Early decline of asparagus in the Netherlands:

etiology, epidemiology and management

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Abstract

Asparagus plants on fields cropped with asparagus before establish well but economic life of the crop is only half of that on fresh land. *Fusarium oxysporum* f.sp. *asparagi* was identified as the main cause of this early decline. Autotoxic compounds were detected in residues of asparagus roots even 11 years after the crop was finished but evidence for a role of these compounds in the etiology of the disease was not obtained. The pathogen was found on asparagus seed, on one-year-old crowns used as planting material and was isolated from soil, frequently even from fields without an asparagus history. In inoculation experiments, the pathogen caused severe root rot in asparagus, mild root rot occasionally in pea and lupin, and it colonized the roots of many plant species that did not show symptoms. Twenty-four isolates of the pathogen were assigned to 18 vegetative compatibility groups indicating the large genetical diversity of the population in the Netherlands. The potential of nonpathogenic isolates of *F. oxysporum* to reduce severity of Fusarium root rot was shown in growth chamber and greenhouse tests but not when plants were grown in the field for one year. A new method for reducing soil infestation with *Fusarium oxysporum* f.sp. *asparagi* and other soilborne pathogens was developed. It is based on induction of fermentative soil conditions. When fresh broccoli or grass was incorporated into soil that was subsequently irrigated and covered with plastic mulch, oxygen in soil was rapidly depleted and redox potential (Eh) reached values as low as -200 mV. After 15 weeks, populations of *F. oxysporum* f.sp. *asparagi*, *Rhizoctonia solani*, *Verticillium dahliae*, and *Globodera pallida* were strongly reduced in inoculum samples buried in soil demonstrating the potential of this approach to control a range of soilborne pathogens.

Stellingen bij het proefschrift

'Early decline of asparagus in the Netherlands: etiology, epidemiology and management'

1 De aanduiding 'herinplantziekte' kan voor asperge verwarring geven, is weinig informatief en dient vervangen te worden door de aanduiding '*Fusarium*-voet- en wortelrot'.
(Dit proefschrift)

2 De stelling van Schofield dat identificatie van autotoxinen uit aspergewortels prioriteit verdient bij het onderzoek naar de bestrijding van herinplantziekte is onjuist.
(P. Schofield, 1991, New Zealand J. Crop Hort. Sci 19:213-220; dit proefschrift)

3 De term 'forma specialis' dient afgeschaft te worden omdat de term geen recht doet aan de vaak grote variatie die er voor belangrijke kenmerken is binnen verschillende formae speciales, en een kennis van de waardplantenreeks suggereert die meestal niet waargemaakt kan worden.
(D.L. Hawksworth, 1994, pp. 93-105 in D.L. Hawksworth (ed.) The identification and characterization of pest organisms, CAB International, Wallingford)

4 Voor het samenstellen van vruchtopvolgingsschema's die tot doel hebben de overleving van pathogene bodemschimmels te minimaliseren gaat men meestal uit van de waardplantenreeks van een soort als *pathogeen*. Het is echter effectiever om uit te gaan van de waardplantenreeks als *parasiet*.

5 Voor de identificatie van potentieel succesvolle antagonisten van pathogene bodemschimmels is kennis van de ecologie van de antagonist met betrekking tot de micro-habitat, belangrijker dan kennis van het werkingsmechanisme.
(J. Deacon, 1991, Biocontrol Science and Technology 1:5-20)

6 Voor het ontwikkelen van producten voor de biologische bestrijding van pathogene bodemschimmels is het van belang dat een onderzoeker of onderzoeksgroep verantwoordelijkheid neemt voor het gehele traject van selectie van antagonisten tot formulering en opschaling van de productie van de antagonist.

7 Bij de bestrijding van pathogene bodemschimmels door middel van cultuurmaatregelen waarbij vers organisch materiaal wordt ingewerkt, wordt het belang van het optreden van anaërobe 'micro-sites' en de daarin optredende processen te weinig onderkend; beide aspecten verdienen nadere studie.

8 Manipulatie van de bodemmicroflora ter verhoging van het algemene ziektewerende vermogen van de grond is in veel gevallen te prefereren boven de introductie van een specifieke antagonist. Voorwaarde voor een effectieve manipulatie is dat meer kennis wordt verkregen van de dynamiek en functie van dat deel van de bodemmicroflora dat niet is te kweken.

9 Forma specialis *asparagi* neemt binnen *Fusarium oxysporum* een bijzondere plaats in omdat er voor deze forma specialis meer vegetatieve-compatibiliteitsgroepen zijn gevonden dan voor andere formae speciales. In phylogenetische studies van *F. oxysporum* dient dan ook meer aandacht aan deze forma specialis geschonken te worden.
(W.H. Elmer & C.T. Stephens, 1989, Phytopathology 79:88-93; dit proefschrift)

10 Het gebruik van computerbestanden voor het opsporen van wetenschappelijke literatuur heeft het gevaar in zich dat wordt voorbijgegaan aan de oudere, waardevolle literatuur, hetgeen kan leiden tot herhaling van onderzoek.

11 Bij het vaststellen van eisen aan en het beoordelen van de productiviteit van wetenschappelijk onderzoek verdient het de voorkeur om de leerstoelgroep als uitgangspunt te nemen. Individuele onderzoekers kunnen vervolgens beoordeeld worden op hun bijdrage aan het functioneren van hun leerstoelgroep. Terwijl voor het functioneren van een leerstoelgroep de kwaliteit en kwantiteit van wetenschappelijke publicaties een logische en belangrijke maat kan zijn, wordt individuele onderzoekers alleen recht gedaan als eveneens bijdragen welke niet in cijfers zijn uit te drukken in beschouwing worden genomen.

12 De sterke ontzuiling van de maatschappij sinds de 60-er jaren is niet zozeer toe te schrijven aan toegenomen tolerantie maar eerder aan een teloorgang van beginselen. Als zodanig is dit verschijnsel geen reden tot verheuging maar eerder tot verontrusting.

13 Stilte is een essentiële levensbehoefte van de mens. In die zin vormen radio en televisie in veler leven een bedreiging.
(R. Guardini, 1962, De gestalte der toekomst, Het Spectrum, Utrecht; J.H. van den Berg, 1963, Leven in meervoud, Callenbach, Nijkerk)

14 Wetenschappelijke kennis verschaft een beperkt inzicht in slechts een deel van de volle werkelijkheid. In dit licht bezien is het onverantwoord dat de algemeen heersende grondhouding ten aanzien van kennen en handelen in overheersende mate bepaald wordt door wetenschap en techniek.

15 De natuur stelt iets voor.

16 Naast vele verschillen vertonen de middeleeuwse mens en de moderne, westerse mens een treffende overeenkomst: beiden kennen een 'plat' wereldbeeld.

17 De wetenschapper die het bestaan van wonderen ontkent, overschrijdt de grenzen van de wetenschap.
(A. v.d. Beukel, 1990, De dingen hebben hun geheim, Ten Have, Baarn; C.S. Lewis, 1994, Wonderen, Van Wijnen, Franeker)

W.J. Blok
Wageningen, 5 december 1997

Ter nagedachtenis aan Arna

Voor Joanne
Voor Simon, Ard, Paul, Jonathan en Geerten

Voorwoord

Het is een bekend feit dat een aspergegewas op grond waar al eerder asperge werd geteeld lagere opbrengsten levert dan een gewas op grond zonder aspergeteelthistorie. In veel gevallen is de economische levensduur van het gewas bij herinplant slechts de helft van die bij teelt op verse grond. Dit verschijnsel staat in de praktijk bekend als herinplantziekte of bodemmoeheid. In de tachtiger jaren was in het belangrijkste teeltgebied van asperge in Nederland, het noorden van de provincie Limburg, herinplantziekte het belangrijkste probleem geworden. In dit gebied werd al gedurende tientallen jaren asperge geteeld en verse grond geschikt voor de teelt van asperge was zodanig schaars geworden dat veel telers werden gedwongen om nieuwe percelen aan te leggen op oud aspergeland. De oorzaak van herinplantziekte was onbekend en bestrijdingsmaatregelen waren niet voorhanden. Dit was voor het bestuur van de Stichting Proeftuin Noord-Limburg reden om Drs. G.J. Bollen te vragen onderzoek te verrichten naar de herinplantziekte. Er werd een vierjarig project geformuleerd waarmee ik aan de slag mocht. Het doel van dit project was het vinden van de oorzaak van de herinplantziekte. Na een succesvolle afronding van dit project volgde een driejarig project gericht op de epidemiologie en bestrijding van de ziekte. De voornaamste resultaten van beide projecten zijn in dit proefschrift beschreven.

Bij het gereedkomen van dit proefschrift wil ik graag hartelijk danken alle mensen die, op welke wijze dan ook, een bijdrage hebben geleverd aan het totstandkomen ervan. Een aantal mensen wil ik persoonlijk noemen.

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In het promovendigroepje van de onderzoekschool Productie Ecologie onder leiding van Aad Termorshuizen werden vele leerzame discussies gevoerd over manuscripten van de leden. Prof. Zadoks dank ik voor het kritisch doornemen van de eerste twee manuscripten. Gerrit Gort van de vakgroep Wiskunde dank ik voor zijn waardevolle adviezen met betrekking tot de statistische verwerking van de proefgegevens. De mensen van de kassendienst van het Binnenhavencomplex dank ik voor de verzorging van de

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Wim Blok
Randwijk, 1 oktober 1997

Contents

Chapter 1

General introduction

Early decline of asparagus crops in the Netherlands is the subject of this thesis. In this chapter some background information to the subject is provided. The first section describes asparagus production in the Netherlands. In the next two sections the phenomenon of early decline is introduced and the objectives of and approach to the study are outlined.

Asparagus production in the Netherlands

Asparagus (*Asparagus officinalis* L.) is a perennial vegetable crop belonging to the Liliaceae. It has a long history as a vegetable cultivated for its young stems. Some 4700 years ago asparagus was cultivated. Pictures with bundles of asparagus stems were discovered in Egyptian pyramids from about 2700 B.C. The Romans developed its cultivation and several handbooks are known from this period. From the Mediterranean the cultivation of asparagus was spread to Western Europe. Records of the cultivation of asparagus are found in several official documents and gardeners handbooks from the sixteenth and seventeenth century, originating from France, Germany and the Netherlands. In the nineteenth century asparagus was produced in the Netherlands at various, relatively small production centres along the coast on the sandy soils behind the dunes. At the end of the nineteenth century the area around the city of Bergen op Zoom in the province of Noord-Brabant became the major production centre (Geldof, 1969). After the first World War asparagus production developed in the northern part of the province of Limburg and this region took over the position of the other production centres completely and holds its position until now. The area under asparagus production was 1266 ha in 1950, increased to a maximum of 5054 ha in 1964 and decreased gradually to an area of 2000 to 2500 ha in recent years with about 80% of the area in the northern part of the province of Limburg and the remaining 20% in the adjacent province of Noord-Brabant.

In the Netherlands, asparagus is typically grown on sands or loamy sands of good agricultural quality. A production field is started by planting one-year-old plants (crowns) of almost exclusively Dutch cultivars. In the second year after planting, plants are harvested for only a few weeks. From the third or fourth year on, plants are harvested from the emergence of the first spears (late April to the beginning of May) till the 24th of June. Maximum yields are obtained in the fifth to seventh year after planting and amount to typically 7000 to 10000 kg of marketable product per ha. The spears are traditionally

harvested white. From the early nineties, green asparagus has been produced on a very limited scale, mainly on the heavier soils outside the main production centre.

Major pests and diseases in Dutch asparagus production are early decline of asparagus crops planted on former asparagus land, asparagus fly (*Platyparea poeciloptera* Schrank), asparagus beetles (*Crioceris duodecimpuntata* L. and *C. asparagi* L.), and purple spot (*Stemphylium vesicarium* (Wallr.) Simmons). The early decline, also known as replant disease, has become the major problem in the southeastern part of the Netherlands, the main production centre.

Early decline

The problem

On fresh land, asparagus production is highest in the fifth to seventh year. Then the production decreases gradually each year till a level is reached at which it is no longer profitable to maintain the crop, which is typically after 10 or 11 years under Dutch conditions. This period is satisfactory from an economical point of view. An extra argument to abandon a crop after about ten years is that after such a period new varieties, superior in quality and yield to the old varieties, have become available.

When asparagus is planted on former asparagus land, crop establishment is normally as good as on fresh land for Dutch crops. However, after only a few years with normal harvests, crop growth declines, spear diameter and number decrease, and each year an increasing number of plants die showing a poor root system with most of the secondary roots collapsed. As a result, annual yields decrease much earlier than on fresh land. This phenomenon, called replant disease or soil sickness by the farmers, is encountered hardly without exception and results in an economic life that is typically five to six years only. In first instance the phenomenon was called *replant disease* (Chapter 2) but later it was realized that this can be misleading as it suggests problems with crop establishment, as occurs with apple replant disease (Hoestra, 1968). The term *early decline* better describes the phenomenon.

Early decline has been reported from most of the major asparagus growing regions (Grogan & Kimble, 1959; Gindrat *et al.*, 1984; Young, 1984; Schofield, 1991; Schreuder *et al.*, 1995; Elmer *et al.*, 1996). In many reports it is mentioned that early decline occurs in asparagus crops on fresh land and that crop establishment is not successful when asparagus is planted in fields where plantings have declined. In the Netherlands, the situation is different in that early decline is confined to replanted crops and that problems with crop establishment rarely occur. Therefore the phenomenon was termed *replant-bound early decline* to tentatively distinguish it from early decline reported from other regions.

The causal factor of replant-bound early decline is very persistent (Van Bakel and Kerstens, 1970). Even after 25 years, replanting is not as profitable as planting on fresh soil (Boonen *et al.*, 1977; Poll and Huiskamp, 1992). In many areas with an important asparagus industry and a limited amount of fresh soil suitable for asparagus production, early decline has caused the area planted with asparagus to decrease substantially (Hartung, 1987; Elmer *et al.*, 1996). It is the main problem of the growers in the traditional asparagus-producing area in the southeast of the Netherlands.

Etiology

In most studies early decline of crops on former asparagus land has not been distinguished from that on fresh land and is regarded as one and the same phenomenon. Although a large number of biotic or abiotic factors can contribute to an early decline of the asparagus crop, most researchers mention soilborne fungal diseases as the major causal factor (Schofield, 1991; Elmer *et al.*, 1996). Besides these diseases, autotoxic compounds have received considerable attention as a causal factor in early decline (Laufer and Garrison, 1977; Hartung *et al.*, 1989).

Fusarium oxysporum (Schlecht.) emend. Snyd. & Hans. f.sp. *asparagi* Cohen and *F. moniliforme* Sheld. are primarily reported as being responsible for early decline (Cohen and Heald, 1941; Grogan & Kimble, 1959; Endo and Burkholder; 1971; Johnston *et al.*, 1979; Gindrat *et al.*, 1984). Both pathogens cause crown and root rot of asparagus and can be isolated from roots, crowns and stems. *F. moniliforme* has been taxonomically separated into several different species of which *F. proliferatum* (Mats.) Nirenb. was identified as being mainly responsible for attack of asparagus (Nelson *et al.*, 1983; Elmer *et al.*, 1996). *F. oxysporum* f.sp. *asparagi* was found to predominate in young stands, whereas *F. moniliforme* was the dominant colonist in older stands (Johnston *et al.*, 1979; Damicone and Manning, 1985).

In Europe, besides *Fusarium* spp., *Rhizoctonia violacea* Tul. (Gindrat *et al.*, 1984; Molot and Lombard, 1986) and *F. culmorum* (W.G. Sm.) Sacc. (Weise, 1939; Van den Broeck and Van Assche, 1966; Gindrat *et al.*, 1984) were also associated with declining asparagus crops.

In the Netherlands, Van Bakel and Kerstens (1970) distinguished four soilborne diseases, foot rot being one of them. After an extensive analysis of fungi present on stem bases of plants with foot rot symptoms originating from more than 100 different asparagus fields, they concluded that foot rot was caused by *F. oxysporum* f.sp. *asparagi*. They did not isolate other pathogens from bases of stems with foot rot symptoms. They mentioned a strong decline in plant growth when fields had been replanted with asparagus and called the phenomenon soil sickness. The symptoms were the same as described above for replant-bound early decline. The authors mention that the cause is not known but factors involved might include depth of rooting, exhaustion of minor elements, a poor soil

structure and attack by pathogenic fungi including *Fusarium* spp..

In addition to pathogenic fungi, allelopathic compounds present in asparagus roots have been suggested to be involved in early decline. Laufer and Garrison (1977) were the first to report toxicity of asparagus tissue to seedlings of asparagus and other vegetable crops. Yang (1982) showed that extracts of both field-grown and tissue-cultured plants inhibited root and shoot growth of asparagus seedlings grown in growth pouches, and that the autotoxic compounds are heat-stable and water-soluble. Growth inhibition of asparagus seedlings by soil-incorporated asparagus root tissue was reported by several authors (Hartung and Putnam, 1986; Shafer and Garrison, 1986). In the search for the active compounds in root extracts, various toxic compounds were isolated including methylenedioxycinnamic acid (Hartung, Nair and Putnam, 1990), caffeic acid (Miller *et al.*, 1991), and tryptophan (Lake *et al.*, 1993). However, each single compound could account for only part of the toxicity of the extract which suggests that more than one compound is involved. In addition to direct growth inhibition, asparagus root tissue was also found to cause an increase in root rot caused by *F. oxysporum* f.sp. *asparagi* and *F. moniliforme* (Hartung and Stephens, 1983; Hartung *et al.*, 1989; Peirce and Colby, 1987). Hartung *et al.* (1989) provided data suggesting that water-soluble compounds from asparagus roots predispose asparagus roots to infection by *Fusarium* spp. by causing physiological and biochemical changes. Hartung and Stephens (1983) and Hartung (1987) found indications that asparagus root tissue selectively affects the soil microflora as root extracts inhibited the *in vitro* growth of *Pythium* spp. and *Bacillus* spp., but not that of *F. oxysporum* f.sp. *asparagi*, *F. moniliforme*, and *Rhizoctonia solani*.

Several authors pointed out that fusarium crown and root rot is a stress-related disease. Any factor causing plant stress was found to increase the incidence and severity of the disease, thus limiting economic production and longevity of the crop. Recognized stress factors, in addition to autotoxic compounds mentioned already, are virus diseases (Evans and Stephens, 1989), damage by insects (Damicone *et al.*, 1987), fungal diseases including rust, purple spot, and Cercospora blight (Elmer *et al.*, 1996), and poor soil structure (Stahl, 1970).

Management

Currently, methods that effectively control early decline are not available to the farmer. Means for reducing damage of early decline have been reviewed recently by Schofield (1991) and Elmer *et al.* (1996). They are directed mainly against fusaria, and include the use of resistant or tolerant varieties, chemical control, cultural practices and biological control.

Despite extensive screening of cultivars and breeding lines of *A. officinalis* for resistance to *F. oxysporum* f.sp. *asparagi* or *F. moniliforme*, true resistance was not found in this species (Stephens *et al.*, 1989). The resistance of *A. densiflorus* 'Sprengeri' and

'Myersii' cannot easily be transferred to *A. officinalis* because the species are sexually incompatible. The greater tolerance or field resistance that is reported for the modern varieties, especially the all-male ones, is the result of increased vigour rather than true genetic resistance.

Chemicals are used to treat seeds and planting material, and to disinfest the soil. Eradication of pathogenic fusaria from seed is possible with a high concentration of benomyl in acetone (Damicone *et al.*, 1981). Soil fumigation and preplant crown soaks can be effective in situations with a high soil infestation. They can give a short-term protection thus ensuring that the crop becomes established (Manning and Vardaro, 1977; Lacy, 1979; Lill and Tate, 1982; Di Lenna *et al.*, 1988). For the delay of early decline, however, chemicals are of limited value due to the perennial nature of the crop and its deep rooting.

Cultural practices successful in managing fusarium crown and root rot include the use of fresh soil when available and prevention of plant stress by proper control of weeds and diseases (Elmer *et al.*, 1996). Recently, Elmer (1992) reported on the application of NaCl (common rock salt) on asparagus beds. Spring applications of 560-1120 kg NaCl ha^{-1} suppressed fusarium crown and root rot, retarded decline and increased marketable yields. Currently, rock salt is tested for its efficacy in the field by commercial growers (Elmer *et al.*, 1996).

The potential for the use of antagonistic organisms to protect asparagus plants has been tested on a limited scale only. Damicone and Manning (1982) could reduce fusarium crown rot on plants grown in the field for 8 weeks by application of a nonpathogenic isolate of *F. oxysporum*.

About this thesis

Background of the research

Growers are well aware that producing asparagus on land that has been planted to asparagus before is always less profitable than production on land virgin to asparagus. As mentioned earlier, the southeastern part of the Netherlands has been the major production centre for several decades. As a result, fresh land suitable for asparagus production has become more and more limited in this region, which means that growers can no longer avoid early decline by using fresh soil but are forced to replant former asparagus land if they want to stay in asparagus. Currently, some farmers have replanted several times with a further decline in longevity of the crop each time. Early decline is, together with a shortage in manpower in the harvest period, the main reason for the decline in the area under asparagus production.

In the seventies, experiments were conducted on the experimental farm 'Noord-

Limburg' aiming at destruction of the causal factor by chemical soil disinfestation. However, these treatments had either no or only a short-lived effect (P. Boonen, personal communication).

The increasing importance of early decline in the major production centre and the absence of a means to control it prompted the board of the Regional Research Centre Noord-Limburg at Horst, to initiate the research described in this thesis.

Objectives, approach, and outline of the thesis

The ultimate request of the farmers is an effective management strategy for replant-bound early decline. However, a prerequisite for developing a management strategy is knowledge of the main causal factors. The etiology of replant-bound early decline was not understood as we had reasons to tentatively distinguish the early decline of Dutch plantings from that in most other countries. In the Netherlands, early decline is only found in replant situations and *F. moniliforme*, recognized as a main causal organism of early decline in other countries, had not been isolated from Dutch plantings (Van Bakel and Kerstens, 1970). Furthermore, *F. oxysporum* f.sp. *asparagi*, the other pathogen reported as a main causal organism of early decline, was found in plantings on both fresh and asparagus land, whereas early decline occurs only in the latter. Therefore, the first objective was to determine the causal factor(s).

It was hypothesized that the factors mentioned in Figure 1 might be involved as main causal factors.

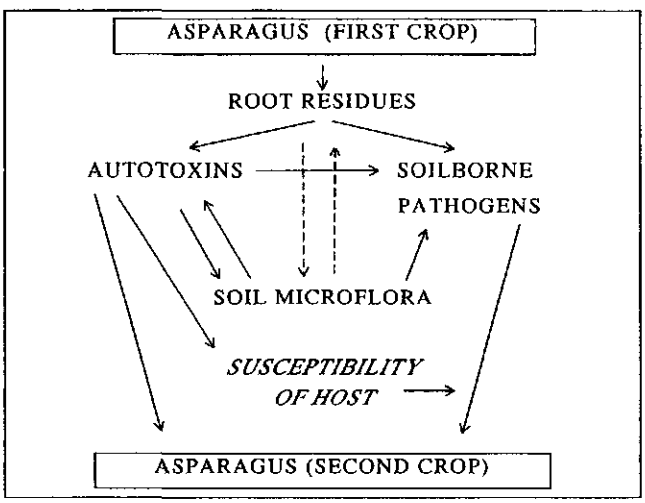


Figure 1. Factors and their effects that might be involved in the etiology of early decline of asparagus in replanted fields.

Soilborne pathogens and autotoxins can both directly cause damage to the asparagus plant. In addition to a direct action, the autotoxins might also indirectly influence plant growth by increasing disease severity. First, by increasing the susceptibility of the asparagus plant to soilborne pathogens, and second, by reducing antagonism of the soil microflora to soilborne pathogens.

The following topics were studied:
1. the potential of autotoxic compounds from asparagus root residues left over in soil from the first crop as a direct cause of growth inhibition in the next asparagus crop (Chapter 2)
2. an analysis of soilborne pathogens on roots and stem bases from plants growing in fresh soil or asparagus soil (Chapter 3)
3. the relative contribution of autotoxicity and pathogens to replant-bound early decline as reproduced in pot experiments (Chapter 4)
4. the role of indirect effects of autotoxins viz. predisposing the plant to infection by *F. oxysporum* f.sp. *asparagi*, and their effects on the soil microflora (Chapter 5).

The main conclusion from the etiological studies was that *F. oxysporum* f.sp. *asparagi* is the major causal factor in replant-bound early decline in Dutch asparagus plantings. A second objective was then to study those aspects of the epidemiology of the disease that are relevant to the development of control practices. The aspects studied were:
1. major inoculum sources in Dutch asparagus production (Chapter 6)
2. host specificity and vegetative compatibility of Dutch isolates of *F. oxysporum* f.sp. *asparagi* (Chapter 7).

The objective of the last part of the project was devoted to the management of early decline, including both the evaluation of existing methods for their efficacy and the development of new methods. Firstly, the potential of biological control by application of nonpathogenic isolates of *F. oxysporum* was studied (Chapter 8). Secondly, a new method involving the incorporation of easily decomposable plant material and plastic mulching, was developed and its potential to control various soilborne pathogens, including *F. oxysporum* f.sp. *asparagi*, was investigated (Chapter 9).

Chapter 2

The role of autotoxins from root residues of the previous crop in the replant disease of asparagus

Abstract

Replant disease is a common phenomenon in asparagus growing in The Netherlands. It is distinct from the decline phenomenon reported from many other asparagus producing areas. The involvement of autotoxins from root residues of former asparagus crops was evaluated. Residues of asparagus roots decompose extremely slowly. At two locations, each with fields where asparagus production was terminated one and ten years before, biomass of root residues was 4180 and 11060 kg dw ha^{-1} after one year and 420 and 1140 kg dw ha^{-1} after ten years. Although ten-year-old residues were for the greater part decomposed, crude aqueous extracts inhibited root growth of asparagus seedlings significantly and even more of garden cress. In root observation boxes with field soil mixed with non-sterilized or sterilized asparagus root fragments, growth of secondary roots was inhibited. Non-sterilized strawberry roots did not inhibit root growth, suggesting that effects of organic matter were not involved. In a pot experiment, sterilized asparagus root fragments inhibited root growth when added at a rate of 20 g l^{-1}, but not at 2 g l^{-1}. Addition of non-sterilized root fragments strongly inhibited root growth at both levels. This was probably due to simultaneous infection by *Fusarium oxysporum* present in these residues. When an asparagus field is replanted, the amount of root residues left behind in soil after termination of the crop in the previous season is about 2 g dw l^{-1}, that corresponds to approx. 11000 kg dw ha^{-1}. This level is too low for considering direct growth inhibition by autotoxins as a major factor. Their possible indirect effects are briefly discussed.

Introduction

In the Netherlands, asparagus (*Asparagus officinalis* L.) is grown on an area of about 3000 ha. For optimal growth asparagus requires a soil in which it can easily root to a depth of one meter or more. The ground water table should be lower than one meter but at the same time water supply should meet the relatively high water demand of the crop. The production of white asparagus, which is by far the most important type in The Netherlands, requires sandy soils. In the asparagus producing area, many growers do not have land available without an asparagus history that meets these requirements. Therefore, replanting is a common practise. Replanting never gives a crop which is as commercially profitable as a crop on fresh land. In most cases, establishing the crop on asparagus land rarely causes problems. However, the economical life of a crop on replanted fields averages only half of that of a crop on fresh land. After a few years with normal yields, crop growth declines and consequently yield and diameter of harvested spears gradually decrease. The root system is poorly developed and has many dead secondary roots. Within a few years, continuation of the crop is no longer

profitable. Replant disease is currently, next to problems with the supply of labour, one of the major reasons that the area under asparagus production can not be increased.

Replant disease is reported from many asparagus growing regions (Grogan and Kimble, 1959; Young, 1984; Hartung, 1987; Schofield, 1991). A remarkable feature is the persistence of the causal factor(s). Replanting, even after 20-25 years, never is as profitable as planting on fresh land (Boonen *et al.*, 1977).

