hij met de studie Plantenveredeling aan Wageningen Universiteit (toen nog Landbouwuniversiteit geheten). De studie omvatte 2 hoofdvakken, fytopathologie en plantenveredeling. Bij de vakgroep Fytopathologie werden resistentiefactoren van kool tegen de ringvlekkenziekte (*Mycosphaerella brassicicola*) onderzocht. Bij de vakgroep Plantenveredeling werd de ontstaanswijze van 2n-gameten in aardappel bestudeerd. Zijn stage aan het Everglades Research and Education Center in Belle Glade, Florida, richtte zich op de veredeling van bleekselderij, sla en suikermaïs. Na het behalen van zijn diploma, is hij in 1994 werkzaam geweest op het ATO-DLO in Wageningen. Hier is bewaaronderzoek gedaan aan bladgroenten, ten behoeve van het verlagen van het nitraatgehalte. In 1995 en 1996 zijn op *freelance*-basis werkzaamheden verricht voor Eucarpia (de Europese vereniging voor plantenveredelingsonderzoek) en de Stichting Stimulering Aardappelonderzoek. Voor laatstgenoemde instantie werd een literatuuroverzicht gemaakt over de veredelings-mogelijkheden tegen *Rhizoctonia solani* in aardappel. In 1997 is hij als AIO begonnen bij de vakgroep Fytopathologie van Wageningen Universiteit met het onderzoek dat in dit proefschrift beschreven is. Eind 1999 is het AIO-project voortgezet bij de leerstoelgroep Biologische Bedrijfssystemen van Wageningen Universiteit. Vanaf begin 2000 is hij bij deze leerstoelgroep werkzaam als 'pre-doc' onderzoeker in het Europese samenwerkingsproject '*Verticillium wilt in trees*'. Hierin wordt verder onderzoek verricht op het gebied van de detectie en ziektebeheersing.

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