In most cases, replant disease is considered in direct relation to an early decline of the preceding asparagus crop. Consequently, research has been concentrated on the decline phenomenon. Replant disease as such has not been a separate subject of research. Decline is a premature decrease in productivity of asparagus plantings on land without an asparagus history. It is associated with a reduced number and diameter of stems, brown lesions on stem bases, crowns and roots, and collapse of feeder and storage roots (Grogan & Kimble, 1959). It is a common phenomenon in many asparagus producing areas (Hartung, 1987). However, in The Netherlands, although it might occur, it is not recognized as a problem. On fertile, fresh land, suitable for asparagus production, most crops have an economical life of 10-12 years. This period is considered satisfactory by the growers. The start of a new planting enables them also to change to new varieties with higher yield and better quality. The difference in problems makes it reasonable to distinguish between the decline phenomenon and replant disease for the Dutch situation.

The decline phenomenon is attributed to a variety of factors. In most cases *Fusarium oxysporum* (Schlecht.) emend. Snyd. & Hans. f.sp. *asparagi* Cohen and *F. moniliforme* Sheld. were suggested as its cause (Cohen and Heald, 1941; Endo and Burkholder, 1971; Johnston *et al.*, 1979; Gindrat *et al.*, 1984; Fantino and Fantuz, 1990; Hartung, Nair and Putnam, 1990). In Europe, *Rhizoctonia violacea* Tul. (Gindrat *et al.*, 1984; Molot and Lombard, 1986) and *F. culmorum* (W.G. Sm.) Sacc. (Weise, 1939; Van den Broeck en Van Assche, 1966; Gindrat *et al.*,1984) were also reported as causal agents. In New Zealand, a root rot incited by *Phytophthora megasperma* Drechsl. var. *sojae* Hildebrand was shown to cause establishment failures, particularly under wet conditions and in a replant situation (Schofield, 1991).

Besides pathogens, autotoxins are also supposed to be involved. Compounds released from living, senescing or dead asparagus roots were supposed to cause inhibition of root growth, resulting in yield decline (Laufer and Garrison, 1977; Yang, 1982; Young, 1984; Young and Chou, 1985; Hartung and Putnam, 1986; Shafer and Garrison, 1986). Several cinnamic acids were identified as being partly responsible for the autotoxicity of extracts of asparagus roots (Hartung, Nair and Putnam, 1990; Miller *et al.*, 1991).

The presence of root residues of asparagus in soil can enhance incidence of root rot caused by *F. oxysporum* f.sp. *asparagi* and *F. moniliforme* (Hartung and Stephens, 1983; Hartung *et al.*, 1989; Peirce and Colby, 1987).

F. oxysporum f.sp. *asparagi* and *F. culmorum* were isolated from root and stem lesions

in Dutch asparagus crops (Van Bakel and Kerstens, 1970; Van Bakel and Krom-Kerstens, 1974). A recent survey by the first author (unpublished) showed that both pathogens commonly occur replantings, but in first plantings also. The difference between the problems in other countries and those in The Netherlands, viz. decline of first plantings versus replant disease, might have two reasons. First, in the traditional asparagus-growing region in The Netherlands, asparagus is grown on fertile land that is well suitable for growing this crop. Under optimal conditions decline will not develop as fast as when the crop is grown under marginal conditions, as is the case in some asparagus producing areas. Second, *F. moniliforme*, reported from many areas to be a major factor, has never been detected in Dutch asparagus plantings.

In order to develop effective control methods, research was started to identify the factors involved in the replant disease in Dutch asparagus fields. The present study focuses on the involvement of autotoxins. Hartung *et al.* (1989) suggested that root residues of the standing crop are a source of autotoxins that inhibit root growth of the same crop under field conditions. The aim of the present study is to assess whether autotoxins from root residues of the preceding asparagus crop can be a source of autotoxins that are involved in asparagus replant disease. Other aspects of the etiology of replant disease which were studied will be dealt with in other publications.

Materials and methods

Biomass of asparagus root residues in former asparagus fields

To estimate the level of biomass of asparagus root residues in former asparagus fields, root residues were collected on two locations in the centre of the asparagus growing region. On each location, two fields were sampled: one field where production was stopped one year ago and another field where production was stopped ten years ago. On each field two holes (0.5 x 0.5 m) were dug. The soil was dug out in layers of 30 cm up to 90 cm. Preliminary observations revealed that no root residues were present deeper than 90 cm in these fields. The soil of each layer was sieved over a gauze tray with a 5-mm mesh. The asparagus root residues could be recognized easily. They were collected, dried during seven days at 50°C and weighed.

***In vitro* toxicity of asparagus root material to seedlings of asparagus and garden cress**

Collection and treatment of root material. Asparagus roots and root residues were collected from different locations (Table 1). Roots and root residues were thoroughly washed in tap water and oven-dried at 50°C during 7 d before they were used for preparation of extracts.

Bioassays. Toxicity of extracts was assessed in two different bioassays, a Petri-dish test and

Table 1. Samples of asparagus plant material used in the various experiments.

Sample	Type of plant material	Origin
1	Storage roots	Ten-year-old standing crop at Horst (province of Limburg)
2	Storage roots	Six-year-old standing crop at Helden (province of Limburg)
3	Root residues	Field at Helden, production was terminated 5 years ago
4	Root residues	Field at Horst, production was terminated 1 year ago
5	Root residues	Field at Horst, production was terminated 10 years ago
6	Root residues	Field at Helden, production was terminated 1 year ago
7	Root residues	Field at Helden, production was terminated 10 years ago

a culture-tube test. Seeds of asparagus were surface-sterilized by immersion for 5 min in 0.2% $HgCl_2$, washed in running tap water for 30 min, immersed for 5 min in 2 % NaClO and rinsed three times in sterile distilled water. For the culture-tube test asparagus seeds were pregerminated under sterile conditions on moist filter paper at 23°C until radicles were 1-2 mm long.

Petri-dish test. Glass Petri dishes (diam. 9 cm) with two filter papers were autoclaved. The filter paper was moistened with 3 ml extract or with sterile distilled water as a control. In each dish ten surface-sterilized asparagus seeds (cv. Gynlim) were placed between the two filter papers, or ten non-sterilized garden cress seeds (*Lepidium sativum*, cv. Gewone) were placed on the filter paper. Garden cress is a plant species frequently used in tests for phytotoxicity. Five Petri dishes were used for each treatment. The dishes were sealed with parafilm and incubated at 23°C. After 3-4 days (garden cress) or 10 days (asparagus) radicle lengths were measured.

Culture-tube test. Culture tubes (diam. 22 mm, height 150 mm) were filled with 20 ml perlite, capped and autoclaved. To each tube 2 ml filter-sterilized Steiner nutrient solution and 8 ml extract or sterile distilled water were added. In each tube one surface-sterilized, pregerminated asparagus seed (cv. Gynlim) was placed. After a three-week incubation period in the growth chamber at 20°C, plants were removed from the tubes and lengths of primary roots were measured.

Data analysis. Data were subjected to analysis of variance. The means were separated by the multiple comparison test of Tukey (Sokal & Rohlf, 1981).

Experiments. Expt 1. Three treatments were included in both the Petri-dish test and the culture-tube test: (1) an aqueous extract of sample 1, mentioned in Table 1 (2) the same extract autoclaved during 20 min and (3) sterile distilled water. In the Petri-dish test both asparagus and garden cress were used as test plants, in the culture-tube test only asparagus

was used. The extracts were made of 5 g dry root tissue per 100 ml distilled water. The root material was cut in pieces of approx. 2 cm and pulverized in water in a blender. The slurry was shaken for one hour on a rotary shaker, filtered through two layers of cheese cloth and successively filtered through membrane filters (Schleicher and Schuell) of pore size 8.0, 0.45 and 0.22 μm.

Expt 2. Extracts of samples 2-7 were prepared as in experiment 1 except that after filtration through cheese cloth, the extracts were centrifuged during 30 min at 3400 g before filtration. The extracts were centrifuged to prevent that the filters became clogged up too quickly. Toxicity of extracts was tested in a Petri-dish test with garden cress and a culture-tube test with asparagus.

Expt 3. The amount of root material used in experiments 1 and 2 to prepare the extracts could have been too high to detect differences in concentrations of toxic compounds in root material in different stages of decomposition. The effect of the concentration of root material was measured by preparing a dilution series and assessing the effect of each dilution. Extracts of 5 g dry root material in 100 ml distilled water were diluted 1:2 and 1:5 with distilled water (corresponding to 2.5 and 1 g dry roots per 100 ml, respectively). Extracts were prepared of sample 1, a mixture of samples 4 and 6 and a mixture of samples 5 and 7 (Table 1). For this experiment all root samples were ground in a mill (1-mm mesh). The ground material was shaken in water for one hour on a rotary shaker and the extracts were further prepared as in experiment 2. Toxicity of extracts was tested in a Petri-dish test with garden cress as the test plant.

In vivo toxicity of asparagus root material

The effect of toxic compounds present in asparagus root material on plants grown in field soil was assessed in two experiments. In the first experiment asparagus plants were grown in root observation boxes where root growth could be followed continuously. The second experiment was a pot experiment in which root and shoot biomass was determined.

Experiment with root observation boxes. The boxes consisted of a perspex holder with two sheets of glass (height 30 cm, width 20 cm; 3.5 cm apart). The space between the sheets was filled with field soil in the following way. Part of the soil (two columns, width 5 cm, height 15 cm) was mixed with root fragments (1:4, v/v). The surrounding soil was left unamended. This setup allowed direct observation of the response of root growth to root residues. The soil was collected from a field on the experimental farm 'Meterikse Veld' at Horst (province of Limburg), which had never been planted with asparagus. The soil was a loamy sand with a pH-KCl 6.5 and an organic matter content of 2.3%. Four types of root material were tested: 1) sterilized asparagus roots, 2) non-sterilized asparagus roots, 3) sterilized strawberry roots, and 4) non-sterilized strawberry roots. Strawberry root material was included to test whether effects of asparagus roots were specific, due to the presence of toxic compounds, or just

caused by the relatively high amount of organic material added. Asparagus roots were storage roots of two-year-old plants of breeding material that was strongly related to modern Dutch cultivars. Strawberry roots came from one-year-old plants, cv. Elsanta. Roots were sterilized by gamma-irradiation (2.5 Mrad). Roots of both species were thoroughly washed in tap water, air-dried for two days and cut into pieces of 1-2 cm. The boxes were wrapped in black plastic sheets and placed under an angle of 45° in a greenhouse where temperatures were between 20 and 25°C. Asparagus seeds (cv. Gynlim), disinfected with thiram, were pregerminated on moist filter paper in Petri dishes at 25°C in an incubator. Seeds with radicle length 3-4 mm were transferred to the boxes, three seeds per box. There were two boxes for each treatment. Plants were watered daily. Root growth was observed weekly for eight weeks.

Pot experiment. In this experiment sterilized and non-sterilized asparagus root material was incorporated into field soil at rates of 0, 2 and 20 g air-dry root material per litre soil. A fourth treatment with 20 g l^{-1} air-dry sterilized strawberry root and rhizome material served as a control for the effect of a high amount of organic matter. The soil was the same as used in the root observation boxes. The asparagus roots were collected from a field where a six-year-old crop was ploughed under two weeks earlier. Strawberry roots and rhizomes were collected from an experimental field at Wageningen. Roots and rhizomes were washed thoroughly with tap water, air-dried and cut in pieces of 1-2 cm. The roots were sterilized by gamma-irradiation (2.5 Mrad). After the root materials had been mixed through the soil, the mixtures were put in six black plastic 4-litre pots per treatment. Pots were arranged in a completely randomized block design with six blocks. The soil was left unplanted for four weeks to allow the mixtures to stabilize. After this period the pots were planted with three five-week-old asparagus plants (cv. Gynlim) grown from disinfected seeds in a sterilized commercial potting mixture. The seed was disinfected by immersion in 0.2 % $HgCl_2$ for 5 min followed by washing for 30 min in running tap water. The plants were fertilized every ten days during the first 13 weeks, each pot with 100 ml of a solution containing 1.67 g Nutriflora-t and 2.0 g calcium nitrate per litre, pH 6.7. This solution is used in soilless cultures as a complete nutrient solution. After 17 weeks, plants were harvested and sprout and root dry weights per pot were determined. The data were subjected to an analysis of variance. The means were separated by the multiple comparison test of Tukey (Sokal & Rohlf, 1981).

Results

Biomass of asparagus root residues in field soil

Asparagus root residues were found in all fields sampled. In the two fields where the

asparagus crop was ploughed under the year before, only the cortex of the roots was partly decomposed, the stele and the exodermis were still intact. In the two fields where the asparagus crop was ploughed under ten years ago, only the exodermis was left. Because of their appearance as tubes, they were easily recognized.

One year after termination of the crop, biomass of asparagus root residues amounted to 4180 and 11060 kg (dw) ha^{-1} for the two locations sampled. After ten years, biomass of root residues amounted to 420 and 1140 kg (dw) ha^{-1} (Table 2). The amount of root residues decreased with depth in all fields sampled (Table 2).

Table 2. Biomass of asparagus root residues in former asparagus fields at various depths.

Field[1]	Soil layer (cm)	Biomass	
		g(dw) l^{-1} soil	kg(dw) ha^{-1}
A 1	0 - 30	0.78	2340
	30 - 60	0.45	1360
	60 - 90	0.16	480
	In total	4180	
A 10	0 - 30	0.07	200
	30 - 60	0.05	140
	60 - 90	0.03	80
	In total	420	
B 1	0 - 30	2.57	7720
	30 - 60	0.85	2540
	60 - 90	0.27	800
	In total	11060	
B 10	0 - 30	0.21	620
	30 - 60	0.15	440
	60 - 90	0.03	80
	In total	1140	

[1] On each of two locations (A and B) root residues were dug up in two fields, one where an asparagus crop was ploughed under one year ago (A1 and B1) and one where an asparagus crop was ploughed under ten years ago (A10 and B10).

In vitro toxicity of asparagus root material

Experiment 1. Crude aqueous extracts of living asparagus root tissue strongly inhibited radicle growth of asparagus. Autoclaving the extract did not eliminate toxicity (Table 3). Garden cress was even more sensitive than asparagus, both the autoclaved and non-autoclaved extract inhibited radicle growth totally. Growth of the hypocotyl was not completely inhibited. The

Table 3. Mean lengths of radicles of asparagus and garden cress following exposure during ten and four days, respectively, to a crude aqueous extract of asparagus roots (Petri-dish test).

Treatment	Mean radicle length (mm)	
	Asparagus	Garden cress
Distilled water	25.4 a [1]	39.3
Root extract, non-autoclaved	9.8 b	0
Root extract, autoclaved	9.4 b	0

[1] Means with different letters are significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).

asparagus seedlings in the culture tubes showed a similar response (Table 4). Roots exposed to both autoclaved and non-autoclaved root extract had less feeder roots than those exposed to distilled water and root tips were brown.

Table 4. Mean length of primary root of asparagus seedlings grown from germinated seeds for 14 days in perlite with a crude aqueous extract of asparagus roots (culture-tube test).

Treatment	Mean root length(mm)
Distilled water	32.5 a [1]
Root extract, non-autoclaved	9.0 b
Root extract, autoclaved	11.6 b

[1] Means with different letters are significantly different according to Tukey's multiple comparison test ($P \leq 0.05$).

Experiment 2. The asparagus root residues from fields where asparagus production was stopped five to ten years ago were decomposed for the greater part. Only the exodermis was left. However, extracts of these decomposed roots inhibited root growth. In the Petri-dish test with garden cress, all extracts caused significant inhibition of root growth (data not shown). Extracts of samples 2-6 did not differ significantly, resulting in a mean inhibition of root length of 97%. The extract of sample 7 inhibited root length with 68 %, and was significantly less inhibitory than the extracts of the other samples. The culture-tube test with asparagus gave corresponding results (Table 5).

Table 5. Mean length of primary root of asparagus seedlings grown from germinated seeds for 14 days in perlite with crude aqueous extracts of asparagus root material in different stages of decomposition (culture-tube test).

Treatment[1]	Sample[2]	Root length (mm)	Inhibition (%)[3]
Distilled water	-	48.1 a[4]	
X 1	6	12.3 bc	74
X 10	7	17.9 b	63
Y 0	2	6.5 c	86
Y 5	3	14.9 bc	69
Z 1	4	7.1 c	85
Z 10	5	9.6 bc	80

[1] The letter indicates the location, the number indicates the number of years since asparagus production was stopped.
[2] See Table 1 for description.
[3] Inhibition is given as a percentage of root length with distilled water.
[4] Means without a letter in common differ significantly at $P \leq 0.05$ according to the range test of Tukey.

Experiment 3. The Petri-dish test with garden cress as a test plant and different dilutions of extracts of asparagus root material in different stages of decomposition showed that the oldest residues contained significantly less inhibitory compounds than the roots of living plants and than the residues from fields where asparagus production was stopped only one year ago (Table 6).

Table 6. Mean lengths of radicles (mm) of garden cress following exposure during three days to different concentrations of crude aqueous extracts of asparagus root material in different stages of decomposition (Petri-dish test).

Treatment[1]	Concentration (g dry root material l^{-1} distilled water)		
	50	25	10
Distilled water	19.5 a[2]	19.5 a	19.5 a
LR	1.2 b	1.9 c	4.6 b
RR 1	1.6 b	2.5 bc	8.9 b
RR 10	3.9 b	7.0 b	18.0 a

[1] LR = living roots from standing field crop, RR 1 = root residues from fields where asparagus production was stopped one year ago, RR 10 = root residues from fields where asparagus production was stopped ten years ago.
[2] Means in the same column without a letter in common are significantly different at $P \leq 0.05$ according to the range test of Tukey.

***In vivo* toxicity of asparagus root material**

Experiment with root observation boxes. Growth of main roots was not inhibited. However, growth of secondary roots was markedly inhibited in soil amended with asparagus root fragments. When main roots grew just along the borderline of soil with asparagus root fragments and soil without root fragments, secondary root growth was inhibited at the side exposed to the soil with root fragments but not at the other side (Fig. 1). This inhibition was

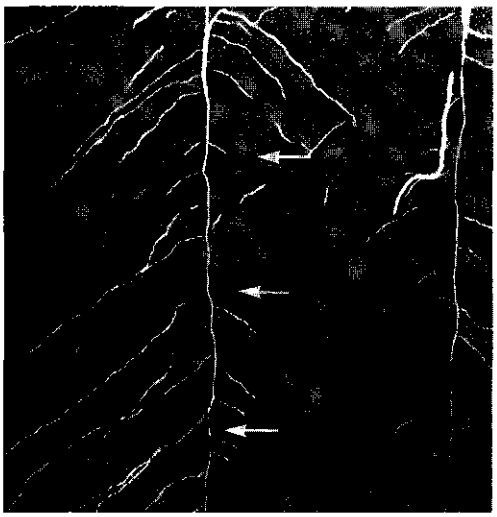


Fig. 1. Detail of a root observation box. The arrows indicate the borderline between non-amended field soil (left) and field soil amended with gamma-irradiated asparagus root fragments (right). In the latter, growth of secondary roots is inhibited.

caused by asparagus root fragments only. Strawberry root fragments had no visible adverse effect on asparagus root growth. Inhibition was observed with both sterilized and non-sterilized asparagus root fragments, which indicates that inhibition was not caused by oxygen depletion due to increased microbial activity after the introduction of sterile root material but rather by the presence of autotoxic compounds.

Pot experiment. In soil with non-sterilized asparagus root fragments at 2 and 20 g l^{-1}, root dry weight was significantly less than in soil without root fragments (Fig. 2). In the first soil, the asparagus roots had many brown lesions. From these lesions mainly *Fusarium oxysporum* was isolated. Addition of sterilized root fragments at 20 g l^{-1} also resulted in significant inhibition of root growth, but it was not associated with lesions on the roots. The sterilized strawberry roots did not affect asparagus root growth.

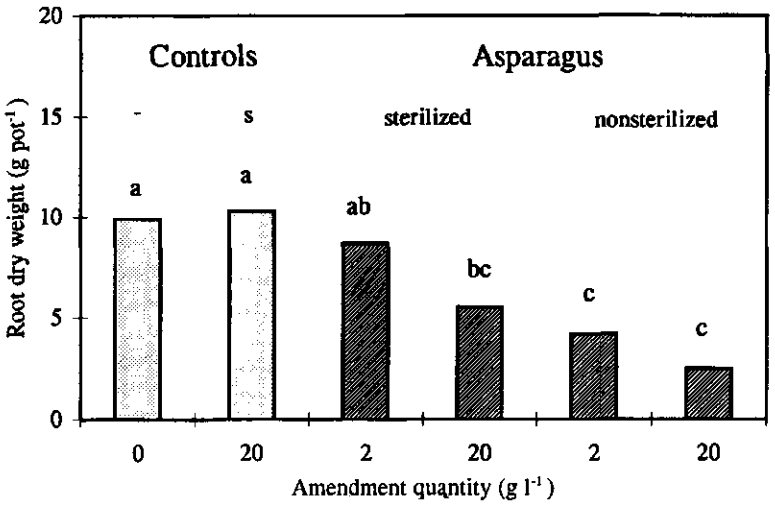


Fig. 2. Root weight (total dry weight of three plants per pot) of asparagus seedlings grown in non-amended field soil or in field soil amended with root fragments of asparagus or strawberry (pot experiment). Control treatments: - = non-amended soil; S = soil amended with sterilized strawberry root fragments (20 g/l). Columns without a letter in common are significantly different at $P \leq 0.05$ according to the range test of Tukey.

Discussion

Asparagus roots decompose slowly in soil. Ten years after asparagus production was stopped an amount of 420-1140 kg (dw) ha^{-1} was still present. Even in fields where production was stopped 25 years ago, asparagus root residues could be found, although at lower amounts (Schönherr and Blok, unpublished).

Both, a non-autoclaved and an autoclaved aqueous extract of living asparagus roots were strongly inhibitory to radicle growth of asparagus and garden cress (Tables 3 and 4). These results are in agreement with previous findings (Yang, 1982; Hartung *et al.*, 1989; Hazebroek, 1989). The culture-tube test allowed to observe the formation of secondary roots. Growth of both primary and secondary roots was inhibited by the extracts. Enhanced root branching or secondary root formation, as was observed by Yang (1982), was not found.

Although only the exodermis of the roots was left in root residues in fields where asparagus production was stopped ten years ago, the residues still contained compounds toxic to radicle growth of asparagus and garden cress. In the culture-tube test extracts of root material in different stages of decomposition were not or only slightly different in their toxicity to asparagus seedlings (Table 5). The Petri-dish test with garden cress and different dilutions of the extracts showed that toxicity of the oldest root residues was significantly

lower than that of living roots or of young root residues (Table 6). Apparently, the concentration of 5 g root tissue per 100 ml water, used in the culture-tube test, was too high to reveal these differences. In conclusion, asparagus root tissue (especially the exodermis) and the autotoxins it contains are very persistent in soil.

The decrease in concentration of toxins can be due to breakdown and to leaching from the residues. Several authors found the toxins to be polar and water-soluble. Therefore, it is likely that the toxins can be readily leached from the residues (Hartung and Putnam, 1986; Yang, 1986). Hartung and Putnam (1986) provided evidence that the toxins can be broken down by the soil microflora. Shafer and Garrison (1986) incorporated asparagus root tissue in soil at rates up to 60 g (dw) per kg dry soil and tested its effect on seedling emergence of lettuce, tomato and asparagus. Inhibition of emergence was found for all three species when the root material was incorporated a short time before seeding. When incorporated 90 days before seeding there was no inhibition. Contrary to our findings, these results indicate that allo- and autotoxic compounds in asparagus roots are inactivated in soil in a fairly short time. However, the root material used by Shafer and Garrison was macerated before incorporation in soil which makes the toxins readily accessible to microbial breakdown or leaching from the soil. In the field autotoxins are incorporated in the root tissue, which is very persistent and probably protects them from microbial breakdown and leaching.

In the root boxes where root growth in soil could be followed, an effect of asparagus root fragments was clearly demonstrated. Secondary roots were formed in similar numbers in amended and non-amended soil, but in the first soil growth was inhibited by autotoxins.

The pot experiment allowed to quantify the effect of soil-incorporated asparagus root fragments on root growth. Sterilized asparagus root fragments at 20 g l^{-1} caused a significant decrease in root weight. Since sterilized strawberry material did not affect root growth, the inhibition cannot be just a matter of a high amount of organic material or of a physical effect, but is caused by autotoxins. A quantity of 2 g l^{-1} of sterilized asparagus roots did not inhibit root growth. This amount was chosen because it corresponds to the maximum amount of root residues present in soil when a former asparagus field will be replanted (Table 2).

The non-sterilized roots caused a significant inhibition of root growth, even in the lowest amount (fig. 2). Since *F. oxysporum* was often isolated from the lesions, this inhibition was most probably due to infection by this pathogen combined with an effect of autotoxins.

Our data are in contrast with those of Hartmann and Wuchner (1972). They incorporated amounts of non-sterilized asparagus roots of up to 60 g l^{-1} soil in three different soils. Asparagus plants were grown in these soils in 15-l pots for three or four years. They did not find inhibitory effects on shoot weights. A possible explanation is that when asparagus plants are grown for such a long period in pots, growth conditions are suboptimal and inhibitory effects of the root material will not be expressed. Our findings are in agreement with those of Hartung *et al.* (1989), who incorporated different amounts of sterilized asparagus roots in steamed sand and obtained growth inhibition with 20 g l^{-1} soil but not with 10 g l^{-1}. Our data

show that asparagus seedling growth was also inhibited in non-sterilized field soil with sterilized asparagus root material at 20 g l^{-1}.

In conclusion, the results demonstrate that autotoxins are present in soil during a long time after termination of an asparagus planting. Because the causal factor of replant disease is persistent too, involvement of autotoxins is suggested at first sight. However, direct growth inhibition by autotoxins can not be considered to be a major factor in the replant disease, because the biomass of asparagus root residues found in former asparagus fields is too low for such an effect. Another argument against an important role of direct growth inhibition by autotoxins is the aspecificity of asparagus root extract toxicity and the specificity of the replant disease. Asparagus root extracts are toxic to many plant species (Yang, 1982; Shafer & Garrison, 1986; Hazebroek *et al.*, 1989). Replant disease, however, is specific as was shown in pot experiments (Schepman & Blok, unpublished). Moreover, we failed to find any record on inhibition of other crops grown after asparagus.

Although direct growth inhibition of asparagus by autotoxins of the previous asparagus crop can not be considered an important factor, these compounds might indirectly affect the next crop. First, residual autotoxins may predispose asparagus roots to infection by root pathogens. Second, these products can affect the soil microflora interacting with pathogens. Hartung and Stephens (1983) found that extracts of asparagus roots inhibited growth of *Pythium ultimum* but not of *F. oxysporum* f.sp. *asparagi* and *F. moniliforme*. The effect of residual autotoxins on infection by *F. oxysporum* f.sp. *asparagi* and on the soil microflora will be dealt with in a next publication.

Chapter 3

Fungi on roots and stem bases of asparagus in the Netherlands: species and pathogenicity

Abstract

A survey was made to identify the most important soilborne fungal pathogens of asparagus crops in the Netherlands. Ten plants were selected from each of five fields with a young (1-4 y) first planting, five fields with an old (6-13 y) first planting and five fields with a young replanting. The analysis included fungi present in the stem base and the roots of plants with symptoms of foot and root rot or showing growth decline without specific disease symptoms. Isolates of each species were tested for pathogenicity to asparagus on aseptically grown plantlets on Knop's agar. Symptoms were caused by *Fusarium oxysporum*, *F. culmorum*, *Botrytis cinerea*, *Penicillium verrucosum* var. *cyclopium*, *Cylindrocarpon didymum*, *Phialophora malorum*, *Phoma terrestris* and *Acremonium strictum*. *F. oxysporum* was by far the most common species and was isolated from 80% of the plants. Not all of its isolates were pathogenic to asparagus. Symptoms were caused by 67%, 78% and 93% of the isolates obtained from young first plantings, old first plantings and replantings, respectively. *F. culmorum* was isolated from 31% of the plants. Two other notorious pathogens of asparagus, *F. moniliforme* and *F. proliferatum*, did not occur in our samples.

Species causing symptoms in the *in vitro* test that were found on more than 5% of the plants were additionally tested for their pathogenicity in pot experiments. *F. oxysporum* f.sp. *asparagi* caused severe foot and root rot, significantly reduced root weights and killed most of the plants. *F. culmorum* caused lesions on the stem base often resulting in death of the plant. *P. terrestris*, a fungus only once reported as a pathogen of asparagus, caused an extensive root rot, mainly of secondary roots that became reddish. The fungus was isolated in only a few samples and is not to be regarded as an important pathogen in Dutch asparagus crops. *P. malorum* caused many small brown lesions on the stem base and incidentally also on the upper part of small main roots. This is the first report of its pathogenicity to asparagus. The fungus is one of the organisms inciting spear 'rust' and it reduced crop quality rather than crop yield. *P. verrucosum* var. *cyclopium* and *C. didymum* did not cause symptoms in pot experiments.

Because of its predominance on plants with foot and root rot and its high virulence, *F. oxysporum* f.sp. *asparagi* was considered to be the main soilborne pathogen of asparagus in the Netherlands.

Introduction

Asparagus (*Asparagus officinalis* L.) is one of the main vegetable crops grown in the southeastern part of the Netherlands. The asparagus produced is almost exclusively white asparagus. In this area, asparagus has been grown for several decades and many growers do not have fresh land available. Therefore, replanting of former asparagus fields is common practice. Plantings on former asparagus land are, however, never as profitable as

plantings on fresh land. Crop establishment occurs without problems, but after a few years with normal yields growth declines. An increasing number of plants suffer from early dying and, consequently, within a few years yields decrease, quantitatively and qualitatively, to a level at which maintaining the crop is no longer profitable. The economic life of a crop on a replanted field averages half that on fresh land. Initially, the phenomenon was termed 'asparagus replant disease' (Blok and Bollen, 1993). However, this term can be misleading because it suggests problems in crop establishment. As the disease is expressed as an early decline rather than a failure of establishment, a more appropriate term is replant-bound early decline. The disease is currently a major factor impeding the increase of the area under asparagus in the southeastern part of the Netherlands.

Problems after replanting former asparagus land are reported from many regions (Grogan and Kimble, 1959; Young, 1984; Schofield, 1991). In most cases, it is a matter of establishing a commercially profitable stand. In almost all reports on replant problems, the phenomenon is related to an early decline of the preceding asparagus crop. Early decline is attributed to two major factors. The first one is an increased infection by soilborne pathogens, mainly *F. oxysporum* f.sp. *asparagi* and *F. moniliforme*. The other one is growth inhibition by autotoxic compounds from roots and root residues of the standing crop (Schofield, 1991). Most authors do not discriminate between early decline and replant problems. For the Dutch situation, it is relevant to distinguish between early decline and replant-bound early decline.

In order to develop methods to control replant-bound early decline, a project was started to identify the causal factors. Growth inhibition of asparagus by autotoxins from root residues of a previous asparagus crop was shown to be not a major factor in the etiology of replant-bound early decline (Blok and Bollen, 1993). The aim of the present study is to identify the soilborne fungal pathogens in Dutch asparagus fields. We focused on fungal pathogens because several soilborne fungi are known to cause considerable losses in asparagus (Schofield, 1990), whereas nematode damage or symptoms of bacterial diseases were not yet observed. An analysis was made of fungi present in diseased stem bases and roots. Their pathogenicity was tested under *in vitro* conditions and in the greenhouse.

Material and methods

Analysis of fungi present in stem base and root tissue

Collection of plant material. The area under asparagus production is mainly concentrated in the northern part of the province of Limburg. In this area, 15 representative fields were selected. On all fields white asparagus was produced. The size of the fields ranged

between 0.5-1.5 ha. In september 1988, plant material was collected from five asparagus fields with an old (6-13 years) first planting, five fields with a young (1-4 years) first planting and five fields with a young replanting (Table 1). On each field, ten plants showing foot and root rot symptoms (yellowing, wilting or dying of stems) or growth decline without specific disease symptoms were selected. The plants were randomly selected from the whole field. From each of the plants a 10-cm piece from the subsoil part of two stems and a 4-8 cm piece of each of three storage roots were collected. The samples were stored in plastic bags at 4°C. Isolations were made within two weeks.

Table 1. Description of the asparagus fields from which plant material was sampled. On all fields white asparagus was produced. Samples were taken in 1988.

Field no.	Location	Asparagus history	Year of planting	Category[1]	Cultivar
1	Meterik	First planting	1975	2	Limbras-26
2	America	" "	1979	2	Limbras-26
3	Heythuysen	" "	1979	2	Lucullus
4	Helden	" "	1981	2	Venlim
5	Helden	" "	1982	2	Franklim
6	Castenray	" "	1984	1	Gynlim
7	Helden	" "	1985	1	Gynlim
8	Meterik	" "	1986	1	various
9	Grubbenvorst	" "	1987	1	Boonlim
10	Panningen	" "	1987	1	Gynlim
11	Grubbenvorst	Second planting	1982	3	Limbras-18
12	Grubbenvorst	" "	1983	3	Limbras-26
13	Castenray	" "	1984	3	Gynlim
14	Meterik	" "	1984	3	Boonlim
15	Helden	" "	1988	3	Boonlim

[1] Category 1: young (1-4 years), first planting; category 2: old (6-13 years), first planting; category 3: young (1-6 years), second planting.

Isolation of fungi. The stem and root pieces were thoroughly washed under running tap water. At both ends of each piece 1-2 cm was discarded to get rid of most of the micro-organisms which had invaded the tissue after collection. The remaining parts were externally disinfested in 2% sodium hypochlorite (20% household bleach) for 2 min and rinsed three times in sterile distilled water. Tissue pieces from the border zone between healthy and diseased tissue were plated onto two media. Mycophil agar (BBL, pH 4.7) with 50 μg ml^{-1} oxytetracycline was used to isolate non-oomycetous fungi and wateragar (Oxoid, 1.5%) with 100 μg ml^{-1} pimaricin was used to isolate oomycetous fungi. Fungi growing from the tissue pieces were subcultured to potato dextrose agar (PDA, Merck)

slants, allowed to grow for one week and stored at 4°C for later identification and pathogenicity testing.

One of the species that were pathogenic to asparagus could initially not be identified because none of its isolates sporulated. Colony morphology and reddish discoloration of asparagus roots led us to the identification of the isolates as *Phoma terrestris* (syn. *Pyrenochaeta terrestris*). Fifteen isolates, including two well-sporulating reference strains of *P. terrestris* from *Calathea crocata* and rice (*Oryza sativa*) that were kindly provided by Mr. J. de Gruyter, Plant Protection Service, Wageningen, the Netherlands, were grown on five media. The media were: (1) malt extract agar (Oxoid), (2) oatmeal agar with sterilized lupin stem pieces, (3) oatmeal agar with sterilized grass leaves, (4) a basal mineral salts agar (3 g $NaNO_3$, 1 g $MgSo_4$. $7H_2O$, 20 g agar, 1 l dist. water) with sterilized chopped wheat straw sprinkled on the agar just before solidification (Watson, 1961), and (5) the same medium as (4) but with sterile lupin stem pieces. The plates were incubated at 28°C in the dark for one week and then placed under near-UV light.

For identification of *Fusarium* spp. the system of Nelson *et al.* (1983) was followed. Most of the other fungi were identified using the keys of Domsch *et al.* (1980).

***In vitro* pathogenicity test**

The first step in the assessment of pathogenicity to asparagus was a test on plants grown under aseptic conditions in culture tubes according to Stephens and Elmer (1988) with a few modifications. Seeds of asparagus cv. Gynlim were surface-disinfested in 2% sodium hypochlorite for 30 min, rinsed in sterile distilled water, placed in an Erlenmeyer flask with 50 ml aceton plus 1.25 g benomyl (2.5 g Benlate 50 WP) and shaken in an orbital shaker at 20°C for 20-24 h. To remove the fungicide, seeds were washed twice in aceton and three times in sterile distilled water. Subsequently, the seeds were placed under sterile conditions on moist filter paper in glass Petri dishes sealed with parafilm. After 5-7 days the seeds had germinated and single seeds with radicles of 1-5 mm were aseptically placed in culture tubes (height 150 mm, diam. 22 mm) containing 12 ml sterile Knop's solution plus 0.4% agar. The tubes were placed in a growth chamber at 20°C and 16-h light period. After 10-20 days, the seedlings were inoculated by placing a PDA-plug with growing mycelium at the stem base of the seedling. Three seedlings were inoculated with each isolate. After an incubation period of 4-5 weeks in the growth chamber, the seedlings were examined for the presence of symptoms on roots or stem bases. An isolate was rated as pathogenic when it caused lesions on one or more seedlings.

Fusarium oxysporum includes numerous formae speciales defined by their ability to cause disease symptoms on one or more host species. *Fusarium oxysporum* (Schlecht) emend. Snyd. & Hans. f.sp. *asparagi* Cohen is defined by its ability to cause disease symptoms on *Asparagus* spp. but not on many other plant species. To estimate the relative frequency of f.sp. *asparagi* within the population of *Fusarium oxysporum* isolates

obtained, one isolate of *F. oxysporum* from each plant from which this species was obtained, was tested *in vitro* for pathogenicity on asparagus. When typical symptoms were caused, the isolate was regarded as a member of f.sp. *asparagi*. When *F. oxysporum* was isolated from roots and stems as well, an isolate obtained from the root was tested. For other fungal species isolated from more than 5% of the plants three isolates for each category of fields were included, so a total of nine isolates was tested. For the remaining species one isolate was tested.

Greenhouse pathogenicity test

Fungal isolates. Species which caused severe symptoms in the *in vitro* test and which were isolated from more than 5% of the plants were further tested for their pathogenicity in pot experiments under greenhouse conditions. The species were *F. oxysporum, F. culmorum, Phoma terrestris* (syn. *Pyrenochaeta terrestris*), *Phialophora malorum, Penicillium verrucosum* var. *cyclopium* and *Cylindrocarpon didymum*. Two isolates of each species were tested. An isolate of a *Rhizoctonia* species obtained from large, irregular, sunken lesions on harvested asparagus spears was included too. Of all isolates single-spore cultures were produced, except for *P. terrestris* and *Rhizoctonia* sp. of which hyphal-tip cultures were produced.

Plant material. Seeds of asparagus cv. Gynlim were surface-disinfested as described before and sown in flats with a commercial potting mixture (Trio-17; pH-KCl 5.4; org. matter content 63%). The plants were raised at 21-25°C. After seven weeks they were carefully uprooted and the roots were washed free of soil. After selection for health and uniformity, the plants were used in the experiments.

Inoculum production and inoculation procedures. Pathogenicity was tested with two different procedures, one in which the roots of the plants were dipped in a suspension of spores or mycelial fragments (root-dip procedure) and one in which plants were planted in soil infested with a soil-meal inoculum (soil-infestation procedure).

For the root-dip procedure inoculum was produced in Erlenmeyer flasks with 100 ml malt extract broth (Oxoid). The flasks were incubated at 23°C in an orbital shaker for 17 days. Conidial suspensions were prepared by filtering the cultures through glass wool. The suspensions were washed by centrifugation and adjusted to $1x10^6$ microconidia ml^{-1}. Cultures of fungi that did not sporulate (*P. terrestris* and *Rhizoctonia* sp.) or showed poor sporulation (*C. didymum*) were chopped in a blender (Ultraturrax, 30 sec at 4000 rpm) and washed by centrifuging (30 min at 3400 g). Plants were inoculated by immersing their roots and stem bases in suspensions of conidia or mycelial fragments during 20 min. Thereafter, plants were planted in 1.5-l pots filled with autoclaved soil (loamy sand, pH-KCl 7.7, organic matter content 3.3 %). For each treatment seven pots with two plants

each were used. A treatment in which plant roots were immersed in tap water served as a control.

For the soil-infestation procedure, inoculum was produced in a soil-meal medium. Potting mixture (Trio-17) was amended with 15% (d.w./d.w.) oatmeal. Tap water was added to a pF-value of 1.8. Erlenmeyer flasks of 500 ml with 200 ml of the soil-oatmeal mixture were autoclaved for 30 min on two consecutive days. The flasks were inoculated with two PDA-plugs with growing mycelium and incubated at 25°C for three weeks. Then the culture was mixed thoroughly with autoclaved soil at a rate of 0.75% (f.w./f.w.). The soil was the same as used for the root-dip procedure. For each treatment seven 1.5-l pots were filled and planted with two plants per pot. A treatment in which autoclaved soil-meal inoculum of one of the two isolates of *F. oxysporum* was mixed through soil, served as a control.

In both experiments, pots were placed on dishes and were watered carefully to prevent cross-infections. The pots were arranged in a randomised block design with seven blocks. The experiments were carried out in two separate greenhouse compartments at 20-25°C. After 12 weeks observations were made on the presence of symptoms. When symptoms were present, pieces of tissue with typical symptoms were sampled. After external disinfestation (1 min in 2% sodium hypochlorite) the pieces were plated on PDA with 50 ppm vendarcine and outgrowing fungi were identified. Dry weights of roots and ferns were determined after 24 h drying at 105°C.

Statistical analysis

Relative frequencies of isolation were calculated as fractions of plants from which a species was isolated. These fractions and the fraction of pathogenic isolates of *F. oxysporum*, were transformed (arcsin $\sqrt{\text{fraction}}$) and tested for normality and for homogeneity of variances between treatments before subjection to ANOVA. Significance of differences for isolation frequencies and for fraction pathogenic isolates was tested with the Studentized range test of Tukey. Root weight data obtained in the greenhouse tests were subjected to ANOVA without transformation. Significance of differences between the inoculated treatments and the noninoculated control treatment was tested with Dunnett's procedure (Steel and Torrie, 1980).

Results

Oomycetous fungi did not appear on the media. On Mycophil agar many fungi were isolated. The species and the isolation frequencies for the most common species are given in Tables 2,3 and 4. *F. oxysporum* was the most common fungus followed by *F. culmorum*. Isolation frequencies for the six most common species were not significantly

Table 2. Frequency of isolation of fungi from stem bases or storage roots of asparagus plants showing symptoms of foot and root rot or showing growth decline. The species mentioned here were isolated from more than 10% of the total number of plants sampled (150).

Fungi	First planting, 1-4 y old (Category 1)		First planting, 6-13 y old (Category 2)		Second planting, 1-6 y old (Category 3)	
	# Fields[1]	Isol. freq.[2]	# Fields	Isol. freq.	# Fields	Isol. freq.
Fusarium oxysporum	5	0.78 a[3]	5	0.82 a	5	0.80 a
F. culmorum	4	0.38 ab	4	0.33 bc	4	0.45 ab
Botrytis cinerea	4	0.35 b	4	0.30 bc	4	0.18 b
Penicillium verrucosum var. *cyclopium*	4	0.20 b	3	0.50 b	3	0.27 b
Cylindrocarpon didymum	1	0.40 b	4	0.18 c	4	0.28 b
Phialophora malorum	4	0.33 b x	5	0.16 c y	3	0.13 b y

[1] Number of fields from which the species were isolated (maximum is 5).
[2] Isolation frequency = Mean fraction of plants from which the fungus was obtained (this mean was calculated with the data of those fields only from which the fungus was isolated).
[3] Means without a letter in common are significantly different at $P \leq 0.05$ according to Studentized range test of Tukey. Letters a,b and c are used for comparisons within columns, letters x and y are used for comparisons within rows, but only for *P. malorum* as frequencies for the other species did not differ significantly between categories of fields.

different for the three categories of fields except for *P. malorum*.

The two reference strains of *P. terrestris* readily formed pycnidia that appeared in large numbers on all five media. Four out of the 13 isolates from asparagus formed a few sporulating pycnidia on wheat straw (medium No 4) within 4 weeks. In this way, identification as *P. terrestris* was confirmed. After 4 months, an additional six isolates had formed pycnidia without conidia on wheat straw.

Table 3. Frequency of isolation from root and stem base tissue for the six most common fungi. For both substrate types, the fraction of the total number of plants (n=150) from which the fungus was isolated, is given.

	Root	Stem
Fusarium oxysporum	0.56	0.61
F. culmorum	0.11	0.16
Botrytis cinerea	0.15	0.07
P. verrucosum var. *cyclopium*	0.13	0.13
Cylindrocarpon didymum	0.14	0.01
Phialophora malorum	0.08	0.11

Table 4. Fungi isolated from stem base or storage root tissue of asparagus plants showing symptoms of foot and root rot or showing growth decline. The species listed here were isolated from less than 10% of the total number of plants sampled (150).

Acremonium strictum	*Paecilomyces marquandi*
Alternaria alternata	*Penicillium* sp. II
A. tenuissima	*Penicillium* sp. III
Aureobasidium pullulans	*Phoma leveillei*
Cylindrocarpon destructans	*P. terrestris*
C. olidum	*Phoma* sp.
Exophiala sp.	*Rhizoctonia solani*
Fusarium equiseti	*Sesquicillium candelabrum*
Geotrichum sp.	*Trichoderma harzianum*
Gliocladium catenulatum	*Trichosporiella cerebriformis*
G. roseum	*Ulocladium atrum*
G. virens	*Verticillium tricorpus*
Humicola fuscoatra	*Volutella ciliata*
Mucor racemosus	*Zygorrhynchus moelleri*
M. hiemalis	Sterile mycelia

The species which caused symptoms in the *in vitro* test are mentioned in Table 5. Most species did not cause any disease symptoms in the *in vitro* test. The percentages of isolates of *F. oxysporum* that were pathogenic on asparagus were 67, 78 and 93% for category 1,2 and 3, respectively. These percentages were not significantly different according to the Studentized range test of Tukey ($P \leq 0.05$).

In the greenhouse test symptoms were caused by *F. oxysporum*, *F. culmorum*, *P. malorum* and *P. terrestris*. These fungi could be re-isolated from diseased tissue. *C. didymum*, *Penicillium* sp. and *Rhizoctonia* sp. did not cause any disease symptom.

F. oxysporum caused brown lesions on roots and stem bases, resulting in dying off of stems and roots, and significantly lower root weights (Table 6). When stem bases were heavily attacked in an early stage, the plants died. The attack was more severe with the soil-infestation procedure than with the root-dip procedure.

F. culmorum caused mainly stem base lesions resulting in stem death. Inoculation by the root-dip procedure resulted in a heavy attack of the stem base in an early stage and death of most plantlets. With the soil-infestation procedure, the lesions on the stems developed less rapidly and all plants, except one, survived, but root weights were significantly lower than those of the control plants (Table 6). On most plants, a few brown lesions were formed on the roots.

P. malorum caused many small, elongated, brown lesions on the stem base. Root lesions were formed only incidentally on the upper part of small main roots (Fig. 1).

Table 5. Fungi causing disease symptoms on asparagus plantlets in the pathogenicity test *in vitro*.

Fungal species	# Isolates tested	# Pathogenic isolates	Description of symptoms
F. oxysporum	120	95	Many brown oval lesions on roots, sometimes also on stem bases. Most plantlets died.
F. culmorum	9	9	Most plantlets died rapidly, with or without formation of brown oval lesions on roots and stem bases.
B. cinerea	9	6	A small number of lesions on the stem base. Sometimes small root lesions were formed. Plantlets were not killed.
P. verrucosum cyclopium	9	9	Rot of upper part of main root. Browning of root var. system. A few plantlets died.
C. didymum	9	7	Browning of roots and inhibition of root growth. No lesions formed, no plantlets died.
P. malorum	9	9	Whole root system and stem base covered with small, elongated brown lesions. Secondary root growth inhibited. Plantlets were not killed.
P. terrestris	3	3	Strong inhibition of root growth. Many elongated brown lesions formed on roots and stem bases. Large parts of the root system were discoloured red.
A. strictum	1	1	Many elongated brown lesions on the roots. None of the plantlets died.

P. terrestris caused a root rot, mainly of the secondary roots. The main roots showed brown to redbrown irregular, superficial lesions and the secondary roots became reddish (Fig. 1). The discoloration of the roots was far more extensive with the soil-infestation procedure than with the root-dip procedure.

Discussion

Among the many fungi isolated from diseased stem base and root tissue, only eight species were pathogenic to asparagus. Apparently, most of the isolates had colonized the diseased tissue without causing symptoms.

Two *Fusarium* species which are notorious pathogens of asparagus, *F. moniliforme* and *F. proliferatum*, were not isolated from the plants in our survey. *F. moniliforme* is associated with asparagus decline in many regions (Johnston *et al.*, 1979; Fantino, 1990). *F. proliferatum* was recently described as a causal agent of crown and root rot of asparagus (Elmer, 1990). The latter species was often isolated from carnations in Dutch greenhouses (Aloi and Baayen, 1993). The temperature requirements of *F. proliferatum* may explain why it was not isolated from asparagus fields. Another explanation can be

Table 6. Survival and root dry weight of asparagus plants inoculated with seven soil fungi using two procedures of inoculation.

Inoculum	Isolate	Soil infestation		Root dipping	
		Surviving plants[1]	Root dry weight[2]	Surviving plants[1]	Root dry weight[2]
None		2.0	9.3	2.0	8.7
F. oxysporum	CWB 1	1.4	1.0 *	2.0	3.5 *
	CWB 6	1.9	2.0 *	1.6	3.4 *
F. culmorum	CWB 500	2.0	4.3 *	0.1	0.9 *
	CWB 501	1.9	4.2 *	0.9	1.6 *
P. verrucosum	CWB 515	2.0	9.7	2.0	10.1
var. *cyclopium*	CWB 516	2.0	10.3	2.0	10.1
C. didymum	CWB 510	2.0	7.6	2.0	11.0
	CWB 511	2.0	9.4	2.0	9.8
P. malorum	CWB 505	2.0	9.7	2.0	10.9
	CWB 506	2.0	9.8	2.0	10.1
P. terrestris	CWB 520	2.0	7.4 *	2.0	8.4
	CWB 521	2.0	6.9 *	2.0	7.2
Rhizoctonia sp.	CWB 530	2.0	10.1	2.0	8.7
LSD (Dunnett, P=0.05)		1.7			3.0

[1] Mean number of surviving plants per pot (per pot two plants were planted).
[2] Mean total root dry weight per pot (g). Means followed by an asterisk differ significantly from the control according to the test of Dunnett ($P \leq 0.05$).

that isolates of *F. proliferatum* from carnation are not pathogenic to asparagus. *F. proliferatum* was isolated only once from asparagus by Mrs. Veenbaas-Rijks of the Plant Protection Service, Wageningen, the Netherlands. This isolate was not pathogenic to asparagus in our tests. *F. moniliforme* and *F. proliferatum* had not been recorded in an earlier survey for fungi on stem bases of plants with foot rot symptoms in the Netherlands (Van Bakel and Kerstens, 1970).

Among the eight pathogens, *F. oxysporum* f.sp. *asparagi* was by far the most important one because of its high frequency of isolation and its virulence. This applied to plantings on fresh soil as well as to replantings. *F. oxysporum* f.sp. *asparagi* was reported worldwide as the cause of foot and root rot and as one of the main factors in asparagus

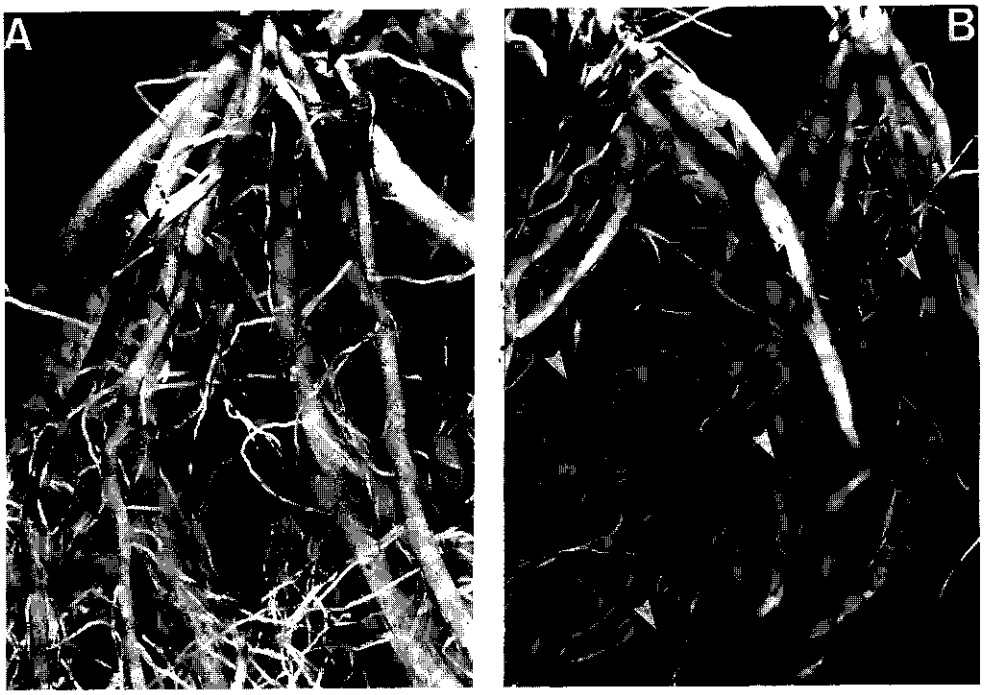


Fig. 1. Symptoms on the roots of asparagus plants caused by *Phialophora malorum* (A) and *Phoma terrestris* (B). *P. malorum* caused similar lesions on the stem base. Arrows show specific symptoms (see text).

decline (Schofield, 1991). *F. culmorum* is known as the cause of foot rot and is also mentioned in relation to asparagus decline in European countries (Weise, 1939; Gindrat *et al.*, 1984; Fantino, 1990). Both pathogens have earlier been reported from asparagus crops in the Netherlands (Van Bakel and Kerstens, 1970; Van Bakel and Krom-Kerstens, 1974). In the greenhouse experiments, *F. oxysporum* f.sp. *asparagi* attacked both roots and stem bases, whereas *F. culmorum* attacked mainly the stems and only rarely caused lesions on the roots. This corresponds with the descriptions of the symptoms in the literature (Weise, 1939). Its presence in roots and stem bases in similar frequencies (Table 3) is worth mentioning.

The plantlets used for the pathogenicity tests had only thin stems. *F. culmorum*, being a parasite on the stem base, rapidly killed the plantlets. Therefore, these tests probably overestimate its pathogenicity. Under field conditions normally only one to a few stems per plant are attacked by *F. culmorum* and damage remains limited. On the other hand, *F. oxysporum* f.sp. *asparagi* attacks both stems and roots which weakens the plant more strongly and can lead to plant death, especially under stress conditions.

P. terrestris is well-known as the cause of pink root of *Allium* spp. (Punithalingam and Holliday, 1973) and root rot of maize in warmer regions (Rouhani *et al.*, 1979; Campbell *et al.*, 1991). From its low frequency of isolation it is concluded that it is of minor importance as a pathogen under the cool conditions of the Netherlands. Its pathogenicity was clearly demonstrated in the greenhouse test with temperatures 20-25°C, where it caused a severe root rot. The typical reddish discoloration of the secondary roots was also observed in plants grown in former asparagus soil. From these roots the fungus could readily be isolated. The low frequency of its isolation in our analysis is most probably due to its high temperature requirements, the optimum temperature for growth and disease expression being at 28°C (Punithalingam and Holliday, 1973). The fungus was only rarely recorded on asparagus. Grogan and Kimble (1959) tested 20 isolates of *P. terrestris* on asparagus and found that all of them induced reddish discolorations of roots. The fungus was also isolated from asparagus roots by Messiaen and Lafon (1970), but these authors did not provide data on its pathogenicity. The low number of records suggests that the fungus is not widespread in asparagus-growing regions. It might be that it is overlooked on isolation plates, as it is easily overgrown because of its low growth rate. Moreover, the fungus is not easily recognized because of poor sporulation of most isolates.

The pathogenicity of *P. malorum* was clearly demonstrated in the greenhouse test. The fungus is known as a causal agent of storage rot of apple and pear (Sugar and Spotts, 1992). To the best of our knowledge, this is the first account of its pathogenicity to asparagus. The numerous small lesions on the stem bases and, to a far less extent, on the roots did not result in lower root weights. In the field, significant growth inhibition by this pathogen is not to be expected but the lesions on the spears severely reduce the quality of the product. In asparagus production, spears covered with brown, small lesions are often harvested and the disease is called 'rust' or 'physiological rust' (Blanchard and Faure, 1988). *P. malorum* was, together with other fungi, e.g. *Fusarium oxysporum* and *C. didymum*, isolated from 'rusty' spears.

P. verrucosum var. *cyclopium*, *C. didymum* and *A. strictum* caused symptoms under *in vitro* conditions only and were, therefore, not regarded as true pathogens. *P. verrucosum* var. *cyclopium* is a synonym of *P. martensii* (Samson *et al.*, 1976). The latter species was described by Menzies (1955) as the cause of crown rot of injured asparagus seedlings. This symptom did not appear in our inoculation experiments, which is in line with Menzies' observation that inoculation was only followed by infection after wounding the plants. In previous trials we isolated the fungus from rotten primary roots of asparagus seedlings. Another variety of the same species, var. *corymbiferum*, was mentioned by Gordon-Lennox and Gindrat (1987) as being highly pathogenic to aseptically grown plantlets. *C. didymum* was reported by Brayford (1987) as a weak pathogen of asparagus but details on symptoms were not given.

Notable is the high frequency of isolation of *B. cinerea*. This fungus infects shoot tissue

and, if not controlled, can cause considerable losses by inciting premature shoot dying. In our samples it was frequently isolated from stem bases and roots.

In conclusion, *F. oxysporum* f.sp. *asparagi* is the most important soilborne pathogen of asparagus in the Netherlands because of its predominance on plants with foot and root rot symptoms and its high virulence. *F. culmorum* is less frequently isolated and under field conditions less virulent than *F. oxysporum* f.sp. *asparagi*. The other pathogenic species do not cause significant growth inhibition. They can, however, cause a decrease in quality of harvested spears. The exact role of soilborne pathogens in the replant disease will be dealt with in a next publication.

Chapter 4

Etiology of asparagus replant-bound early decline

Abstract

Asparagus replant-bound early decline (ARED) was characterized and its etiology was elucidated in experiments under greenhouse and field conditions. Selective soil treatments were used to differentiate between autotoxic compounds and soil-borne pathogens as causal agents.

In greenhouse experiments, there were symptoms of ARED within 12-15 weeks. Asparagus plants grown in soil formerly used for asparagus (asparagus soil) showed brown lesions on primary and secondary roots, and many secondary roots had rotted. Root weights of plants grown in asparagus soil were lower than those of plants grown in fresh soil. *Fusarium oxysporum* f.sp. *asparagi* (Foa) was by far the most common species among the fungi isolated from roots with lesions. Under greenhouse and field conditions, there were similar symptoms, which indicates that the results obtained under greenhouse conditions are similar to those in the field.

The vertical distribution of the ARED-causing factor(s) was studied in a greenhouse experiment in which plants were grown in soil from three layers: 0-30, 30-60, and 60-90 cm. For all four asparagus soils tested, there were ARED symptoms and similar disease severity in samples from all three depths. The causal factor persisted at least 11 years after soil was no longer used for asparagus.

When asparagus soil was diluted with fresh soil to give mixtures with 100%, 80%, 50%, 20% and 0% asparagus soil, disease severity did not decrease with increasing dilution of the asparagus soil from 100% to 20%. Disease severity of all mixtures with asparagus soil was significantly higher than that for fresh soil. The results imply that ARED is caused by a pathogen colonizing the soil rather than inhibition by autotoxins released from residues of the preceding asparagus crop. This conclusion is supported by the results of greenhouse and outdoor experiments with heat and fungicide treatments of soil. ARED was nullified by heat treatments of 30 min at 55 or 60°C but not 45 and 50°C, eliminating autotoxins as an important cause of ARED because they are heat-stable. Foa is eliminated by a 30-min soil treatment at 55-60°C but not 50°C. Prochloraz, known for its toxicity to *F. oxysporum*, also nullified ARED. Disease severity level was related to the density of Foa in soil. The results provide conclusive evidence that *F. oxysporum* f.sp. *asparagi* is the main cause of ARED in the Netherlands, which largely removes the need to discriminate between early decline and replant-bound early decline, because Foa is the main cause of both diseases.

Introduction

In the Netherlands asparagus production is concentrated mainly in the southeast, where it is common to replant former asparagus land because the amount of fresh land (without a history of asparagus cropping) is limited. However, asparagus production on former asparagus land is less profitable than that on fresh land. Crop establishment generally is not affected, but after a few years of normal yields, growth and yield decline. The economic life of a crop on asparagus land averages only half that of a crop on fresh land.

The symptoms of this phenomenon are similar to those described for asparagus early decline (Grogan & Kimble, 1959). However, whereas early decline occurs on fresh soil as well as asparagus soil, problems in the Netherlands are confined to replant situations. Because of this difference and because the etiology of the phenomenon was not clear for the Dutch situation, the phenomenon was distinguished tentatively from early decline and termed asparagus replant-bound early decline (ARED) (Blok and Bollen, 1995). Replant-bound early decline is currently one of the main threats to asparagus production in the traditional asparagus-growing region of the Netherlands.

A number of factors have been associated with early decline of asparagus: increased infestation of the soil with soil-borne pathogenic fungi, mainly *Fusarium* spp. (Schofield, 1991), and direct or indirect effects of autotoxic compounds emanating from asparagus roots (Hartung, 1987; Wacker *et al.*, 1990; Peirce & Miller, 1993). *F. oxysporum*, *F. moniliforme* and *F. proliferatum* are often mentioned as the major causal agents (Grogan & Kimble, 1959; Johnston *et al.*, 1979; Gindrat *et al.*, 1984; Elmer, 1990; Schreuder *et al.*, 1995). However, studies in which the contribution of each of these factors to the etiology of the disease is estimated are lacking. For this reason and because it is unclear whether ARED is different from early decline, definitive conclusions about the etiology of the disease in the Netherlands could not be drawn.

In earlier studies, root residues of the preceding asparagus crop were shown to be a source of autotoxins, and the main soil-borne fungal pathogens in Dutch asparagus fields were identified (Blok and Bollen, 1993, 1995). *Fusarium oxysporum* f.sp. *asparagi* (Foa) was the main pathogen because of its prevalence and virulence. To characterize ARED further and to estimate the relative contribution of various causal factors, a method was needed to reproduce the symptoms of the disease. In greenhouse experiments, Huiskamp and Poll (1990) and Poll and Huiskamp (1992, 1994) compared growth of asparagus plants in fresh and (former) asparagus soil from 15 locations. They found that plants grown in asparagus soil could be distinguished easily from those grown in fresh soil. The former had fewer secondary roots and had brown lesions, whereas the latter had healthy root systems with many secondary roots. Root weight of plants grown in asparagus soil was lower than that in fresh soil. The researchers demonstrated that ARED symptoms can be reproduced in greenhouse experiments within 9-15 weeks.

Our goal was to characterize ARED further and elucidate the etiology. We used soil samples from a limited number of locations of the asparagus-growing area in the Netherlands. We decided that this sample number would suffice because Poll and Huiskamp (1994; personal communication) tested a large number of samples from different locations in the Netherlands and found that the root systems were affected identically in all asparagus soils.

The vertical distribution of ARED was studied by comparing asparagus growth in fresh and asparagus soils collected from different depths. A possible dilution effect was studied

in a series of mixtures of asparagus soil and fresh soil. A dilution effect suggests involvement of toxins or slow-growing organisms as major factors in ARED development. Since the autotoxins tolerate autoclaving (Yang, 1982; Blok and Bollen, 1993) and fungal pathogens are sensitive to heat, ARED soils were subjected to heat treatments to discriminate between these factors as causes of the disease. We attempted to characterize fungi involved in ARED further by treatment of soil with products with a different antifungal spectrum.

Materials and methods

General procedures

Plant material and nutrition. As all Dutch asparagus cultivars suffer from ARED, the current cv. Gynlim was used throughout all experiments. Seeds were surface-disinfected in 2% sodium hypochlorite for 30 min and then in a suspension of benomyl in acetone (25 mg ml^{-1}) for 20-24 h, after which they were rinsed in acetone twice and tap water three times (Stephens and Elmer, 1988). The seeds were sown in a commercial potting mixture (Trio-17, a decomposed sphagnum peat with some clay and marl; pH-KCl 5.4; organic-matter content 70%). After several weeks at 20-25°C in the greenhouse, seedlings were uprooted, culled to a uniform size and planted in pots. Unless stated otherwise, every 10-14 days an application of 100 ml of a solution containing 1.67 g Nutriflora-T l^{-1} and 2.0 g calcium nitrate l^{-1} was made in each 4-l pot. This solution at pH 6.7 is used in soilless cultures as a complete nutrient solution. It contains the following elements (concentrations in mg l^{-1}): N, 344; P, 78; K, 560; Mg, 50; Ca, 340; S, 206; Fe, 1.2; Mn 2.2; B, 0.7; Zn, 0.5; Cu, 0.020; Mo, 0.116.

Disease rating. At the end of the experiments roots were washed free of soil carefully and rated for disease symptoms. Root dry weights (DWR) were determined after drying for 24 h at 105°C. Disease symptoms on primary (DPR) and secondary roots (DSR) were rated combined (Expts 1 and 2) or separately (Expts 3 and 4) on a scale of 0-5 where 0 = no lesions, 1 = 0-5% of the root length covered with lesions, 2 = 6-20%, 3 = 21-60%, 4 = 61-95%, and 5 = >95% or dead plants. In many cases secondary roots were rotted completely and not retrieved when root systems were washed, which implies that the number of secondary roots is also a measure of disease severity. The loss of secondary roots (LSR) was rated on an arbitrary scale from 0 (abundant secondary roots, no loss) to 10 (no secondary roots present, total loss). Based on the ratings for DPR, DSR, and LSR, a disease index (DI) ranging from 0-10 was calculated. To equalize scales and give the

three components the same weight, DI was calculated as DI = (2*DPR + 2*DSR + LSR) / 3.

Isolation from infested material. Root parts showing symptoms were washed free of adhering soil under running tap water and disinfested externally by washing, depending on the thickness of the root parts, in 1-2% sodium hypochlorite for 1-2 min and then three times in sterile distilled water. Small sections of secondary roots or tissue parts excised from the edge of a lesion on a primary root were plated onto potato dextrose agar or malt extract agar, both amended with oxytetracycline (50 μg ml^{-1}), or Komada's agar (Komada, 1975). Fungi growing from these sections were subcultured on fresh PDA plates for further identification. *Fusarium* spp. were identified according to Nelson *et al.* (1983). Other fungi were identified using the keys of Domsch *et al.* (1980). Some of the *F. oxysporum* isolates were tested for pathogenicity to asparagus on plantlets grown aseptically in culture tubes containing Knop's agar, as described previously (Blok & Bollen, 1995).

Estimation of the population density of Fusarium oxysporum *f.sp.* asparagi *in soil.* Soils were assayed for *F. oxysporum* by placing 10.0-g samples (dry-weight equivalent) in Erlenmeyer flasks with 90 ml sterile distilled water. The flasks were shaken for 1 h on an orbital shaker, and the soil suspension then was diluted further by adding 10 ml of it to 90 ml sterile distilled water. Of each soil suspension, 0.5 ml was spread on each of four or five replicate plates with Komada's selective medium. After incubation for two weeks at 25°C in the light, the number of colonies of *F. oxysporum* was counted and expressed as numbers of colony forming units (cfu) per gram of dry soil. To determine the proportion of isolates of *F. oxysporum* that belonged to f.sp. *asparagi*, a number of isolates was selected randomly on the Komada plates and subcultured onto PDA slants. The pathogenicity of these isolates to asparagus was tested *in vitro* as described above.

Experiments

Vertical distribution of ARED-causing factor(s) in soil (Expt 1). To study the occurrence of the causal factor(s) of ARED at various depths, soil samples were collected from two different locations in the asparagus- growing region and tested in a bioassay. Soils from both locations were loamy sands with an organic-matter content of 2-3%. Location 1 was on the experimental farm Meterikse Veld at Horst, and location 2 was on a commercial farm at Castenray. A control field with fresh soil and two former asparagus fields were sampled at both locations. Five sites in each field were selected randomly. Six litres of soil was collected at each site from each of the following depths: 0-30, 30-60, and 60-90 cm. For each soil layer, the soil from the five sites was bulked and mixed well, creating a total of 18 samples (six fields x three layers) of 30 l each. For each sample the pH (KCl)

Table 1. Data of soil samples used in the study of the occurrence of the causal factor in the soil profile (Expt 1).

Location	Field	Asparagus history	Soil layer (cm)	Amount of asparagus root residues (g dw kg^{-1} dry soil)	pH-KCl
1	1	none	0-30	0	5.7
			30-60	0	4.8
			60-90	0	4.5
1	2	Production field,	0-30	0.32	5.8
		terminated 2 y before	30-60	0.26	5.1
			60-90	0.23	4.7
1	3	Production field,	0-30	0.03	6.0
		terminated 11 y before	30-60	0.10	5.5
			60-90	0.04	4.8
2	4	None	0-30	0	5.3
			30-60	0	4.4
			60-90	0	4.6
2	5	Production field,	0-30	0.18	6.3
		terminated 2 y before	30-60	0.16	6.1
			60-90	0.06	5.6
2	6	Production field,	0-30	0.39	5.6
		terminated 5 y before	30-60	0.16	5.6
			60-90	0.08	5.9

and the amount of asparagus root residues were determined (Table 1). For the latter, a subsample of 6 l was sieved through a sieve with 5-mm mesh. Dry weight of root residues was determined after 24 h at 105°C.

For each sample, six pots were filled with 4 l of soil, and each pot was planted with three 5-wk-old plants. The pots were placed in a greenhouse compartment at 20 ± 2°C in a randomized complete block design with six blocks. The experiment was concluded after 22 weeks.

Effect of dilution of infested soil on disease severity (Expt 2). A series of mixtures of asparagus soil and fresh soil was used to study the effect of dilution of infested soil on disease severity. On the experimental farm Meterikse Veld at Horst, soil was collected from the upper 25 cm of a field without an asparagus history (fresh soil) and from a field where asparagus production was terminated two years before (asparagus soil). In former experiments, soil from the latter field showed typical symptoms of ARED. Soil from both fields had similar soil type (loamy sand), pH (5.8), and organic-matter content (2.8%). Both soil samples were sieved through a screen with a 5-mm mesh to remove stones and soil clods. The root residues that remained were returned to the soil. The following

mixtures of fresh and asparagus soil were made: 1:0, 4:1, 1:1, 1:4 and 0:1. For each mixture, eight pots were filled with 4 l of soil each. Every pot received three 9-wk-old plants. The experiment was set up as a randomized complete block design with eight blocks and carried out in a greenhouse at 20-24°C. The experiment was concluded after 15 weeks.

Effects of selective heat treatments of soil in a greenhouse experiment (Expt 3). In order to discriminate between the contribution of autotoxins and fungal pathogens as causal agents of ARED symptoms, soil was heated at different temperatures. For this experiment samples of fresh and asparagus soil were collected at the same locations as those for the dilution series (Expt 2). Sieved fresh and asparagus soil, with asparagus residues returned and mixed with the soil, were heated with aerated steam and kept for 30 min at 55, 60, or 65°C (± 1.5°C) or left untreated (control). The soil was pasteurized in a slightly modified version of the apparatus described by Aldrich and Nelson (1969). After treatment the soil was stored in open plastic boxes, kept moist, and turned several times. After six weeks, seven 4-l pots for each soil type-treatment combination were filled. From each pot a soil sample of about 15 ml was collected with a sterile spoon. The samples were bulked for each soil type-treatment combination and assayed for *F. oxysporum*. For the untreated fresh and asparagus soil, 25 isolates of *F. oxysporum* were selected randomly from the soil dilution plates and tested for pathogenicity on asparagus *in vitro*. In each pot three 6-wk-old asparagus plants were planted. The pots were placed in a randomized complete block design with seven blocks. The experiment was carried out in a greenhouse at 22-25°C. The experiment was finished after 14 weeks.

Effects of selective heat and fungicide treatments of soil in an outdoor pot experiment (Expt 4). To identify the causal factor of ARED further and to check whether its symptoms could be reproduced under field conditions, the following experiment was conducted. Fresh and asparagus soil was collected from the upper 25 cm at Meterikse Veld. The fields were different from those in which soil was collected for Expts 1-3, but soil type, pH, and organic-matter content were similar. The asparagus soil came from a field where asparagus had been grown for 10 years and the crop was abandoned the year before. Both soils were sieved through a screen with a 5-mm mesh. The asparagus root residues were returned and mixed through the soil. Details on treatments of soil are given in Table 2. Heat treatment of soil was made as in Expt 3. The fungicides were applied at the start of the experiment by mixing the products through soil in a concrete mixer. Later applications were given as soil drenches (100 ml per cylinder). Three samples each of the untreated, heat-treated, and irradiated soils were taken and assayed for *F. oxysporum*.

Table 2. Selective soil treatments applied in the outdoor pot experiment (Expt 4).

1. Blank (untreated)
2. Untreated and not fertilized
3. Heat treatment for 30 min at 45°C
4. Heat treatment for 30 min at 50°C
5. Heat treatment for 30 min at 55°C
6. Heat treatment for 30 min at 60°C
7. Metalaxyl (Ridomil-5G, 5% granulate), one application of 3.2 mg a.i. kg^{-1} dry soil at the start of the experiment
8. Quintozene (Luxan PCNB, 70% WP), one application of 16.0 mg a.i. kg^{-1} dry soil at the start of the experiment
9. Benomyl (Benlate, 50% WP), applications at 0, 4, and 8 weeks of 54.0, 75.0 and 75.0 mg a.i. kg^{-1} dry soil, respectively
10. Prochloraz (Sportak, 450 g l^{-1}), applications at 0, 4, and 8 weeks of 64.0, 30.0 and 30.0 mg a.i. kg^{-1} dry soil, respectively
11. Captafol (Rhodifolatan-4F, 480 g l^{-1}), applications at 0, 4, and 8 weeks of 32.0, 20.0 and 20.0 mg a.i. kg^{-1} dry soil, respectively
12. Irradiation (2.5 Mrad)

From both the untreated fresh soil and the untreated asparagus soil, twenty isolates of *F. oxysporum* were randomly selected and tested for pathogenicity on asparagus *in vitro*. After treatment the soil was put in bags constructed from polypropylene cloth impenetrable to roots. Each bag was filled with 13 l of soil and placed in a pvc cylinder (height 50 cm, diameter 20 cm). The cylinders were sunk into the field to the soil surface. For each soil type-treatment combination, eight cylinders were filled. The cylinders were placed 125 x 80 cm apart in a randomized complete block design with eight blocks. In each cylinder two 15-wk-old asparagus plants were planted. The outer rows of cylinders were surrounded by border rows of pairs of plants planted in free soil. During the first eight weeks, plants were watered by sprinklers as needed. After eight weeks plants were watered by drip irrigation. Each cylinder, except those of treatment 2, was fertilized directly after planting and after 4, 8, and 12 weeks with 600 ml of a solution containing 1.67 g Nutriflora-T l^{-1} and 2.0 g calcium nitrate l^{-1}. After 15 weeks each cylinder received 1200 ml of the same solution. After 17 weeks needles started to yellow and plants were fertilized for the last time by spraying with a solution containing 10 ml of the compound leaf fertilizer Wuxal l^{-1}. The solution contained the following elements (concentrations in mg l^{-1}): N, 2070; Mg, 663; Fe, 13.8; Mn, 6.9; B, 2.8; Zn, 8.0; Cu, 2.8; Mo, 0.069. Plants of treatment 2 received tap water instead of nutrient solution. After 24 weeks the shoots had completely died and the experiment was finished.

Statistical analysis

Data for DWR and DI were subjected to analysis of variance after a check for normality and homogeneity of variances. To make preplanned comparisons, linear contrasts were evaluated with normal F-tests. Comparisons that were not preplanned were evaluated with either *LSD* (pairwise comparisons of treatments) or Dunnett's multiple-comparison test (pairwise comparisons of treatments with the control)(Steel and Torrie, 1960). Linear regression analysis was performed with data of all individual experimental units to study the relationship between DI and DWR. Adjusted R^2 values were calculated for the relationship between DWR and the individual components of the DI and compared with the adjusted R^2 value of the relationship between DWR and DI.

Results

Reproduction of ARED symptoms

There was a consistent difference between plants grown in fresh soil and those grown in asparagus soil. In the fresh soils, root systems were the normal pale cream with many secondary roots and low numbers of lesions. In the asparagus soils, however, although all plants survived during the experimental period, many of the secondary roots had rotted, giving the root systems a sparse appearance. The root system was brown because of the many smaller and larger lesions on the main as well as the secondary roots. Thus, ARED symptoms were reproduced easily in all experiments.

In all instances in which fungi were isolated from roots with symptoms, *F. oxysporum* predominated among the isolates, on the selective medium and the general media. *Phoma terrestris* was isolated from a few plants of the outdoor pot experiment. The fungus was found on reddish discoloured rootlets (which were encountered occasionally) and brown rootlets. Pathogenicity tests showed *Phoma terrestris* and almost all isolates of *F. oxysporum* to be pathogenic on asparagus. Other fungi, which were isolated occasionally, were not pathogenic.

ARED symptoms were indicative of a lower root weight. The shoot weight was influenced far less, and therefore these data are not presented.

Vertical distribution of ARED-causing factor(s) in soil (Expt 1)

ARED symptoms were present on roots from all asparagus soil samples, irrespective of depth. The DI values are given in Fig. 1. There were some lesions on roots in fresh soil. The numbers were highest for the 0- to 30- cm layer, and in the deepest layer, there were no lesions. These results were reflected in the DI values of the fresh soils, which were slightly higher for the upper soil layers than the deeper ones.

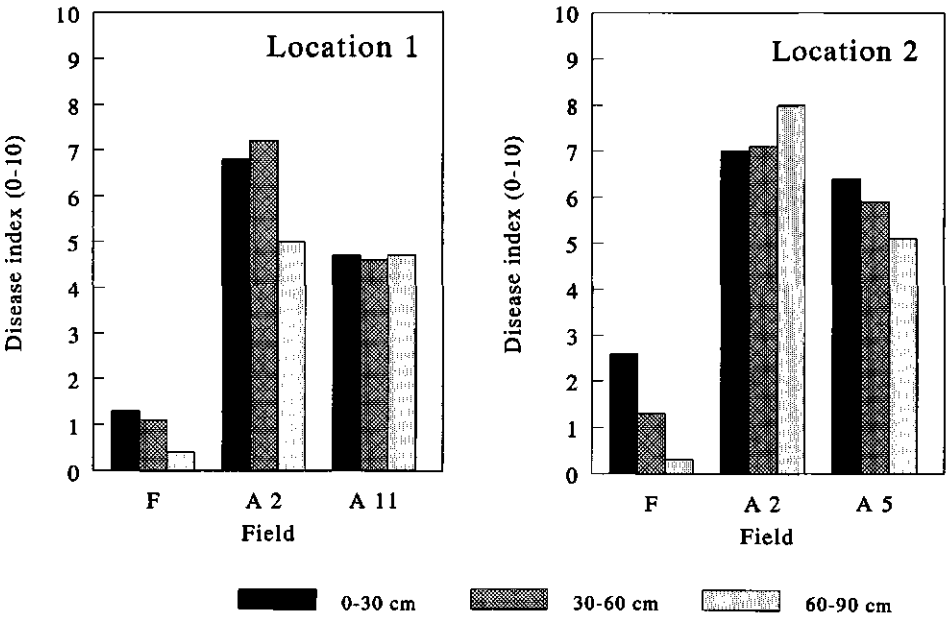


Figure 1. Occurrence of ARED-causing factor(s) in the soil profile (Expt 1). Disease severity for location 1 (left) and location 2 (right).
F= fresh soil; A= asparagus soil; the numbers indicate the number of years since asparagus production was stopped.

Effect of dilution of infested soil on disease severity (Expt 2).

There were typical ARED symptoms on plants in all four treatments with asparagus soil (asparagus treatments), whereas in fresh soil, few lesions were on the roots. For all asparagus treatments, the means for the DI were significantly higher, and for DWR significantly lower, than those for fresh soil (Table 3). Among the various asparagus treatments, the differences in DI were small and DWR were not significantly different. An indication of decreasing disease severity with decreasing rates of asparagus soil (dilution effect) was not found.

Effects of selective heat treatments of soil in a greenhouse experiment (Expt 3).

Results for DI and DWR are given in Fig. 2, and significance levels for both variables are given in Table 4. Disease severity of plants grown in untreated asparagus soil was significantly higher than that of plants grown in fresh soil. After a heat treatment at 60 or 65°C, disease severity in asparagus soil did not exceed that in fresh soil.

Table 3. Means for disease index and root dry weight of the soil dilution series experiment (Expt 2).

Treatment[1] A (%) : F (%)	Disease index (0 - 10)	Root dry weight (g pot^{-1})
100 : 0	4.9 [2]	7.79 [2]
80 : 20	6.0	7.30
50 : 50	5.7	7.79
20 : 80	4.8	6.36
0 : 100	2.5 *	11.91 *
LSD (Dunnett, α = 0.05)	1.2	3.94

[1] A = asparagus soil; F = fresh soil.
[2] Means followed by an asterisk are significantly different from the nondiluted asparagus soil (100% A), according to Dunnett's test ($P \leq 0.05$).

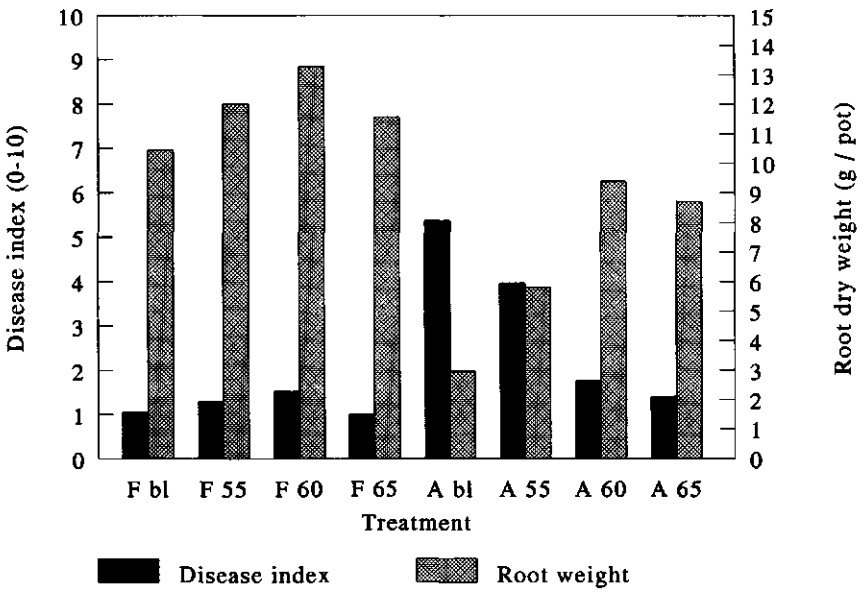


Figure 2. Effect of heat treatments of soil on disease index and root dry weight (Expt 3).
F = fresh soil; A = asparagus soil; bl = blank; 55 = 30 min at 55°C; 60 = 30 min at 60°C; 65 = 30 min at 65°C. For significance levels of contrasts, see Table 4.

Table 4. Significance levels (P) from analysis of variance for disease index and root dry weight for plants grown in heat-treated soil (Expt 3).

Source	Disease index	Root dry weight
Soil	< 0.001	< 0.001
Treatment	< 0.001	0.009
Block	0.003	0.946
Soil * Treatment	< 0.001	0.287
Contrasts:		
F bl vs. A bl	< 0.001	< 0.001
F 55 vs. A 55	< 0.001	0.002
F 60 vs. A 60	0.492	0.045
F 65 vs. A 65	0.145	0.146

From each of the eight treatments, three soil samples were assayed for *F. oxysporum*. After the 55°C treatment, the density of *F. oxysporum* had decreased to 10% of that in the untreated soil; after the 60°C treatment, the fungus was no longer detectable in the fresh soil and had decreased to very low levels in the asparagus soil (Table 5).

Table 5. Density of *Fusarium oxysporum* (Fo) in soil and fraction of isolates pathogenic to asparagus for the greenhouse experiment with heat treatments of soil (Expt 3).

Treatment	Cfu Fo g^{-1} soil Mean*	S.D.	# Pathogenic isolates / # Isolates tested
F blank	212.0	± 61.5	1 / 23
F 55	20.0	± 12.0	
F 60	< 4		
F 65	< 4		
A blank	1029.3	± 348.3	6 / 24
A 55	98.7	± 10.0	
A 60	17.3	± 12.2	7 / 13
A 65	< 4		

* The lower limit of detection was 4 cfu g^{-1} of soil; '< 4' means that no colonies were detected on the plates.

Effects of selective heat and fungicide treatments of soil in an outdoor pot experiment (Expt 4)

DI values are given in Fig. 3. For DI and DWR significance levels for factors, interaction, and contrasts are given in Table 6. We conclude from the results that

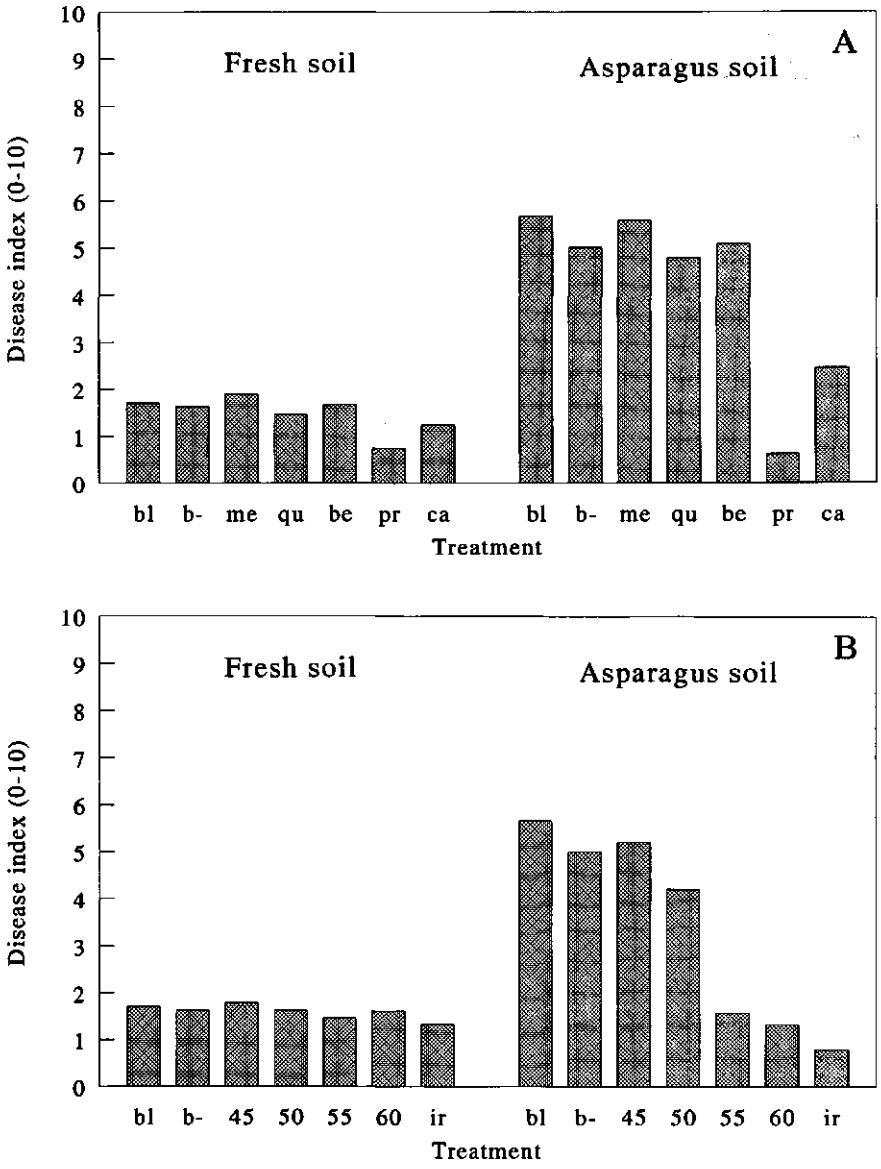


Figure 3. Effect of fungicide, heat treatments, and irradiation on disease index (Expt 4).
A: effects of fungicide treatments; B: effects of heat treatments and irradiation.
bl = blank (untreated); b- = blank, nonfertilized; me = metalaxyl; qu = quintozene; be = benomyl; pr = prochloraz; ca = captafol; 45 = 30 min at 45°C; 50 = 30 min at 50°C; 55 = 30 min at 55°C; 60 = 30 min at 60°C; ir = irradiation. For significance levels of contrasts, see Table 6.

Table 6. Significance levels (P) from analysis of variance for disease index and root dry weight data of the outdoor pot experiment with fungicide and heat treatments of soil (Expt 4). For a description of treatment codes, see Figure 3.

Source	Disease index	Root dry weight
Soil	< 0.001	0.163
Treatment	< 0.001	< 0.001
Block	< 0.001	0.009
Soil * Treatment	< 0.001	0.014
Contrasts:		
F bl vs. A bl	< 0.001	0.017
F b- vs. A b-	< 0.001	0.555
F me vs. A me	< 0.001	0.134
F qu vs. A qu	< 0.001	0.408
F be vs. A be	< 0.001	0.167
F pr vs. A pr	0.647	0.407
F ca vs. A ca	< 0.001	0.023
F 45 vs. A 45	< 0.001	0.047
F 50 vs. A 50	< 0.001	0.280
F 55 vs. A 55	0.647	0.877
F 60 vs. A 60	0.260	0.037
F ir vs. A ir	0.046	0.709

symptoms of ARED can be reproduced under field conditions. Plants grown in untreated asparagus soil were affected the same way as those grown under greenhouse conditions. ARED did not occur after application of prochloraz, heating at 55 or 60°C, or irradiation of the soil. Captafol reduced the DI significantly (*LSD*, $\alpha = 0.05$).

After the 50°C treatment, the density of *F. oxysporum* had decreased considerably; after the 55°C and 60°C treatment, *F. oxysporum* was no longer detectable (Table 7).

Relationship between disease severity and root dry weight

For Expt 1 DWR for the fresh soil from location 1 were lower than expected. From information obtained afterward, we concluded that the most probable cause was herbicide residues. Therefore, data from this field were excluded from the regression analysis. For all four experiments, the linear regression of DWR on DI was highly significant ($P < 0.001$), with adjusted R^2 at 0.50 ($n = 87$), 0.34 ($n = 35$), 0.37 ($n = 55$), and 0.07 ($n = 188$) for Expts 1, 2, 3 and 4, respectively. The slopes of the regression lines for the three greenhouse experiments (Expts 1-3) were not significantly different at $P = 0.05$ and, therefore, data from these experiments were pooled and a linear regression line was fitted to the pooled data (Fig. 4d). The calculated regression lines for the three experiments also

Table 7. Density of *Fusarium oxysporum* (Fo) in soil and fraction of isolates pathogenic to asparagus for the untreated soil and the heated asparagus soil of the outdoor pot experiment (Expt 4).

Treatment	Cfu Fo g^{-1} soil Mean*	S.D.	# Pathogenic isolates / # Isolates tested
F blank	687.0	± 20.7	0 / 20
A blank	773.3	± 21.2	4 / 20
A 45	1049.7	± 167.8	
A 50	114.3	± 54.6	
A 55	< 4		
A 60	< 4		

* The lower limit of detection was 4 cfu g^{-1} of soil; '< 4' means that no colonies were detected on the plates.

are shown separately (Fig. 4a, b, and c). The regression line obtained for the outdoor pot experiment (Expt 4) when DWR was regressed on DI was different from that for the greenhouse experiments (Fig. 4e), with an explained variance of only 7%. A main difference between the outdoor pot experiment and the greenhouse experiments is the higher overall level of DWR in the outdoor pot experiment.

For the relationship between DWR and DI or the single components of DI, generally DI explained variation in DWR best. Only for Expt 3 did the component LSR give a higher percentage explanation (57%) than the DI (38%).

Discussion

When asparagus soil was diluted with fresh soil, disease severity remained at the same level and there was no increase in root weight (Expt 2). These results provide evidence for involvement of a pathogen colonizing the soil and virtually exclude as an important ARED factor, direct growth inhibition by autotoxins of the preceding asparagus crop. This hypothesis is substantiated by the effects of selective heat treatments. Autotoxins of asparagus are very heat-stable, as was reported by Yang (1982). The fact that ARED was nullified by heat treatments at 55-60°C and treatment with prochloraz excludes autotoxins as a cause of ARED. In all experiments Foa was isolated from brown lesions, which corresponds with the results of an analysis of the most common soil-borne fungal pathogens of asparagus in the Netherlands, where Foa was the most prevalent and virulent

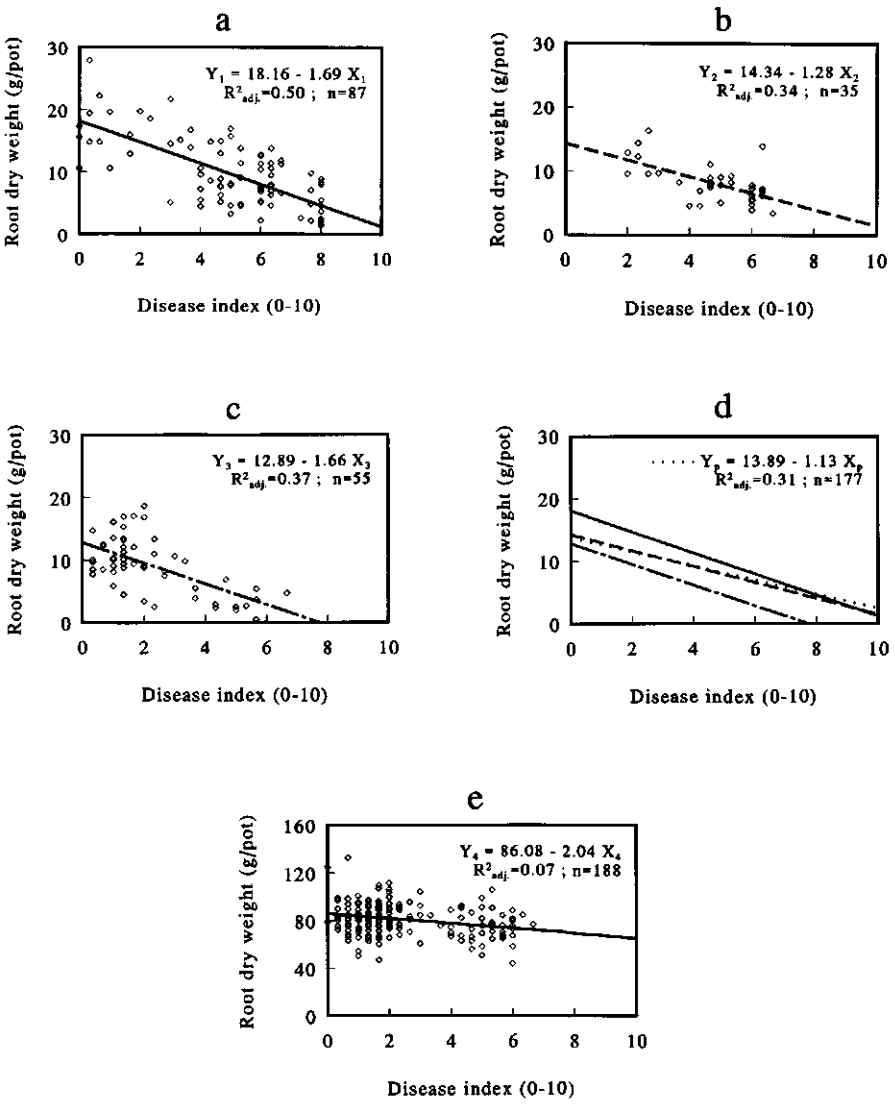


Figure 4. Relationship between root dry weight and disease index.
a, b, and c: scatter plots with linear regression lines for Expts 1, 2, and 3, respectively; d: regression lines for Expts 1, 2, and 3 and for the pooled data of these experiments (Y_p); e: scatter plot with linear regression line for Expt 4.

species (Blok & Bollen, 1995). All selective soil treatments that prevented brown lesions caused by Foa resulted in healthy root systems similar to those produced in fresh soil. The fact that ARED symptoms were reproduced under greenhouse and field conditions indicates that results obtained in the greenhouse are indicative of what occurs in the field. These arguments provide conclusive evidence that Foa is the main cause of ARED.

In a previous study we concluded that direct growth inhibition by autotoxins cannot be considered a major cause of ARED (Blok and Bollen, 1993); adding enough sterilized asparagus roots to field soil to equal that found after termination of an asparagus crop did not result in a significant yield decrease. However, it is possible that autotoxic compounds are more persistent than root residues and that after decomposition of the roots, they remain bound to soil organic matter. In the present study asparagus soils were used, and autotoxins in root residues as well as those bound to organic matter could have affected the plants. The amount of asparagus root residues retrieved from the soils (Table 1) is similar to that found previously (Blok and Bollen, 1993).

We realize that assessments of nutrient deficiencies and deterioration of soil structure, two factors that are mentioned frequently as causes of replant diseases (Savory, 1966), were excluded from this study. Because of the meticulous asparagus-field preparation practised in the Netherlands, it is unlikely that these factors would be involved in the cause of ARED. It is common practice to plough the soil to a depth of 80 cm before asparagus is (re)planted to allow deep rooting. To restore soil fertility, one or two green manure crops are grown and soil is amended with large amounts of compost before planting. During crop growth organic and inorganic compound fertilizers are given each year.

The conclusion that Foa is the main cause of ARED implies that ARED and early decline are closely related phenomena. A difference that remains is that *F. moniliforme* and *F. proliferatum* are not involved in ARED (Blok & Bollen, 1995; this study) whereas these pathogens are reported to play a major role in early decline (Endo & Burkholder, 1971; Johnston *et al.*, 1979; Gindrat *et al.*, 1984; Elmer, 1990; Bousquet, 1993; Schreuder *et al.*, 1995). Another difference is that ARED as it occurs in the Netherlands, is confined to replant situations whereas early decline occurs also in fields with a first asparagus crop (Grogan & Kimble, 1959). That ARED is confined to replant situations is probably because *F. moniliforme* and *F. proliferatum* are not present in these situations. A second factor could be that in the Netherlands, asparagus is grown almost exclusively on fields that are very suitable for this crop. Suitable soil allows vigourous crop development, making problems with Foa less severe and economic life satisfactory. It is only when young plants are planted in heavily infested soil, as is the case in a replant situation, that problems become severe and economic life is significantly shortened. The conclusion that early decline and ARED are closely related, largely removes the need to maintain the term ARED; it can be substituted adequately by: asparagus crown and root rot caused by Foa.

The effect of heat treatments on the density of *F. oxysporum* was different in Expts 3 and 4. Whereas 10% of the Foa population survived the 55°C treatment in Expt 3, the same treatment completely eliminated the population in Expt 4. An explanation might be a difference in metabolic activity of the soil microflora caused by a difference in nutrient status of the soil. A high metabolic activity renders microorganisms more sensitive to heat (Bollen, 1974). For both experiments the DI was related to the density of *F. oxysporum*. From the densities of *F. oxysporum* obtained from untreated asparagus soil and the fraction of isolates pathogenic to asparagus (Tables 5 and 7), the density of Foa in field soil one to two years after asparagus production is estimated at 150-250 cfu g^{-1} dry soil. The Foa population in the root residues was not quantified in the assay.

It is remarkable that benomyl did not prevent lesions caused by Foa, which was also found in an experiment conducted under greenhouse conditions (unpublished data). In the Netherlands, benomyl and the related compound carbendazim are used widely to disinfest crowns before planting. Its efficacy, based on the present results, is questionable. In the literature, successful and unsuccessful benomyl control of fusarium diseases have been reported. Successful control was obtained by Manning and Vardaro (1977), who used preplant crown soaks against Foa, and by Greenhalgh and Clarke (1985), who used soil drenches to prevent selectively fusarium root rot of subterranean clover. Smiley and Craven (1979), however, were not able to control fusarium with benomyl soil drenches in turfgrass.

The DI used to combine the three different parameters of disease severity is satisfactory because, for most experiments, the percentage variance explained in DWR is higher for the index than for single parameters. When young asparagus plants were tested under similar conditions in the greenhouse, the regression lines obtained when DWR was regressed on DI were similar, with percentage variance explained ranging from 34-50%. With an overall higher DWR in the outdoor pot experiment, the percentage was only 7%. In contrast to what generally was found in the greenhouse experiments, relative differences in root weight between fresh and asparagus soil for the outdoor pot experiment were slight. The difference may have been caused by the larger pot volume available in the outdoor pot experiment, which allowed plants to grow vigorously during the whole experimental period, delaying infection and colonization and extending the period that the roots actively took up water and nutrients. This explanation is consistent with several authors' opinion that fusarium foot and root rot is stress-related (Damicone and Manning, 1985; Nigh, 1990).

Phoma terrestris was isolated from a few plants grown under field conditions (Expt 4), which confirms our earlier finding (Blok and Bollen, 1995) that this warm-region pathogen is present in the Netherlands.

ARED has been reported for fields in which the asparagus crop was abandoned some 20 to 25 years previously (Boonen *et al.*, 1977). High persistence of ARED was established

also in Expt 1, in which ARED symptoms appeared on plants grown in soil from a field where asparagus production was terminated 11 years before. Poll and Huiskamp (1992) also detected ARED in samples from fields where asparagus production was terminated 20 to 25 years before.

Of the four asparagus soils used in Expt 1, ARED was detected in all three soil layers. There was no decrease in disease severity with increasing soil depth. The fact that ARED-causing organisms are found to at least one meter deep needs to be considered when control strategies are developed. Grogan and Kimble (1959) found Foa, which they considered a major cause of early decline, at a depth of 1.5 m.

The conclusion that Foa is the main cause of ARED in the Netherlands prompts us to concentrate our research on epidemiology and control methods for this pathogen.

Chapter 5

Interactions of asparagus root tissue with soil microorganisms as a factor in early decline of asparagus

Abstract

Sterilized root residues of asparagus added at a rate of up to 20 g kg^{-1} fresh soil did not influence severity of root and crown rot caused by *Fusarium oxysporum* f.sp. *asparagi* (Foa). Root residues accumulated in field soil during asparagus growing for 10 y did not influence disease severity either. Inoculation of this soil with laboratory-prepared Foa after treatment at 65°C (30 min), at which the indigenous pathogen was killed but toxic substances present in asparagus root residues were left undamaged, led to the same disease severity as inoculation of similarly-treated fresh soil.
On soil extract agar, aqueous root extracts of asparagus but not those of other crops retarded growth of 31 out of 112 fungal isolates from a range of taxa. Sensitive fungi included *Gliocladium* spp. and *Trichoderma harzianum*, but not Foa.
Colonization of Foa infested soil by *Fusarium* species was greatly enhanced by addition of root material from asparagus, Brussels sprouts, and chicory, but not by that from strawberry and perennial rye-grass. As the fraction of Foa among the *Fusarium* population was small, it is concluded that competitive saprophytic ability of the pathogen is far less than that of the nonpathogenic *Fusarium* species. Fungistasis to Foa was not or was only slightly reduced in soils amended with root residues.

In contrast to data reported in the literature, the present results do not suggest an appreciable increase of Foa root rot, or of the Foa population in soils, due to substances present in root residues.

Introduction

Early decline of asparagus (*A. officinalis*) plantings is a common phenomenon in asparagus production (Grogan and Kimble, 1959; Nourisseau *et al.*, 1982; Damicone and Manning, 1985; Gordon-Lennox and Gindrat, 1987; Schreuder *et al.*, 1995; Blok and Bollen, 1996a). The disease occurs generally in plantings on former asparagus land but can also occur in plantings on fresh land. In regions where the area of fresh land is limited, early decline is a real threat to economic asparagus production.

The main factor in early decline is root and crown rot (Schofield, 1991) caused by *Fusarium oxysporum* f.sp. *asparagi* (Cohen and Heald, 1941; Graham, 1955), *F. moniliforme* (Johnston *et al.*, 1979) or *F. proliferatum* (Elmer, 1990).

In addition to pathogens, toxic compounds have been suggested as involved in early decline. Asparagus root tissue contains auto- and allotoxic compounds that inhibit growth of seedlings of asparagus and other plant species (Laufer and Garrison, 1977; Yang, 1982; Young and Chou, 1985; Hartung *et al.*, 1989; Hazebroek *et al.*, 1989). Shafer and Garrison (1986) found that inhibitory effects of macerated root tissue added to soil were

nullified within 90 days after incorporation, indicating that the phytotoxic compounds are inactivated or leached fairly soon in soil. Blok and Bollen (1993), however, found that extracts of asparagus root residues from fields where production was terminated 10 years before sampling still inhibited radicle growth, demonstrating that autotoxic compounds were persistent under field conditions as they were protected from microbial breakdown or leaching when embedded in root tissue.

Hartung and Stephens (1983) and Hartung *et al.* (1989) found that addition of sterilized asparagus roots to soil increased severity of root rot caused by *F. oxysporum* f.sp. *asparagi* (Foa) and *F. moniliforme*. Peirce and Colby (1987) reported that autotoxins in root extracts predispose radicles to infection by Foa and that, at the same time, nutrients in the extracts enhance pathogen growth in the rhizosphere.

Asparagus root tissue selectively affects growth of the microflora. *In vitro*, it inhibited growth of *Pythium* spp. and *Bacillus* spp., but not that of Foa, *F. moniliforme* and *Rhizoctonia solani* (Hartung and Stephens, 1983; Hartung, 1987). Chemicals in asparagus roots may also exert a selective effect on microbial antagonists of Foa and other pathogens. Several research workers have tried to identify the autotoxic substances. In bio-assays, methylenedioxycinnamic acid (Hartung, Nair and Putnam, 1990), caffeic acid (Miller *et al.*, 1991) and tryptophan (Lake *et al.*, 1993) were toxic to asparagus. Probably, autotoxicity is induced by a combination of compounds.

A study on the etiology of the disease under Dutch conditions revealed that crown and root rot incited by Foa was the main cause and that growth inhibition by autotoxins from root residues of a preceding asparagus crop plays a minor role at most (Blok and Bollen, 1993, 1996a). Root residues which remain in soil after an asparagus crop is terminated are a major source of Foa inoculum (Blok and Bollen, 1996b). The question addressed in the present study is whether an interaction between residual root tissue and Foa or the soil microflora is an important factor determining the severity of *Fusarium* crown and root rot in replant situations.

Materials and methods

General methods

Plant material and nutrition. Seeds of asparagus cv. Gynlim were disinfested in a solution of benomyl in acetone (Damicone *et al.*, 1981). The seeds were sown in a commercial potting mixture (Trio-17, decomposed sphagnum peat with clay and marl; pH-KCl 5.4; organic-matter content 70%), either directly or after pregermination on moist filter paper under sterile conditions at 22-25°C for 5-7 days. After several weeks at 20-25°C in the greenhouse, seedlings were uprooted, culled to a uniform size and planted in pots. Unless stated otherwise, each pot received 100 ml of a solution containing 1.67 g Nutriflora-T l^{-1}

and 2.0 g calcium nitrate l^{-1} (pH 6.7) every 10-14 days. The solution contains in mg l^{-1}: N, 344; P, 78; K, 560; Mg, 50; Ca, 340; S, 206; Fe, 1.2; Mn 2.2; B, 0.7; Zn, 0.5; Cu, 0.020; Mo, 0.116.

Inoculum production. Inoculum was produced in a soil-meal medium consisting of commercial potting mixture (Trio-17) and 5% (fresh wt/fresh wt) oat meal. Erlenmeyer flasks (500 ml) containing 250 ml of soil-meal mixture were autoclaved twice on consecutive days, inoculated with three plugs from growing mycelium of Foa on potato dextrose agar (PDA), and incubated for two to four weeks at 23°C. The same isolate of Foa was used for all experiments (CWB 1).

Assessment of contamination of root material with Fusarium *spp.*. To confirm that root material introduced into the soil was free of *Fusarium* spp., some material of each sample was sprinkled on plates (five per sample) with *Fusarium* selective Komada's medium (Komada, 1975). After incubation at 23°C for 14 days, plates were assessed for *Fusarium* colonies.

Disease rating. Symptoms on primary (DPR) and secondary roots (DSR) were rated on a scale of 0-5 where 0 = no lesions, 1 = 0-5% of the root length covered with lesions, 2 = 6-20%, 3 = 21-60%, 4 = 61-95%, and 5 = >95% or dead plants. The loss of secondary roots (LSR) was rated on an arbitrary scale from 0 (abundant secondary roots, no loss) to 10 (no secondary roots present, total loss). Based on the ratings for DPR, DSR, and LSR, a disease index (DI) ranging from 0-10 was calculated (Blok and Bollen, 1996a). DI was calculated as: DI = (2.DPR + 2.DSR + LSR) / 3. Dry weight of roots was determined after drying for 24 h at 105°C.

Assay of fungistatic activity of the soil microflora (fungistasis test). Fungistasis was determined by measuring the inhibition of mycelial growth according to Davet (1976), who slightly modified the method described by Williams and Willis (1962). Cellophane membrane (Cuprophan, Enka; thickness 17.5μm) was tightened over aluminium rings (diameter 75 mm, height 8 mm) with a rubber band. The rings were autoclaved in glass Petri dishes in a shallow layer of distilled water. Then the water was removed and the rings were filled with 25 ml soil and, subsequently, 30 ml lukewarm water agar (1.2%) to form a solid cake, sticking to the membrane. To allow compounds to diffuse to the membrane, the rings were incubated with the membranes downward, for one to two days at 23°C. Thereafter, the rings were turned and the membranes centrally inoculated with a 5-mm PDA disc with growing mycelium of Foa, isolate CWB 1. After five to seven days at 23°C, outgrowth of Foa was determined by measuring the colony diameter on two perpendicular lines. For all samples tested, a control with autoclaved soil (twice on

consecutive days) was included. Each sample was tested in five rings. A fungistasis index was calculated as: 1 - [(diameter of colony on nonautoclaved soil) / (diameter of colony on autoclaved soil)]. This index ranges from 0 (no fungistasis) to 1 (complete fungistasis).

Determination of soil colonization by Fusarium *spp.* In order to select for presence of *Fusarium* as mycelium rather than conidia a soil washing technique (Van Emden, 1971) was used. Approx. 80 ml soil was washed over sieves with 0.75- and 0.45-mm mesh, respectively. Soil particles retained on the latter sieve were washed three times for five minutes; the first two occasions in tap water and finally in sterile distilled water. For washing, the sieve (Ø 6 cm) was placed on a wire frame in a large beaker filled with water. The water inside the sieve was agitated by means of a vibrating vertical rod ending in a horizontal plate with wells (Vibro Mischer, Chemap AG, Männedorf, Switzerland). After blotting on sterile filter paper, 100 organic soil particles were placed on Komada's medium with 10 particles per plate. After incubation at 23°C for 7-10 days, the number of particles with outgrowth of *Fusarium* spp. was determined. The isolates were tested for pathogenicity on asparagus plantlets grown under aseptic conditions in culture tubes with Knop's agar (Stephens and Elmer, 1988; slightly modified by Blok and Bollen, 1995).

Experimental procedures: effect of root residues on severity of Foa root rot (Expts 1-3)

Experiment 1. Effect of chopped root residues on disease severity. Asparagus root material was incorporated into soil where asparagus had not previously been grown (fresh soil) at rates of 0, 1.5, and 15 g (dry wt) kg^{-1} dry soil. As a control for the effect of addition of organic matter, a treatment with strawberry roots (15 g l^{-1}) was included. Asparagus roots came from one-year-old field-grown plants, strawberry roots came from one-year-old plants grown in soilless culture in the greenhouse. Roots were thoroughly washed, dried at 60°C for 48 h, and chopped to 1-2 cm pieces.

The soil was a loamy sand from the experimental farm Meterikse Veld at Horst, with pH-KCl 6.0 and an organic matter content of 2.5%. The four treatments with different levels of root material were combined with three levels of Foa infestation. The three levels of Foa infestation were reached by leaving the soil uninfested or incorporating 0.01% or 0.1% (fresh wt/fresh wt) of soil-meal inoculum. For each treatment, soil was divided over seven 4-l pots and planted with three 12-week-old plants per pot. The pots were arranged in a randomized complete block design with seven blocks. After 16 weeks in the greenhouse at 20-30°C the experiment was concluded.

Experiment 2. Effect of milled root residues on disease severity. Asparagus root material was incorporated into fresh field soil at rates of 0, 5, 10 and 20 g (dry wt) kg^{-1} dry soil. A treatment with strawberry roots (20 g kg^{-1} soil) served as a control. Asparagus roots were

storage roots from eight-year-old, greenhouse-grown plants. Strawberry roots came from one-year-old, field-grown plants. The roots were washed thoroughly, cut in 5-10 cm pieces, dried for seven days at 50°C, ground in a mill with 1-mm mesh and sterilized by gamma-irradiation (2.5 Mrad). The soil was a loamy sand with pH-KCl 7.7 and an organic matter content of 3.3%, collected from an experimental field at Wageningen. The five levels of root material were combined with the same Foa levels as in Expt 1. The soil of each treatment was divided over eight 1.5-l pots and planted with two five-week-old plants per pot. The pots were arranged in a randomized complete block design with eight blocks, in a climate chamber at 20°C. Each pot received four applications of 50 ml of the nutrient solution as described for the other experiments. The experiment was concluded after 11 weeks.

Experiment 3. Effect of root residues accumulated in soil after ten years of asparagus production on disease severity. Soil was collected at the experimental farm Meterikse Veld, from a field with fresh soil and from a field where a 10-year-old asparagus crop had been removed two years previously. Both soils were sandy loams and were similar in respect to pH-KCl (6.1-6.4) and organic matter content (2.8-3.2%). The soils were heated for 30 min at 65°C (± 2°C) and subsequently supplied either with 2.0% (fresh wt/fresh wt) soil-meal inoculum of Foa or with 2.0% autoclaved inoculum as a control. Untreated fresh and asparagus soils served as references for health status in field soils. The soils were heated with aerated steam in a slightly modified version of the apparatus described by Aldrich and Nelson (1969). By heating at 65°C *F. oxysporum* will be killed (Bollen, 1985) but the autotoxins will not be inactivated (Yang, 1982). The soil was divided over seven 4-l pots and planted with three 6-week-old plants per pot. Pots were arranged in a randomized complete block design in a greenhouse at 20-25°C. After 14 weeks the experiment was concluded.

Experimental procedures: effects of root tissue extracts on fungal growth on soil extract agar (Expt 4)

This experiment studied the effect of root extracts on *in vitro* growth of fungal isolates from field soil. Soil fungi were isolated from four fields located in the asparagus growing region. Soil dilutions were plated onto plates with malt extract agar supplemented with oxytetracycline (50 μg ml^{-1}) and validamycine (100 μg ml^{-1}, added as Solacol according to Gams and Van Laar, 1982). Visually distinguishable colonies were subcultured onto PDA slants, allowed to grow for one week at 25°C and stored at 4°C for later identification and testing.

Effects of extracts of root materials on the fungi were tested on plates with soil extract agar (SEA). SEA was made by autoclaving 1 kg of a common potting mixture (Trio-17) together with 1 l of tap water, followed by centrifugation during 30 min at 3400 g. The

supernatant was diluted 1:1 (v/v) with tap water supplemented with K_2HPO_4 (0.2 g l^{-1}) and agar (15 g l^{-1}), and autoclaved. In each SEA plate eight wells (∅ 5 mm) were punched in a circle with ∅ 6 cm for fast growing fungi or 3 cm for slow growing species (Fig. 3). Each well received 10 μl of sterile distilled water or an aqueous extract of asparagus, strawberry or chicory roots; samples of the same extract were placed in pairs of opposite wells. The roots came from field grown plants that were untreated with fungicides. Roots were washed thoroughly and healthy roots were selected. These were cut in 3-5 cm pieces, dried for 24 h at 50°C, and milled through a 1-mm mesh screen.

Extracts were produced by shaking 75 g (dry wt) in one l distilled water during 24 h. The suspensions were filtered through four membrane filters with a decreasing mesh size. The extracts were sterilized by filtering through a membrane filter with a 0.22-μm mesh. After addition of the extracts, the plates were inoculated by placing a 5-mm disk of SEA with growing mycelium of the fungal isolate to be tested in the centre of the plate. Each isolate was tested on two plates incubated at 25°C. The effect of the extracts on growth was assessed when the mycelium had reached the control wells which received water.

Experimental procedures: effects of root tissues or their extracts on soil fungistatic activity to Foa and colonization of soil by *Fusarium* spp. (Expts 5-9)

Experiment 5. Effect of milled root material of asparagus, chicory and strawberry on fungistasis to Foa and colonization of soil by Fusarium *spp.*. A sandy loam soil without a history of asparagus cropping, the same as used for Expt 3, was left unamended or amended with milled, dried root material of asparagus, chicory or strawberry at a rate of 2.5 g (dry wt) kg^{-1} soil. The root material came from field grown plants that were free of fungicides. Roots were washed thoroughly, dried for 48 h at 60°C, and milled through a 1-mm mesh screen. For each treatment, three 2.5-l plastic beakers per treatment were filled with 2 l of soil, wrapped in aluminium foil, covered with parafilm to minimize evaporation losses, and incubated at 15°C. At regular intervals, pots were weighed and distilled water added to compensate for evaporation losses. Immediately after incorporation of the root materials, and after 4, 8, 12 and 24 weeks, samples were collected from each beaker and tested for fungistatic activity of the soil microflora.

The effect on colonization of soil by *Fusarium* spp. was studied by preparing the same soil mixtures and inoculating with a spore suspension of Foa CWB 1 at a density of 10^6 spores l^{-1} soil. Colonization of soil by *Fusarium* spp. was determined for the nonamended control pots before inoculation and immediately after inoculation. After 12 weeks, soil colonization was determined for all pots.

Experiment 6. Effect of root extracts of asparagus, chicory or strawberry on colonization of soil by Fusarium *spp.*. Fresh sandy loam soil of the same experimental farm but from another field as that used in Expt 3 was air-dried and supplied with aqueous extracts of

asparagus, strawberry or chicory root material at a rate of 20 g kg^{-1} soil (dry wt/dry wt) equivalent. Sterile, distilled water served as a control. The extracts were made from the same material as in Expt 5 (chicory) or from similar but fresh material (asparagus and strawberry). Milled root material was shaken in distilled water for 24 h. For asparagus and strawberry, 40 g root material was extracted in 220 ml of distilled water. Because root residues of chicory formed a gel at these proportions, a double amount of water was used and, after extraction, the volume of the extract was reduced by half using a rotary evaporator. The extracts were strained through double cheese cloth and added to the air-dried soil. Additional distilled water was added to reach a water content of 12% (pF 1.9). For each treatment, nine plastic beakers were filled with 300 ml of soil, covered with parafilm and incubated at 18°C. The beakers were regularly weighed and distilled water added to compensate for evaporation losses. After 4, 8, and 12 weeks, three beakers per treatment were harvested and the soil assayed for fungistatic activity.

Colonization of the soil by *Fusarium* spp. was studied using the same treatments but with soil inoculated with a spore suspension of Foa CWB 1 at a density of 10^6 spores l^{-1} of soil. The soil was incubated as described. After 14 weeks, soil colonization was determined.

Experiments 7 and 8. Effect of plant extracts of five crops on fungistasis to Foa and on colonization of soil by Fusarium *spp.*. In both experiments, a fresh sandy loam was used, but the samples came from different fields. In Expt 7 samples were collected from the same field as in Expt 3 and in Expt 8 from a neighbouring field. The soil was air-dried and infested with Foa CWB 1 at a density of 2000 colony forming units g^{-1} of soil by incorporating talcum inoculum. Extracts of asparagus, strawberry, chicory, Brussels sprouts and perennial rye-grass were incorporated into the soil at a rate of 20 g kg^{-1} soil (dry wt/dry wt) equivalent. The source material of asparagus, strawberry, and chicory was the same as for Expt 6. The Brussels sprouts material consisted of roots, stems and leaves, that of perennial rye-grass consisted of roots and leaves. Material of Brussels sprouts and perennial rye-grass was dried for two days at 60°C and milled through a 1-mm mesh screen. The plant materials were extracted by shaking them in distilled water (30 g (dry wt) in 200 ml water) during 24 h, centrifuging during 30 min at 3400 g and subsequent straining through a double layer of cheese cloth.

The extracts were mixed through air-dried soil and additional distilled water was added to reach a water content of 12% (pF 1.9). For each treatment, five plastic beakers were filled with 300 ml of soil, covered with a layer of sterilized gravel to decrease evaporation losses, and incubated at 20°C. Evaporation losses were compensated for by regular addition of distilled water. Before infestation with Foa and immediately after infestation, soil colonization by *Fusarium* spp. was determined for the nonamended control pots. In

Expt 7, soil colonization was determined for all pots after 6 weeks; in Expt 8, soil colonization and fungistatic activity was determined for all pots after 12 weeks.

Experiment 9. Effect of accumulated root residues in fields with different history of asparagus cropping on fungistasis to Foa. Soil was collected from six fields on the experimental farm Meterikse Veld. The soil was a loamy sand similar to that described for Expt 3. The fields differed in their history of asparagus cropping: F1 and F2 had no history of asparagus cropping; A1 had a 4.5-year-old standing crop; A2 had a 8.5-year-old standing crop; fA1 had been grown with asparagus for five years and production was terminated 1.5 year before sampling; fA2 had been grown with asparagus for 13 years and production was terminated 3 years before sampling. On F1, F2, fA1 and fA2, five random samples were collected from the 50-60 cm soil layer. On A1 and A2, five random samples were collected between the rows and five within the rows, all from the 50-60 cm layer. For all samples, the fungistatic activity of the soil microflora was determined as previously described.

Data analysis

Experiments were made as completely randomized designs unless indicated otherwise.

After checks for normality and homogeneity of variances, data were subjected to analysis of variance. Preplanned comparisons were evaluated either by testing single linear contrasts with F-tests (Expts 1 and 2) or by decomposing the treatment sum of squares into a set of mutually orthogonal contrasts (Expt 3). Comparisons that were not preplanned were evaluated with either Tukey's (pairwise comparisons of treatment means) or Dunnett's (pairwise comparisons of treatment means with control) multiple-comparison procedure (Steel and Torrie, 1980).

Results

Effect of root residues on severity of Foa root rot (Expts 1-3)

In Expts 1 and 2, root material was added to non-sterilized field soil. None of the samples of the material plated onto Komada's medium yielded *Fusarium* colonies.

The highest level of artificial infestation of the soil with Foa resulted in mean disease-index values ranging from 5.6 to 5.9 for Expt 1 and from 3.8 to 4.6 for Expt 2. These values are rather similar to the disease-index value of the naturally infested asparagus soil used in Expt 3 that was 5.6.

The size of the plant fragments incorporated into the soil might affect results as it affects the rapidity with which autotoxins are made available to the plant and are subjected

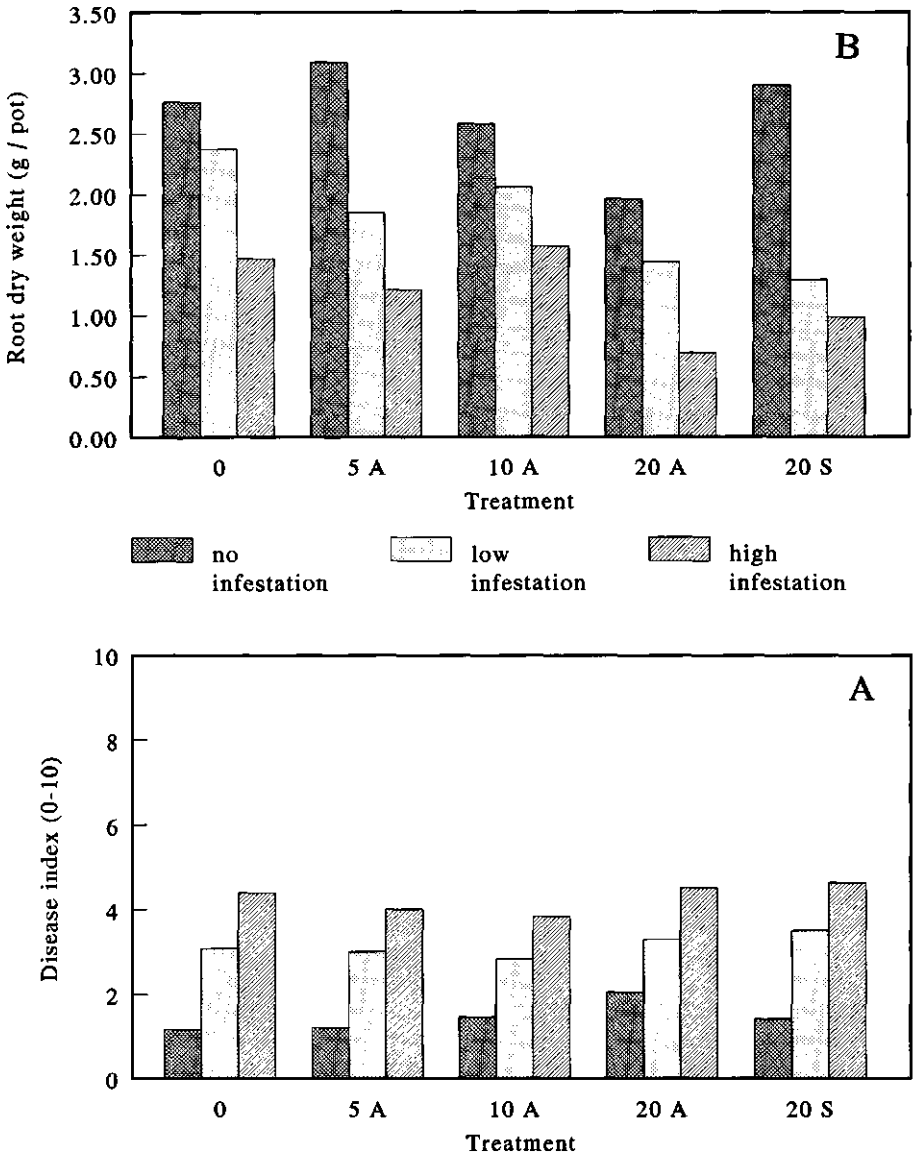


Fig. 1. Disease ratings and root dry weight of asparagus plants grown in fresh field soil supplied with milled, sterilized asparagus or strawberry root material, and either left uninfested or infested at a low or a high level with Foa (Expt 2).
Legend: A = asparagus; S = strawberry; numbers indicate amount of root material in g kg^{-1} soil (dw dw^{-1}).
a) data for disease index **b)** data for root dry weight.
Statistical analyses in Tables 1 and 2, respectively.

Table 1. Summary of analysis of variance and contrasts of disease-index data for Expt 2.

Source of variation and contrasts	Df	F	*P*
Amendments	4	3.83	0.006
Infestation levels	2	188.99	<0.001
Blocks	7	1.48	0.184
Amendments x infestation levels	8	0.95	0.480
Error	98		
Contrasts[1]:			
(asparagus amendments)$_{lin.}$ x no infestation	1	8.74	0.004
(asparagus amendments)$_{quadr.}$ x no infestation	1	0.43	0.514
(20 asp. vs 20 strawb.) x no infestation	1	3.70	0.057
(asparagus amendments)$_{lin.}$ x low infestation	1	0.48	0.490
(asparagus amendments)$_{quadr.}$ x low infestation	1	1.40	0.240
(20 asp. vs 20 strawb.) x low infestation	1	0.41	0.523
(asparagus amendments)$_{lin.}$ x high infestation	1	0.39	0.534
(asparagus amendments)$_{quadr.}$ x high infestation	1	5.12	0.026
(20 asp. vs 20 strawb.) x high infestation	1	0.15	0.699

[1] Linear and quadratic effects were tested for the treatments with 0, 5, 10 and 20 g asparagus root material kg^{-1} soil and 20 g asparagus root material kg^{-1} was compared with 20 g strawberry root material kg^{-1} soil, for each of the three infestation levels.

to leaching and microbial break-down. Therefore, both plant material chopped into 1-2 cm pieces and milled plant material were tested in Expt 1 and 2, respectively. Incorporation of asparagus or strawberry root material into the artificially infested soil did not give higher disease levels in either of the two experiments. In Expt 1 the disease-index values for the highest level of Foa-infestation were 5.6, 5.8, 5.9, and 5.7 for treatments with 0, 1.5, and 15.0 g kg^{-1} asparagus roots, and 15.0 g kg^{-1} strawberry roots, respectively. As the design and results of Expt 1 and 2 were similar, only the results of Expt 2, being the most comprehensive one, are presented in detail (Fig. 1a and Table 1). In the uninfested control treatments, the disease-index value increased with increasing amounts of asparagus roots, but not with strawberry roots. This increase was due to a lower number of secondary roots and not to a higher number of lesions on primary or secondary roots (data not shown) suggesting inhibition of secondary-root growth by autotoxic substances. Such an inhibition was not observed in Foa-infested soils, probably because Foa interfered more with secondary-root growth than did autotoxins. Therefore, the disease index can still be used as a measure for comparing root rot severity in the infested treatments. When the relevant contrasts for amendments at the three infestation levels are studied, it becomes

Table 2. Summary of analysis of variance and contrasts of root dry weight data for Expt 2.

Source of variation and contrasts	Df	F	*P*
Amendments	4	3.74	0.007
Infestation levels	2	29.61	<0.001
Blocks	7	1.79	0.097
Amendments x infestation levels	8	0.91	0.513
Error	98		
Contrasts[1]:			
(asparagus amendments)$_{lin.}$ x no infestation	1	5.40	0.022
(asparagus amendments)$_{quadr.}$ x no infestation	1	1.07	0.305
(20 asp. vs 20 strawb.) x no infestation	1	4.80	0.031
(asparagus amendments)$_{lin.}$ x low infestation	1	3.92	0.051
(asparagus amendments)$_{quadr.}$ x low infestation	1	<0.01	0.969
(20 asp. vs 20 strawb.) x low infestation	1	0.12	0.728
(asparagus amendments)$_{lin.}$ x high infestation	1	2.94	0.090
(asparagus amendments)$_{quadr.}$ x high infestation	1	0.95	0.332
(20 asp. vs 20 strawb.) x high infestation	1	0.46	0.501

[1] Linear and quadratic effects were tested for the treatments with 0, 5, 10 and 20 g asparagus root material kg^{-1} soil and 20 g asparagus root material kg^{-1} was compared with 20 g strawberry root material kg^{-1} soil, for each of the three infestation levels.

clear that the trend of increasing disease index in the uninfested treatments explains largely the significant main effect for amendments (Table 1).

Addition of root material of asparagus but not of strawberry to uninfested soil caused browning of roots and a significant decrease of root dry weight (Fig. 1b, Table 2).

In Expt 3, disease severity of the untreated fresh and asparagus soil differed significantly (Fig. 2, Table 3). When Foa was eliminated by heat treatment at 65°C and the soil left uninfested, the disease severity was not significantly different for fresh and asparagus soil and was similar to that of untreated fresh soil. When the heated soils were infested with Foa, the disease severity was not increased by the presence of root residues in asparagus soil. In conclusion, evidence for a direct effect of substances in asparagus root residues on severity of the disease was not obtained.

Effects of root tissue extracts on fungal growth on soil extract agar (Expt 4)

On soil dilution plates 112 fungal isolates belonging to 48 species were selected for the test on sensitivity of growth to root extracts. Around the wells with asparagus root extract growth was inhibited with 31 isolates belonging to 12 species (Fig. 3, Table 4). Growth was retarded, but not completely suppressed. None of the isolates was sensitive to root extracts of strawberry or chicory. Growth stimulation was not observed.

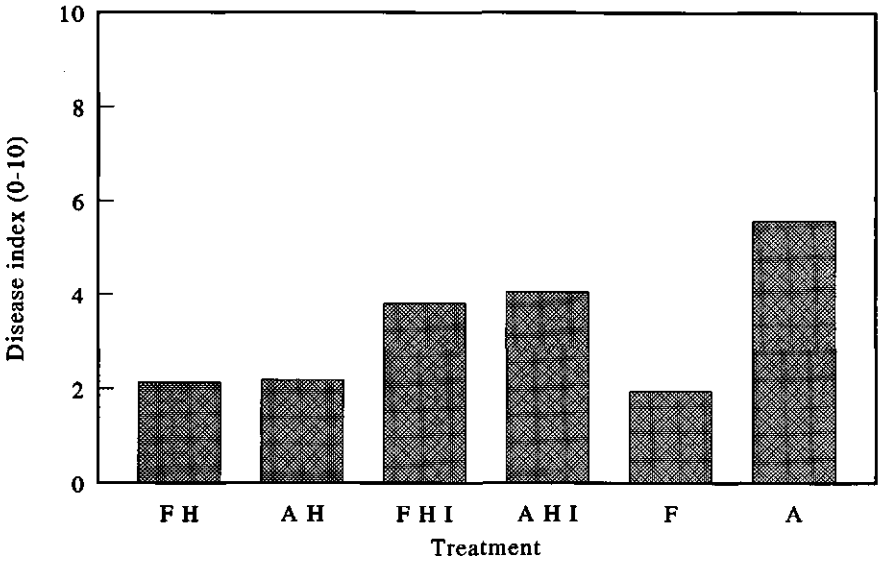


Fig. 2. Disease ratings of asparagus plants grown in heated fresh or asparagus soil, left uninfested or infested with Foa. Untreated fresh and asparagus soils served as controls (Expt 3).
Legend: F= fresh soil; A= asparagus soil; H= heated (30 min at 65°C); I=infested.
Statistical analyses in Table 3.

Table 3. Summary of analysis of variance of disease-index data of Expt 3. The treatment sum of squares has been decomposed into single-df, orthogonal contrasts.

Source of variation	Df	F	P
Blocks	6	1.38	0.257
Treatments[1]	5	31.55	<0.001
FH vs AH	(1)	0.02	0.896
FHI vs AHI	(1)	0.43	0.516
F vs A	(1)	99.70	<0.001
FH, AH vs FHI, AHI	(1)	47.26	<0.001
FH, AH, FHI, AHI vs F, A	(1)	10.36	0.003
Error	30		

[1] Legend: F= fresh soil; A= asparagus soil; H= heated (30 min at 65°C); I=infested.

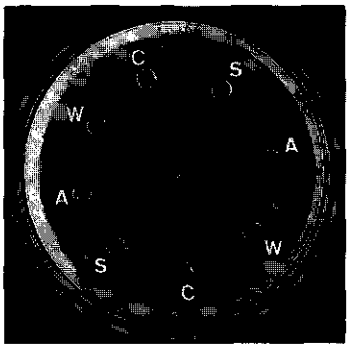


Fig. 3. SEA plate with eight wells filled with sterile distilled water as a control (W), or aqueous extracts of asparagus (A), strawberry (S), or chicory (C)(Expt 4). The fungus *Trichoderma harzianum* was inoculated centrally and shows mycelial growth inhibition near the wells that received asparagus extract.

Table 4. Effect of asparagus root extract on fungal growth on soil extract agar (Expt 4).

Species of which growth was inhibited by the asparagus root extract:

Gliocladium roseum (1/2)[1]	*P. nigricans* (1/1)
G. virens (1/1)	*Pythium* sp. I (1/1)
G. viride (1/1)	*Pythium* sp. II (1/1)
Mortierella alpina (1/1)	*Trichoderma harzianum* (11/11)
Paecilomyces variotii (1/1)	*Zygorrhynchus moelleri* (2/9)
Penicillium sp. I (1/1)	Unidentified species (1/1)

Species of which growth was not inhibited by the asparagus root extract:

Aspergillus fumigatus (1)[2]	*Mucor circinelloides* (1)
Cladosporium cladosporioides (1)	*Paecilomyces marquandii* (1)
Fusarium aquaeductuum (1)	*Penicillium* sp. II (1)
F. equiseti (4)	*Phoma* sp. I (6)
F. merismoides (2)	*Phoma eupyrena* (1)
F. oxysporum (6)	*Phoma terrestris* (8)
F. oxysporum np[3] (4)	*Plectosphaerella cucumeris* (3)
F. oxysporum f.sp. *asparagi* (4)	*Ulocladium atrum* (3)
F. sambucinum (5)	*Verticillium nigrescens* (3)
F. semitectum (1)	*V. tenerum* (1)
Gliocladium catenulatum (4)	*Volutella ciliata* (2)
cf. *Leptosphaeria coniothyrium* (3)	
15 different, not easily identifiable isolates	

[1] Number of isolates of which growth was inhibited on at least one of the test plates / Number of isolates tested.
[2] Number of isolates tested.
[3] Nonpathogenic on asparagus.

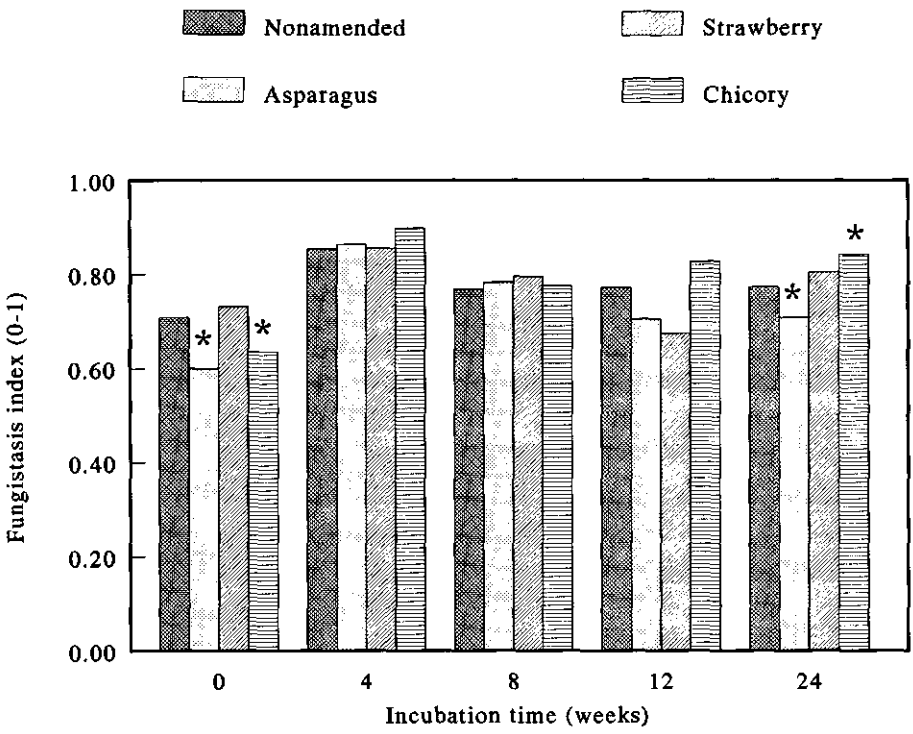


Fig. 4. Fungistatic activity to Foa of nonamended soil and of soil amended with asparagus, strawberry or chicory root material (Expt 5). Incubation was at 15°C. For each incubation time, the treatments which differ significantly from the nonamended control according to Dunnett's test ($P \leq 0.05$) are indicated which an asterisk.

Effects of root tissues or their extracts on soil fungistatic activity to Foa and colonization of soil by Fusarium *spp.* (Expts 5-9)

Incorporation of milled root materials or their extracts only slightly affected fungistatic activity of the soil microflora to Foa (Expt 5). In the first days after incorporation of root material, fungistasis had slightly, but significantly, decreased in soil with asparagus or chicory material (Fig. 4). After 4, 8 and 12 weeks, the treatments with amendments did not differ significantly from the control treatment. After 24 weeks, fungistatic activity was significantly lower in soil with asparagus material and higher in soil with chicory material.

Table 5. Colonization of soil particles by *Fusarium* spp. in soil amended with milled root materials or left unamended, and fraction of *Fusarium* isolates pathogenic on asparagus (Expt 5).

Treatment	Colonization rate *Fusarium* spp. (%)[1]	Fraction path. isolates[2]	Colonization rate Foa (%)[3]
Incubation time: 0 weeks			
Nonamended, noninfested	2.1 x[4]	1 / 12	0.2
Nonamended, infested	4.2 x	1 / 21	0.2
Incubation time: 12 weeks			
Nonamended	3.2 a	1 / 18	0.2
Asparagus	47.6 b	7 / 49	6.8
Chicory	67.6 c	1 / 50	1.4
Strawberry	55.5 bc	2 / 49	2.3

[1] Number of *Fusarium* colonies per 100 organic soil particles; back-transformed values.
[2] Number of pathogenic isolates / number of isolates tested.
[3] Colonization rate *Fusarium* spp. x fraction pathogenic isolates.
[4] Means which have a letter in common do not differ significantly according to the two-sample T-test (x) or Tukey's test (a-c) at $P \leq 0.05$.

The fungistatic effect of the addition of aqueous extracts (Expt 6) showed the same pattern after four weeks as that of milled material after 24 weeks (data not shown) but after 8 and 12 weeks mycelial growth was not significantly different from the control treatment.

In Expts 5, 6, 7 and 8, the level of fungistatic activity in nonamended soil was highly consistent, with an index value around 0.80. Addition of the extracts did not affect fungistasis significantly (Tukey, $P \leq 0.05$). Values of the fungistasis index for the six field soils in Expt 9 were also in the same range as those obtained in Expts 5, 6, 7 and 8. Differences in fungistatic activity between fields were slight and not significant (Tukey, $P \leq 0.05$). Fungistasis was not significantly different either for soil collected in rows and soil collected between rows.

The method used for measuring colonization of soil was shown to be effective in washing the soil particles free of adhering spores as could be inferred from the low colonization rate of the infested soil immediately after infestation (Tables 5 and 7, Fig. 5). In Expt 7, population density of *Fusarium* spp. determined by means of soil dilution plating, was 80 $\pm$ 21.2 colony forming units g^{-1} dry soil before infestation and 1844 $\pm$ 322.4 colony forming units g^{-1} after infestation.

Whereas mycelial growth of Foa measured in the fungistasis test was only slightly affected by products from roots of asparagus or other crops, colonization of soil by the whole population of *Fusarium* species was strongly increased. In Expt 5, root residues of

Table 6. Colonization of soil particles by *Fusarium* spp. in soil amended with aqueous extracts of root materials or left unamended after incubation for 14 weeks, and fraction of *Fusarium* isolates pathogenic on asparagus (Expt 6).

Treatment	Colonization rate *Fusarium* spp. (%)[1]	Fraction path. isolates[2]	Colonization rate Foa (%)[3]
Nonamended	2.5 a[4]	0 / 8	0.0
Asparagus	73.8 b	3 / 30	7.4
Chicory	62.6 b	3 / 30	6.3
Strawberry	5.9 a	0 / 16	0.0

[1] Number of *Fusarium* colonies per 100 organic soil particles; back-transformed values.
[2] Number of pathogenic isolates / number of isolates tested.
[3] Colonization rate *Fusarium* spp. x fraction pathogenic isolates.
[4] Means which have a letter in common do not differ significantly according to Tukey's test ($P \leq 0.05$).

the three crops markedly increased soil colonization by the total *Fusarium* population (Table 5). Asparagus extracts increased colonization of soil by Foa to high levels in Expts 6, 7, and 8 (Table 6, Fig. 5, and Table 7). Those of strawberry had no or only a slight effect, whereas those of chicory promoted colonization to the same level as that of asparagus in Expts 6 and 8 (Tables 6 and 7), but not Expt 7 (Fig. 5). Extracts of Brussels sprouts also promoted colonization to the same extent of that of asparagus in one experiment (Fig. 5) and less in another experiment (Table 7). Extract of perennial ryegrass promoted colonization only slightly or not at all (Table 7, Fig. 5).

The fraction of Foa among the *Fusarium* population was low (Tables 5, 6, and 7).

Discussion

Browning of roots and decrease in root weight in soil with high levels of asparagus root residues confirm previous reports on autotoxicity of asparagus root tissue (Yang, 1982; Shafer and Garrison, 1986; Hazebroek *et al.*, 1989; Blok and Bollen, 1993). However, addition of root material was not followed by an increased severity of root rot caused by Foa (Fig. 1a). We have been unable to confirm the results of Hartung *et al.* (1989) who found an increased disease severity in soil with the same amounts of root residues incorporated as in the present study. The main reason for this discrepancy may be that the root material was incorporated in steamed sand in the study of Hartung *et al.* (1989) and in unsterilized field soil in our experiments. In steamed sand the pathogen can utilize the

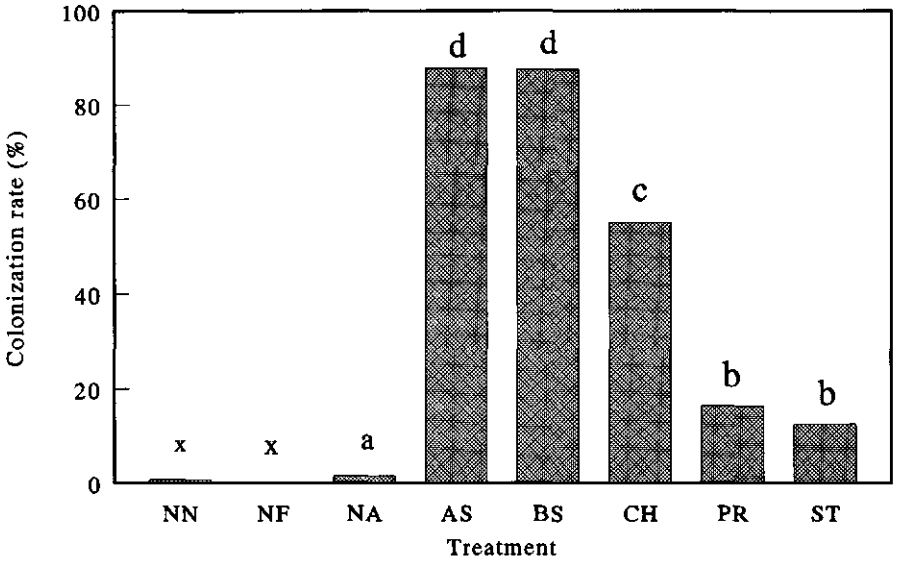


Fig. 5. Colonization rate of *Fusarium* spp. (number of *Fusarium* colonies per 100 organic soil particles; back-transformed values) in soil infested with Foa (2000 cfu g^{-1} soil) and amended with aqueous extracts of root material or left unamended, after incubation for 6 weeks (Expt 7).
Legend: NN= nonamended, noninfested, t=0; NF= nonamended, Foa infested, t=0; NA= nonamended; AS= asparagus; BS= Brussels sprouts; CH= chicory; PR= perennial rye-grass; ST= strawberry.
Bars which have a letter in common differ not significantly according to the two-sample T-Test (x) or Tukey's test (a-c) at $P \leq 0.05$.

nutrients from the sterilized root material in absence of competing soil organisms. The same applies to the results of Peirce and Colby (1987), who found a synergism between asparagus root extract and Foa in depressing seedling emergence in a sterilized peat-vermiculite medium. They obtained substantial evidence that the increased disease severity was mainly due to enhanced pathogen growth. There exists one record of increased root rot severity following incorporation of asparagus root material into a non-sterilized substrate. Hartung and Stephens (1983) added an extremely high amount of milled roots (80 g kg^{-1}) to a mixture of sand and greenhouse soil (2:1). Such a level is probably not relevant for evaluating the significance of the interaction between asparagus root material and Foa under field conditions. After termination of a crop most growers chop the plants and the residues, including dead roots, are mixed with the soil by rotary tilling. One year

Table 7. Colonization of soil particles by *Fusarium* spp. in soil amended with aqueous extracts of root materials or left unamended, and fraction of *Fusarium* isolates pathogenic on asparagus (Expt 8).

Treatment	Colonization rate *Fusarium* spp. (%)[1]	Fraction path. isolates[2]	Colonization rate Foa (%)[3]
Incubation time: 0 weeks			
Nonamended, noninfested	1.0 x[4]		
Nonamended, infested	0.0 x		
Incubation time: 12 weeks			
Nonamended	11.0 a[4]	7 / 47	1.6
Asparagus	92.4 d	2 / 49	3.8
Brussels sprouts	53.1 c	5 / 50	5.3
Chicory	92.5 d	4 / 50	7.4
Perennial rye-grass	8.9 a	2 / 40	0.4
Strawberry	33.2 b	3 / 49	2.0

[1] Number of *Fusarium* colonies per 100 organic soil particles; back-transformed values.
[2] Number of pathogenic isolates / number of isolates tested.
[3] Colonization rate *Fusarium* spp. x fraction pathogenic isolates.
[4] Means which have a letter in common do not differ significantly according to the two-sample T-test (x) or Tukey's test (a-d) at $P \leq 0.05$.

after termination of a crop, we retrieved an amount of 2.57 g (dry weight) l^{-1} soil at most (Blok and Bollen,1993). This level is well below those tested by Hartung *et al.* (1989) and some of those in Expts 1 and 2. Where similar levels were tested, severity of root rot caused by Foa had not increased (Fig. 1a).

It can be argued that assessment of the effect of the amount of root residues retrieved from field soil is inadequate because the active compounds might have leached from root residues into the soil and may still play a role. This possibility was investigated in Expt 3 where it was demonstrated that disease severity was the same after infestation of heated asparagus soil and fresh soil. In an earlier study it was shown that the toxic products were able to withstand heat treatment (Blok and Bollen, 1993).

Hartung *et al.* (1989) suggested that aqueous extracts of asparagus roots alter the plants biochemical processes resulting in a reduced defense against pathogens. Peirce and Miller (1993) studied the effects of cinnamic acids, which are thought to be partially responsible for asparagus autotoxicity, on the epidermis of asparagus radicles. Scanning electron microscopy revealed damage to the epidermal cells and precocious root hair development. The authors hypothesized that these effects enhance root penetration by *Fusarium* in

infested soils. Our results indicate that either the effects mentioned do not occur in unsterilized field soil or if they occur it does not lead to an increased severity of root rot.

Soil fungi differed in the sensitivity of mycelia to asparagus root extracts. This effect was specific for asparagus as growth of no isolate was inhibited by root extracts of strawberry and chicory. A selective effect was also noticed by Hartung and Stephens (1983) and Hartung (1987), who found that growth was inhibited of *Pythium ultimum* and *P. aphanidermatum*, but not of Foa, *F. moniliforme* and *Rhizoctonia solani*. Among the sensitive fungi were several species that are known for their fungistatic activity against pathogens, e.g. *Gliocladium* spp. and *Trichoderma harzianum*. Perhaps selective inhibition of the soil microflora by asparagus root residues affects microbial antagonism against pathogens which may contribute to increased colonization of Foa in soil with root residues.

Antagonistic activity of the soil microflora includes antibiosis, competition, and parasitism. Each of the three mechanisms might be influenced by shifts in the soil microbial community due to selective inhibition by asparagus root extract. The fungistasis test provides information on antibiosis and competition but not on parasitism, as direct contact between the isolate tested and its parasites is prevented by the membrane. The results of the fungistasis test showed clearly the effect of general soil fungistasis on mycelial growth, as the colony diameter on unsterilized soil was only 10-40% of that on sterilized soil. Addition of root material or extract had only a minor effect on the level of soil fungistasis and the effect was not specific for asparagus. Also, after many years of asparagus cultivation, the level of fungistasis of the soil was not significantly affected (Expt 10). Either shifts in the microbial community following additions of asparagus material did not occur or the contribution of the inhibited species to fungistasis was immediately taken over by that of other members of the soil microflora. As mentioned above, effects of possible shifts in the populations of mycoparasites were not measured in these experiments.

Asparagus, Brussels sprouts, and chicory material greatly enhanced colonization by *Fusarium* spp., whereas strawberry and perennial rye-grass did not. Apparently, *Fusarium* spp. are effective competitors for the asparagus, Brussels sprouts and chicory material.

In all experiments where the final population was tested for its pathogenicity, Foa was shown to comprise only a small fraction of the *Fusarium* population. As the density of the resident nonpathogenic *Fusarium* population was much lower than that of the introduced Foa isolate after artificial infestation of soil at the beginning of the experiment (Expt 7), it can be concluded that the pathogen has a low competitive saprophytic ability. As a consequence, it is unlikely that any growth reduction of antagonists such as *Gliocladium* spp. and *Trichoderma harzianum* by asparagus root residues (Table 4, Fig. 3) would be a major factor in development of the pathogen in soil.

Chapter 6

Inoculum sources of *Fusarium oxysporum* f.sp. *asparagi* in asparagus production

Abstract

Fusarium oxysporum f.sp. *asparagi* (Foa) incites crown and root rot of asparagus which causes early decline of asparagus plantings. The aim of the present study was to identify the main inoculum sources of the pathogen in the Netherlands.

As has been reported for foreign seed lots, Dutch seed lots can be infested with Foa at low levels. We found that seed infestation occurs mainly during the seed harvesting process through infested soil adhering to fallen berries.

Soil samples from 59 fields without a history of asparagus growing and differing in their distance from asparagus plantings were tested for infestation with Foa, using a bio-assay with asparagus as a bait plant. A high correlation was found between the incidence of infestation and proximity to asparagus fields; Foa was found in 69% of the samples from fresh fields in an asparagus production centre, and in only 6% of the samples from fields at a distance of one km and more from asparagus fields and outside a production centre.

To evaluate planting material as an inoculum source of Foa, 49 lots of one-year-old crowns from 23 nurseries were collected and rated for disease symptoms. Infestation was found to be common with only two lots free of symptomatic plants. Most of the lots had more than 75% of symptomatic plants. Although most of the plants were infested, they showed only slight root rot symptoms. The procedure for production of Foa-free planting material is discussed.

Persistence and infestation of asparagus root residues in former asparagus fields was assessed by retrieving the residues from eight former asparagus fields with an asparagus-free period of one to 25 years, and three fields with a standing asparagus crop. Even after an asparagus-free period of 25 years asparagus root residues were retrieved from soil, although at low levels. Mean population densities of *Fusarium* spp. declined from 2×10^6 to 1×10^5 colony forming units g^{-1} air-dry root tissue during the first 10 years and were still $> 10^4$ cfu g^{-1} root tissue 20 to 25 years after asparagus production was stopped. The population was dominated by *F. oxysporum*. Eighty-three of the 112 isolates (74%) of *F. oxysporum* belonged to the forma specialis *asparagi*. The proportion of Foa in the population did not decrease in time. It was concluded that persistence of Foa in asparagus root residues is a major reason for its long-term survival.

Introduction

Fusarium oxysporum (Schlecht.) emend. Snyder and Hansen f.sp. *asparagi* (Cohen and Heald) (Foa) is known as one of the incitants of fusarium crown and root rot and causes extensive rotting of all below-ground parts of asparagus (*Asparagus officinalis* L.)(Cohen and Heald, 1941; Graham, 1955). Other *Fusarium* spp. that can be involved in crown and root rot are *F. moniliforme* (Johnston *et al.*, 1979) and *F. proliferatum* (Elmer, 1990). Fusarium crown and root rot is the major cause of early decline of asparagus plantings on fresh and former

asparagus land. Early decline has been reported from many asparagus-growing regions in the world (Caron *et al.*, 1985; Damicone and Manning, 1985; Gordon-Lennox and Gindrat, 1987; Hartung, Stephens and Elmer, 1990; Nourisseau *et al.*, 1992; Schofield, 1991; Schreuder *et al.*, 1995; Tello *et al.*, 1985; Unterecker, 1972). In the Netherlands, only Foa has been isolated from diseased plants thus far (Van Bakel and Kerstens, 1970; Blok and Bollen, 1995; 1996a) and early decline is confined to replant situations. Therefore, early decline was referred to as asparagus replant-bound early decline (Blok and Bollen, 1995; 1996a).

Although Foa is also present in asparagus plantings on fresh land, an economic life of 10 years generally is feasible under Dutch conditions. This period is satisfactory for the growers and Foa is not considered as a threat on fresh land. Nevertheless, effective control of Foa may result in higher yields and in an extended cropping period. Moreover, preventive control of Foa in order to avoid introduction at planting time will delay build-up of inoculum to levels at which subsequent asparagus plantings will be affected. The pathogen can survive asparagus-free periods up to at least 25 years (Blok and Bollen, 1996a).

In the Netherlands, asparagus seed is produced in glasshouses. Berries are hand-picked and the seed is extracted by squashing the berries with a roller, followed by several washings and air-drying. The seed is sown in fields without an asparagus-growing history. One-year-old crowns are used to start a commercial planting. About 60% of the crown material is produced by nurserymen. In order to develop effective control methods, the main inoculum sources of Foa must be known. As has been reported for several countries with asparagus production, sources are infested seed (Lewis and Shoemaker, 1964; Inglis, 1980) and planting material (Unterecker, 1972; Fantino and Fantuz, 1990; Manning *et al.*, 1980; Molot and Lombard, 1986). Assays in which seeds were plated onto fusarium selective Komada's medium or planted in sterilized sand revealed that Dutch seed lots were no exception to this rule (W.J. Blok, unpublished). Some authors mention the possibility of infestation of soil not previously planted to asparagus (Graham, 1955; Hartung, Stephens and Elmer, 1990).

In this study we aimed to establish how asparagus seed becomes contaminated and to explore preventive measures. Besides seed contamination, the sanitary state of one-year-old crowns and the presence of Foa in soils without an asparagus history from different areas in the Netherlands were studied. An analysis of infestation levels in root residues from field soil at different periods after termination of the crop was also included.

Materials and methods

General procedures

Seed disinfestation and pregermination. Seeds were surface-disinfested according to the procedure of Elmer and Stephens (1988). First they were disinfested in 2% sodium

hypochlorite (50% household bleach (AH)) for 30 min. After decanting, seeds were shaken for 24 h in a solution of 25 mg ml^{-1} benomyl in acetone. After washing twice in acetone and three times in sterile distilled water, seeds were pregerminated on moist filter paper under sterile conditions at 22-25°C for 5-7 days.

Isolation from asparagus roots. Storage roots of one-year-old crowns were externally disinfested for 4 min in 2% sodium hypochlorite and rinsed three times in sterile distilled water. Seedling roots were rinsed three times in sterile distilled water only. Tissue pieces from the edge of lesions were excised and plated onto Komada's medium (Komada, 1975).

Identification and pathogenicity testing of Fusarium oxysporum. Asparagus seeds and roots were plated onto Komada's medium unless otherwise stated and plates were incubated in the light at 22-24°C for 10-14 days. The medium was not autoclaved because the red pigmentation, which is one of the characteristics that distinguishes colonies of *F. oxysporum* from those of other *Fusarium* species (Komada, 1975), is subdued when the medium is autoclaved (Schneider, 1984). Colonies were identified as *F. oxysporum* based on colony characteristics on Komada's medium as described by Komada (1975) and supported by platings of reference Foa strains. In each experiment, a number of colonies identified as *F. oxysporum* were randomly collected and their identity verified microscopically using the system of Nelson *et al.* (1983). All colonies examined belonged to *F. oxysporum*. *F. moniliforme* and *F. proliferatum*, two other pathogens of asparagus, were not detected.

Pathogenicity of isolates of *F. oxysporum* on asparagus was tested on seedlings grown under aseptic conditions from disinfested seeds in culture tubes (Stephens and Elmer, 1988; Blok and Bollen, 1995). Only the main details of methods are given here. Disinfested asparagus seeds of cv. Gynlim were placed in sterile culture tubes (Ø 22 mm) with 12 ml Knop's solution with 0.8% agar. When the first shoot had grown halfway up the tube, seedlings were inoculated by placing a PDA-plug with actively growing mycelium at the stem base. After 4-5 weeks incubation at 20°C, seedlings were rated for disease symptoms on a 0-4 scale, where 0 = no disease; 1 = only browning of roots, no lesions; 2 = small number of brown lesions on primary and / or secondary roots, secondary root development hardly or mildly restricted; 3 = many brown lesions on primary roots, secondary root development severely restricted; 4 = death of the plant. Each isolate was tested on three seedlings. Isolates which had a score 2 or more on at least one of the plants were rated as pathogenic and regarded as f.sp. *asparagi*.

Seed contamination

To reveal how asparagus seeds become contaminated with Foa, the contamination level was compared of seed from berries hand-picked from the upper part of plants with that of seed from fallen berries from the ground. The comparison was made for commercially produced

seed of four different cultivars, each grown in a separate glasshouse compartment. In assay 1, seeds of cvs Backlim, Boonlim and Gynlim, and in assay 2, seeds of cv. Thielim were tested.

In assay 1, 100 hand-picked berries and 100 fallen berries were collected for each cultivar. The two lots were kept separate during harvesting. Seed was harvested following a commercial procedure in which the berries were squashed with a heavy roller, the seed and berry residues separated during washing in tap water and the seeds air-dried in a drying cabinet for approx. 1 wk. After storage for 8 months at room temperature the seeds were plated onto Komada's medium. Plates were incubated at 22°C for 10-14 days when the number of seeds with outgrowth of *F. oxysporum* was determined for each sample. The isolates of *F. oxysporum* obtained were tested for pathogenicity on asparagus.

In assay 2 with cv. Thielim, 400 seeds were collected for each of the two samples. After washing, 200 seeds per sample were plated out. The remaining 200 seeds were air-dried for 1 wk according to commercial seed processing practice, and also plated out. After 10-14 days, percentage outgrowth of *F. oxysporum* was determined. Twelve to 17 randomly selected *F. oxysporum* isolates were tested for pathogenicity on asparagus.

One seed lot of cv. Thielim was collected in the commercial way with hand-picked and fallen berries collected together. The slurry of the first washing was collected. Three samples of the slurry were serially diluted in 10-fold dilutions and for each dilution four plates with Komada's medium received 0.25 ml. After 10 days incubation, the number of *F. oxysporum* colonies was counted. Twelve randomly selected isolates were tested for pathogenicity on asparagus.

Soil contamination

Contamination of soils without a history of asparagus cultivation (fresh soils) was assessed in samples collected from different areas of the Netherlands in 1991 and 1992. A bio-assay with asparagus as a bait plant was used.

Most samples were collected by extension workers. The locations of the fields are shown in Fig. 1. In each field, 20 soil samples were randomly taken from the upper 20 cm and bulked into one composite sample. Samples were stored in shade in loosely-tied plastic bags. Bio-assays were performed in two series. Series 1 consisted of 45 samples collected in 1991, series 2 consisted of 14 samples collected in 1992. For the bio-assay, soil was crumbled by hand, mixed thoroughly and divided over 30 (series 1) or 20 (series 2) plastic 150 ml-pots. In each pot a pregerminated seed (cv. Gynlim) was planted. The pots were placed in a glasshouse compartment at 20-28°C. To minimise the risk of cross-contamination between samples, all pots of one sample were placed in a plastic box and the samples were separated by plastic screens. To check for possible cross-contamination three (series 1) and two (series 2) samples of autoclaved field soil were included. Either one (series 1) or two (series 2) samples of (former) asparagus fields were included as references. After 13 (series 1) or 10

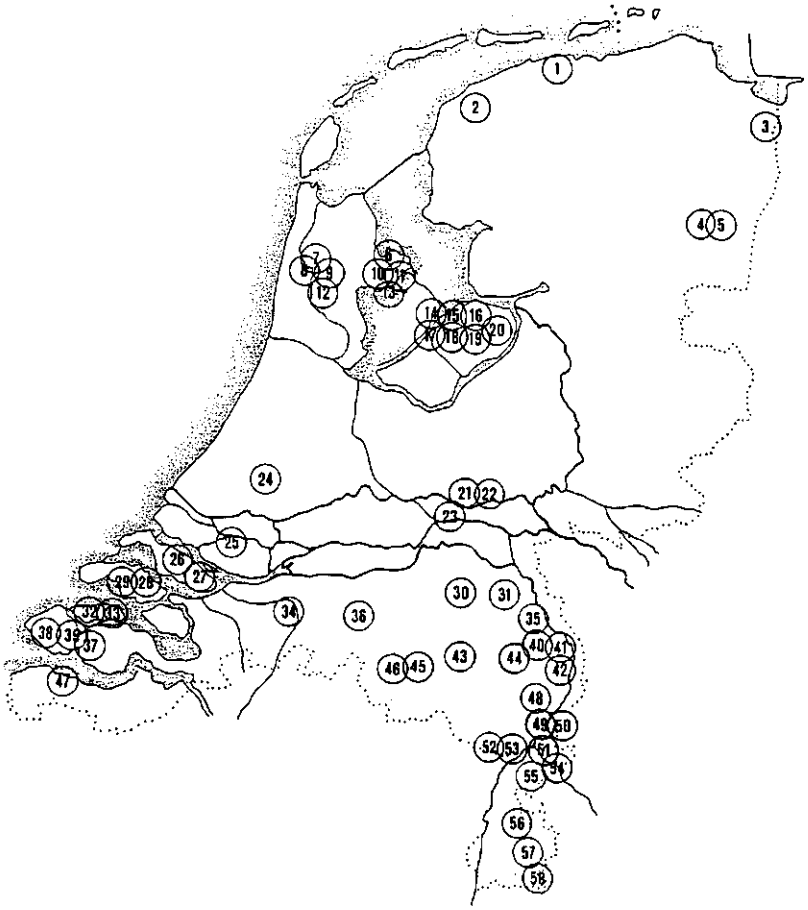


Figure 1. Map of the Netherlands with sample sites for the soil contamination study.

weeks (series 2) roots were carefully washed free of soil and examined for presence of brown root lesions. Roots with brown lesions were plated onto Komada's medium. Outgrowing fusarium colonies were subcultured and tested for their pathogenicity on asparagus.

Each field was, based on its location relative to (former) asparagus fields, assigned to one of three categories of contamination risk. The category with a low contamination risk consists of fields not located in an asparagus production centre and without neighbouring (former) asparagus fields within a distance of 1 km; that with a medium contamination risk consists of fields not located in an asparagus production centre but with neighbouring (former) asparagus fields within a distance of 1 km; the category with a high contamination risk consists of fields located in an asparagus production centre.

Contamination of one-year-old crowns

To evaluate one-year-old crowns as a source of inoculum of Foa, a survey was conducted to determine presence of contamination. In the first half of March 1991, one-year-old dormant crowns were obtained from 23 of the 28 commercial Dutch asparagus nurseries and rated for symptoms of root rot. A total of 49 crown lots were obtained belonging to various cultivars. Crowns which had already been harvested (26 lots) were sampled at random from the large bags in which the crowns are sold. Crowns which had not yet been harvested (23 lots) were collected randomly in the field. As a rule 30 crowns per ha were collected, with a minimum number of 12 and a maximum of 60. After collection, the crowns were taken to the laboratory, washed thoroughly with a high-pressure spray gun and rated for root rot on a 0-3 scale, where 0 = healthy plant; 1 = one or more brown lesions on < 10% of the storage roots; 2 = idem, on 10-50% of the storage roots; 3 = idem, on > 50% of the storage roots. From each of the 49 lots two root pieces with lesions were collected and plated onto Komada's agar. Outgrowing *F. oxysporum* isolates were tested for pathogenicity on asparagus.

Presence of Foa in asparagus root residues

Asparagus root tissue was retrieved from eight former asparagus fields and from three fields with a standing crop. Densities of fusarium population in the root tissue and percentage of *F. oxysporum* isolates pathogenic on asparagus were determined to check whether asparagus root residues were an inoculum source for Foa. The fields were all located in the asparagus growing area in the northern part of the province of Limburg. Asparagus production had been terminated 0, 1, 2, 4, 5, 7, 10, 20 and 25 years ago, respectively. On each field, ten sample sites were randomly chosen and a soil sample was collected with an auger from three depths at each site: 10-30 cm, 30-50 cm, and 50-70 cm. The ten samples for each depth were bulked into one composite sample per depth. The samples were put through a sieve with a 5-mm mesh. Asparagus root residues retrieved on the sieve were washed under running tap water, air-dried for 24 h and weighed. Root residues were cut into 1-cm pieces, suspended in sterile distilled water (1 g air-dry root residues per 100 ml water) and homogenised in a blender. Further 10-fold dilutions were made in sterile distilled water. Aliquots of 0.5 ml were plated onto five replicate plates of modified peptone-PCNB agar (Papavizas, 1967) per dilution. The plates were incubated at 20-25°C for 5 days when colonies of *Fusarium* spp. were counted. From the plates of each sample, 15 randomly selected fusarium colonies were subcultured and identified according to the system of Nelson *et al.* (1983). The isolates identified as *F. oxysporum* were tested for their pathogenicity on asparagus.

Linear regressions were performed for the mean values of the three depths to describe the decline in root biomass and population density of *Fusarium* with time and to test the significance. For these analyses, data were transformed as follows: time was transformed to log (no. of asparagus-free years + 1); biomass of root residues to 1/(biomass + 1), and

population density of fusaria per weight of root tissue and per volume of soil to log (pop. density + 1).

Results

Seed contamination

Numbers of seeds with outgrowth of *F. oxysporum* and the results of the pathogenicity tests with these isolates are given in Table 1. In the slurry of the first washing of seeds of cv. Thielim 453 ± 85 colony forming units of *F. oxysporum* per ml were isolated. Of the 12 isolates tested for pathogenicity, 11 were found to be Foa.

Table 1. Contamination of asparagus seeds with *F. oxysporum* and pathogenicity of isolates on asparagus. In assay 1, dry seeds harvested with a commercial procedure from hand-picked and fallen berries were plated. In assay 2, seeds harvested in the same way from hand-picked and fallen berries were plated; one sample before the final drying, and one sample after drying.

Seed lot	No. seeds with *F. oxysporum* / no. seeds plated	No. pathogenic isolates / no. tested isolates
Assay 1:		
Cv. Backlim, picked berries	0 / 100	-
fallen berries	9 / 100	5 / 9
Cv. Boonlim, picked berries	0 / 100	-
fallen berries	3 / 100	1 / 3
Cv. Gynlim, picked berries	0 / 100	-
fallen berries	0 / 100	-
Assay 2 (cv. Thielim):		
Picked berries, before drying	45 / 200	5 / 12
after drying	0 / 200	-
Fallen berries, before drying	200 / 200	9 / 14
after drying	118 / 200	12 / 17

Soil contamination

The three reference samples from fields with a standing asparagus crop showed a high infestation level. For these samples, all plants were heavily attacked with most of the secondary roots rotten and some of the plants killed. Data for samples of fresh soil are given in Table 2. Many plants grown in the fresh soils showed a few brown-coloured secondary roots or some secondary roots with a few tiny brown lesions. In very few roots was Foa detected by isolation and subsequent pathogenicity testing. Foa was found to be present in

Table 2. Contamination with Foa of soils without a history of asparagus cropping. For each category of contamination risk the number of samples with their codes are given and the number and codes of samples in which Foa was detected using a bio-assay.

Contamination risk[1] category	Samples tested		Samples with Foa		Fraction contaminated (a/b)
	Code[2]	No.(a)	Code[2]	No.(b)	
Low	1-12, 14-27, 32, 33, 36, 37, 39, 47, 57, 58	34	10, 57	2	0.06
Medium	13, 28, 29, 34, 38, 52, 53, 56	8	28, 29, 56	3	0.38
High	30, 31, 35, 40-46, 48-51, 54, 55	16	30, 35, 40-43, 45, 48-50, 55	11	0.69

[1] Low contamination risk: fields not located in an asparagus production centre and without neighbouring (former) asparagus fields within a distance of 1 km; medium contamination risk: fields not located in an asparagus production centre but with neighbouring (former) asparagus fields within a distance of 1 km; high contamination risk: fields located in an asparagus production centre.
[2] See Figure 1 for locations of sample sites.

distinct, brown lesions on primary roots. Infestation levels in samples of fresh soils were low compared with those of samples from asparagus fields, with $<50\%$ of the plants with a few lesions on the primary roots.

Contamination of one-year-old crowns

Storage roots were covered with a layer of very tightly adhering soil. Lesions became visible only after thorough cleaning with a high-pressure spray gun; Foa infestation of the planting material was very common with only two out of 49 plant lots free of Foa symptoms and most of the lots with more than 75% of the plants with symptoms (Table 3). Symptoms on the storage roots ranged from small, elongated brown lesions to large, oval brown lesions. Although the former were atypical, Foa was obtained from both types of lesions. Of the 70 isolates of *F. oxysporum* tested, 40 (57%) were pathogenic on asparagus.

Infestation of asparagus root residues

Asparagus root residues were easily recognised and were retrieved from all fields, even from those where asparagus production was stopped 20 or 25 years ago, although levels were low in the latter (Fig. 2a). The residues harboured large fusarium populations. The population density decreased 20-fold in the first 10 years and even 20 to 25 years after asparagus

Table 3. Status of one-year-old asparagus crowns sampled in 1991. Crown lots were assigned to one of five health classes based on the percentage of plants with symptoms. For each health class the mean and range of root rot rating per crown lot is given based on all plants and based on plants with symptoms only.

Percentage of plants with symptoms	No. of crown lots	Root rot rating[1] (all plants)		Root rot rating[1] (plants with symptoms only)	
		Mean	Range	Mean	Range
0	2	-	-	-	-
1 - 25	2	0.2	0.2-0.2	1.0	1.0-1.0
26 - 50	12	0.5	0.3-0.8	1.1	1.0-1.7
51 - 75	10	0.7	0.6-1.0	1.1	1.0-1.4
76 - 100	23	1.4	0.9-2.3	1.5	1.0-2.3

[1] Root rot was rated on a 0-3 scale, where 0 = healthy plant; 1 = one or more brown lesions on < 10% of the storage roots; 2 = idem, on 10-50% of the storage roots; 3 = idem, on > 50% of the storage roots.

production was stopped the density was still > 10^4 colony forming units g^{-1} root tissue (Fig. 2b). The product of the two variates just mentioned, the population density of fusaria in asparagus root tissue per volume of soil, is presented in Fig. 2c. Over the first 10 years there had been a decline from 8 x 10^6 to 3 x 10^3 cfu l^{-1} soil, after 20 to 25 years the density was still at this level. The linear regressions of the transformed variates with time were all significant at $P \leq 0.05$ (data not shown).

F. oxysporum dominated the fusarium population in the root tissues with 75.1% of the isolates belonging to this species, followed by *F. solani* with 17.8% (Table 4). Of the 112 *F. oxysporum* isolates tested, 83 (74%) were pathogenic on asparagus (Table 5).

Discussion and conclusions

It is concluded that seed lots become contaminated with Foa primarily by means of infested soil adhering to fallen berries (Table 1). These berries are a source of contamination for the whole seed lot during the seed extraction process, as indicated by the high contamination with Foa of the wash water. Inglis (1980) found that contamination of asparagus seed with *F. moniliforme* mainly occurred during the seed harvesting process when wash water was contaminated by diseased host tissue. Although the main contamination occurs during seed harvesting, berries and/or seeds can become contaminated when they are still attached to the

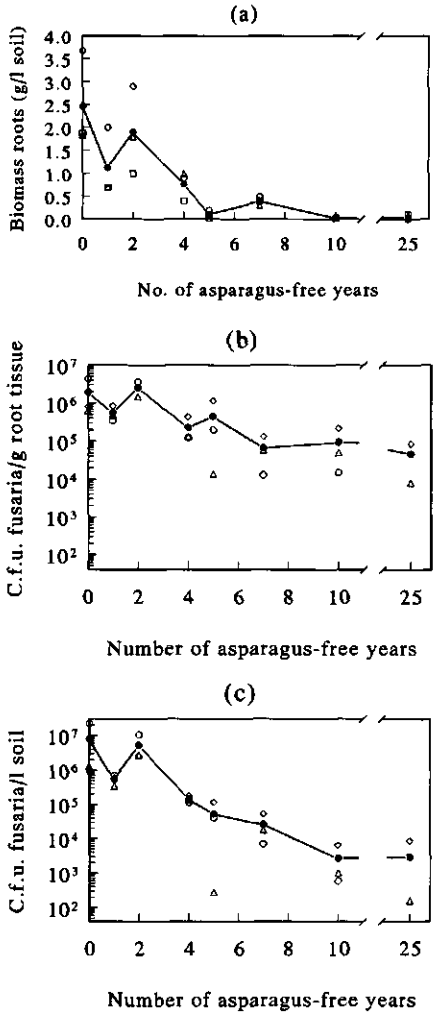


Figure 2. Persistence and infestation with fusaria of asparagus root tissue in field soils with a different number of asparagus-free years.
(a) Biomass of asparagus root tissue, (b) Density of root populations of fusaria per weight of root tissue, (c) Density of root populations of fusaria per volume of soil. For the three fields with a standing asparagus crop (zero asparagus-free years) the mean values are presented. The scale for the population density of fusaria starts at 40 cfu as this was the lower limit of detection.

Table 4. Composition of a population of 433 fusarium isolates selected randomly from PCNB-agar plates with dilutions of all root tissue samples. As a rule, five colonies per sample were subcultured.

Species	Percentage
F. oxysporum	75.1
F. solani	17.8
F. equiseti	3.7
F. culmorum	3.5

Table 5. Pathogenicity of *F. oxysporum* isolates present in root residues from (former) asparagus fields on asparagus seedlings.

Asparagus-free period (yr)	# pathogenic isolates / # isolates tested
0	6 / 7
0	11 / 14
0	11 / 15
1	7 / 7
2	8 / 10
4	10 / 10
5	6 / 13
7	7 / 12
10	7 / 10
20	5 / 9
25	5 / 5
Total	83 / 112 (74% of isolates pathogenic)

plant as is concluded from the contamination of seed from picked berries (Table 1, series 2). This finding is in accordance with results of Manning *et al.* (1980) who isolated *F. oxysporum* from berry and flower surfaces; without, however, performing pathogenicity tests. The findings enable seed producers to take measures for the prevention of seed contamination with Foa.

In many cases, fields without a history of asparagus cultivation were already infested with Foa. Of the 16 fields located in an asparagus production centre, 11 fields were infested. There is a high correlation between incidence of infestation and proximity to asparagus fields (Table 2). This correlation justifies the conclusion that Foa is not a non-specific, ubiquitous pathogen, but a specialised one that is spread to fresh soils from asparagus fields. The

detection of Foa in fresh soils further demonstrates that the pathogen can survive very well in soil in the absence of its host. Contamination of fresh soils is most probably brought about by machinery with adhering infested soil and by wind-blown infested soil. Substantial evidence was obtained that even asparagus grown in soil-less cultures in the glasshouse can become infested through the latter mechanism (W.J. Blok and J. Jeurissen, unpublished). Contamination of non-asparagus soils with Foa was also reported from the USA (Graham, 1955; Hartung, Stephens and Elmer, 1990), Switzerland (Gordon-Lennox and Gindrat, 1987) and France (Nourisseau *et al.*, 1992).

Infestation of planting material was shown to be a common phenomenon. This finding is in accordance with observations in other asparagus-growing areas, including France (Molot and Lombard, 1986; Nourisseau *et al.*, 1992), Spain (Tello *et al.*, 1985), Italy (Fantino and Fantuz, 1990), Germany (Unterecker, 1972), Canada (Caron *et al.*, 1985) and the USA (Manning *et al.*, 1980). Infestation of the crown is not surprising given that the pathogen is seed-borne and most nurseries are located in asparagus production areas, where most of the fresh soils are infested. Whereas most plants were found to be infested, the mean root rot rating was very low. The question arises as to the importance of infestation of planting material for an asparagus crop. When the plants are grown on former asparagus land, slight infestation of the plants will be relatively unimportant as the plants will become infected quickly and extensively by the pathogen population in soil from the previous asparagus crop. However, when a crop is started on fresh land, where soil infestation is absent or only low, infested planting material is a major inoculum source. The damage resulting from infestation of the planting material is twofold. First, there is the direct damage due to lower yields and decreased cropping period in the crop that is started with the infested material. This damage depends on infestation level and growth conditions, especially those during the first period after planting. The latter are important because damage due to Foa infection is stress-related (Nigh, 1990; Damicone and Manning, 1985). Second, build-up of inoculum during the first crop and carry-over of this inoculum to a following asparagus planting causes early decline in this crop. As in most crown lots more than 50% of the plants were infected, planting these crowns in fresh land will result in an even and general infestation that will increase to high levels during the cropping period. This is especially relevant for fields located outside the traditional asparagus-producing areas as these fields are hardly ever infested with Foa (Table 2). Planting Foa-free plants in these fields will largely keep the soil free of Foa during the whole cropping period thus preventing early decline of following asparagus plantings.

The high persistence of asparagus root residues was reported earlier (Blok and Bollen, 1993). In this study similar amounts of root residues were retrieved. The root tissue yielded populations of fusaria higher than reported for fusarium pathogens of other crops (Elmer and Lacy, 1987; Gordon and Okamoto, 1990; Smith and Snyder, 1975) and even after 20 to 25 years the population density was still $> 10^4$ cfu g^{-1} root tissue. Apparently, the decay-resistant asparagus root tissue provides a protective matrix for the pathogen. The fusarium population

consisted for the major part of Foa and, although the number of isolates tested for pathogenicity per sample was low, there was no indication that the fraction of Foa isolates declined with time. Although far less than *F. oxysporum*, also *F. solani* was isolated frequently from root residues. Occurrence of this species in living asparagus roots and stems, albeit at lower frequencies as found in this study, was reported by various authors (Damicone and Manning, 1985; Gordon-Lennox and Gindrat, 1987; Fantino and Fantuz, 1990; Schreuder *et al.*, 1995). The predominance of Foa in the *Fusarium* population can be explained by the ability of Foa to infect and colonise living asparagus roots, compared with nonpathogenic *Fusarium* populations. The persistence of Foa is due either to survival of chlamydospores in a dormant state or a strong competitive saprophytism, as has been reported for other formae speciales of *F. oxysporum* (Park, 1959; Rao, 1959). The latter would enable Foa to renew its population and hold its position during the asparagus-free period in which the root residues were decomposed gradually.

Long-term survival of Foa in the absence of its host has been reported (Poll and Huiskamp, 1992; Blok and Bollen, 1996a). From the high levels of Foa found in asparagus root residues together with the persistence of the latter it can be concluded that survival of Foa in asparagus root residues is one, if not the main, reason for this long-term survival.

In conclusion, one-year-old crowns, the starting point of a new planting, are generally infested with Foa albeit at a low level. This infestation is especially significant for areas that have previously remained free of the pathogen. For these areas especially, planting material that has been tested for Foa contamination, should become available. For crown production, contamination of seed needs to be avoided or seed should be effectively disinfested, and growth media should be free of infestation. A method for disinfestation of seed was described by Damicone *et al.* (1981). Plants should preferably be produced outside an asparagus production area. Infestation of field soils can be tested before planting, using a bioassay as performed commercially in France (Nourisseau *et al.*, 1992).

Chapter 7

Host specificity and vegetative compatibility of Dutch isolates of *Fusarium oxysporum* f.sp. *asparagi*

Abstract

The host range of *Fusarium oxysporum* f.sp. *asparagi* (Foa) was studied in inoculation experiments with 21 plant species. Typical root rot symptoms were incited only in asparagus, in all experiments; lupin and pea were susceptible under *in vitro* conditions but showed only mild symptoms occasionally when tested in soil; none of the other species showed external disease symptoms. Root colonization by Foa was studied for 14 plant species. The pathogen was detected in externally disinfested roots of all species except leek and onion, with asparagus the most extensively colonized species. Asparagus was not susceptible to isolates of *F. oxysporum* f. sp. *pisi*, *lupini*, *cepae*, *lilii*, *gladioli* and *F. sacchari* var. *elongatum*. Naturally-infested field soil was planted twice for 11-13 weeks with eleven plant species, including asparagus and several symptomless hosts, and subsequently with asparagus as a biotest plant. Of these crops, only asparagus greatly increased the severity of Foa root rot. It was concluded that Foa has a narrow host range as a pathogen but a broad host range as a parasite. The consequences of the latter for the epidemiology of Foa are discussed.

Twenty-four Foa isolates were assigned to 18 different vegetative compatibility groups (VCGs), three additional *F. oxysporum* isolates, which were not pathogenic on asparagus, each belonged to a unique VCG. These findings indicate that the Dutch Foa population is very diverse genetically, as was found previously in the USA.

Introduction

Fusarium oxysporum (Schlecht.) emend. Snyder & Hansen f.sp. *asparagi* Cohen & Heald (Foa) incites root and crown rot of asparagus (*Asparagus officinalis* L.) The fungus was identified as the cause of early decline of asparagus plantings in the Netherlands, which occurs generally in replant situations (Blok and Bollen, 1996a). In other countries, together with Foa, *F. moniliforme* and *F. proliferatum* (Johnston *et al.*, 1979; Elmer, 1990; Elmer and Ferrandino, 1992) were identified as causal organisms in early decline. Early decline is a major threat to commercial asparagus production worldwide (Grogan and Kimble, 1959; Nourisseau *et al.*, 1992; Damicone and Manning, 1985; Schofield, 1991; Schreuder *et al.*, 1995).

The pathogen can be present on seed (Inglis, 1980), planting material (Manning *et al.*, 1980; Fantino and Fantuz, 1990; Blok and Bollen, 1996b), and in soil. It was also detected in soil previously not planted with asparagus (Graham, 1955; Hartung, Stephens and Elmer, 1990; Blok and Bollen, 1996b).

Many formae speciales of *F. oxysporum* persist in soil for long periods of time in absence

of their hosts (Garrett, 1970; Burgess, 1981). This persistence has been attributed to production of thick-walled chlamydospores (McKeen and Wensley, 1961; Garrett, 1970), colonization of host and nonhost crop residues (Gordon and Okamoto, 1991), and colonization of symptomless host plants (Armstrong and Armstrong, 1948; Katan, 1971; Gordon *et al.*, 1989). Also Foa can persist for a long period of time in field soil as its presence was demonstrated in fields where asparagus production was terminated some 10-25 years previously (Graham, 1955; Poll and Huiskamp, 1992; Blok and Bollen, 1996a). The pathogen was found to survive in high densities in asparagus root residues for periods up to at least 25 years (Blok and Bollen, 1996b).

Pathogenicity of Foa to plant species other than asparagus was tested several times. Within the genus *Asparagus* the species *A. officinalis*, *A. acutifolius*, *A. plumosus*, and *A. setaceus* were susceptible, whereas *A. densiflorus* was resistant (Graham, 1955; Stephens *et al.*, 1989). Cohen and Heald (1941) reported slight wilting of pea cv. Alaska, whereas others did not find symptoms on pea (Grogan and Kimble, 1959; Armstrong and Armstrong, 1969). Graham (1955) found that Foa caused lesions on gladiolus cv. Picardy. In reciprocal pathogenicity tests of other formae speciales of *F. oxysporum* on asparagus, f.sp. *apii* (Armstrong and Armstrong, 1969; Elmer and Stephens, 1989), *cubense*, race 1, *medicaginis* (Armstrong and Armstrong, 1969), *cepae*, and *gladioli* (Elmer and Stephens, 1989) were found to incite symptoms.

Vegetative compatibility is the capability to form stable heterokaryons after anastomosis. Isolates which are vegetative compatible are placed in the same vegetative compatibility group (VCG) and are genetically more similar than incompatible isolates of the same species (Correll *et al.*, 1987). For most formae speciales of *F. oxysporum* a limited number of VCGs was found. However, with Foa, Elmer and Stephens (1989) identified at least 43 VCGs in a collection of 79 isolates including 67 isolates from the USA, 7 isolates from Europe and 5 isolates from Taiwan. They concluded that a large genetic diversity was present in their Foa population. In the Netherlands, cultivars of almost exclusively Dutch origin have been planted in the last four decades. The seed of these cultivars is produced on one farm only. Because of the predominance of this seed source, it might be expected that the genetic diversity of the Foa population in the Netherlands is narrower than that of the population in the USA. Therefore, a lower number of VCGs might be expected.

Information on host range and the effect of symptomless plant species on the Foa population is needed to determine whether survival of Foa during the years between two asparagus crops might be reduced by a specific choice of rotational crops. In the present study, we investigated the host range of Foa, the ability of Foa to colonize roots of symptomless plants, and the effect of various rotational crop species on Foa inoculum potential. A study on VCG diversity of a limited number of Foa isolates was also included.

Materials and methods

General procedures

Fungal isolates and production of inoculum. Isolates CWB 1, 5, 6, 7, and 10 were isolated from typical lesions on asparagus roots and belong to f.sp. *asparagi*. CWB 14 was isolated from lupin (*Lupinus luteus* L.) grown as a green manure crop after an asparagus crop and, as it caused foot and root rot on asparagus, was also considered to be Foa. CWB 200 and 201 were received from the DLO-Research Institute for Plant Protection, Wageningen, the Netherlands and belong to f.sp. *pisi*, race 1 (IPO 60279) and 2 (IPO 10780), respectively. CWB 205 and CWB 206 belong to f.sp. *lupini* and were kindly provided as W3 and race 1, respectively, by Dr M. Rataj-Guranowska, Plant Protection Institute, Poznán, Poland. CWB 208 and 209 belong to f.sp. *cepae*; the first was received from the Plant Protection Service, Wageningen as PD83/816, and the latter was isolated from a diseased onion with typical symptoms of root rot. CWB 211, 212, and 213 were kindly provided by Dr H. Löffler of the DLO-Centre for Plant Breeding and Reproduction Research, Wageningen and belong to *F. sacchari* var. *elongatum* (Fon 1), *F. oxysporum* f.sp. *lilii* (Fol 4), and *F. oxysporum* f.sp. *gladioli* (Fog 5), respectively.

Soil was infested by incorporation of either a soil meal culture or talcum inoculum. Soil meal cultures were prepared by growing the fungus in Erlenmeyer flasks containing potting mix (Trio-17) amended with 5% (w/w) oat meal, which were autoclaved for 30 min on two consecutive days. The cultures were incubated for 2-3 wk at 23°C. Talcum inoculum was produced by growing the fungus in shaking cultures with malt extract broth. After seven days at 25°C, the cultures were homogenized in a blender, and centrifuged at 3400 x *g* for 30 min. The precipitate was resuspended in sterile distilled water and added to talcum powder (2:1, w/w). This mixture was dried with forced air for 14 days after which the talcum powder was passed through a sieve with 0.36-mm mesh. Population density of the talcum inoculum was determined by placing five replicate 0.25-ml aliquots on malt extract agar amended with oxytetracycline (50 μg ml^{-1}).

Plant material and nutrition. Plant material used is described under the respective experiments. Seeds of asparagus were disinfested in a solution of benomyl in acetone (Damicone *et al.*, 1981). Seeds of other crops were not disinfested. Pregermination of asparagus seeds was done by incubation on moist filter paper under sterile conditions at 22-25°C for 5-7 days.

Fertilization was by application of a nutrient solution containing 1.67 g l^{-1} Nutriflora-t and 2.0 g l^{-1} calcium nitrate. The solution contains in mg l^{-1}: N, 344; P, 78; K, 560; Mg, 50; Ca, 340; S, 206; Fe, 1.2; Mn 2.2; B, 0.7; Zn, 0.5; Cu, 0.020; and Mo, 0.116.

Test for pathogenicity on asparagus and disease rating. Pathogenicity was tested on asparagus plantlets grown aseptically on Knop's agar in culture tubes. The method was adapted from Stephens and Elmer (1988) as described previously (Blok and Bollen, 1995). Disease severity was rated after 4-5 weeks incubation at 20°C on a 0-4 scale as follows: 0, no disease; 1, only browning of roots, no lesions; 2, small number of brown lesions on primary and / or secondary roots, secondary root development hardly to mildly restricted; 3, many brown lesions on primary roots, secondary root development severely restricted; 4, death of the plant. Each fungal isolate was tested on three seedlings. Isolates which had a score of 3 or higher on at least one of the plants were rated as pathogenic and regarded as Foa.

Severity of Foa root rot of plants grown in soil was expressed either as a disease index combining the root rot severity on primary and secondary roots and the loss of secondary roots, or as the mean of the root rot rating for primary and secondary roots. Symptoms on primary (DPR) and secondary roots (DSR) of plants grown in soil were rated on a scale of 0-5 as follows: 0, no lesions; 1, 0-5% of the root length covered with lesions, 2 = 6-20%; 3, 21-60%; 4, 61-95%; 5, >95% or dead plants. The loss of secondary roots (LSR) was rated on an arbitrary scale from 0 (abundant secondary roots, no loss) to 10 (no secondary roots present, total loss). The severity of Foa root rot of the plants was expressed either as the mean of DPR and DSR or as a disease index (DI) ranging from 0 to 10. DI was calculated as (2DPR + 2DSR + LSR) / 3 (Blok and Bollen, 1996a).

Host range and root colonization

Testing in soil. Pathogenicity of Foa isolates to various plant species was tested in soil in four series. Details on plant material tested are given in Table 1. The soil was artificially infested by incorporation of soil meal cultures (0.5-1.5% fwt/fwt) of the pathogen. A treatment with autoclaved soil meal culture served as a control.

In series 1, Foa CWB 1 was tested on eight plant species grown in steamed potting mix in five replicate 1-l pots with three plants per pot. Pots were arranged randomly in a greenhouse, grown at 20-25°C, and evaluated for disease symptoms after 13 weeks.

In series 2, Foa CWB 1, 5, 6, 7, and 10 were tested on 11 species grown in steamed coarse sand in eight replicate pvc boxes (4x4x20 cm) with three seeds or plants per box. Boxes were arranged by isolate to minimize the risk of cross-contamination, and incubated in a climate chamber at 20°C and a 16-h-light period. Seed emergence was determined weekly and plants were evaluated for disease symptoms after four weeks.

In series 3, Foa CWB 1, 5, 7, and 14 were tested on 14 species. In this series root colonization was also studied and plants were grown in unsterilized field soil as being more relevant to field situations than sterilized soil mixtures. The soil was a loamy sand (pH-KCl 7.5, organic matter content 3.3%) collected from a field which had never been planted with asparagus. The plants of most species were planted in plastic pvc boxes (4x4x20 cm) with one plant per box, and 15 boxes, divided over five blocks, per species. Plants of bean, lupin, and

Table 1. Plant species tested in the study on host range of (series 1-4) and root colonization by (series 3) *F. oxysporum* f.sp. *asparagi*.

Latin name and cultivar	Common name	Series and plant age at inoculation
Allium cepa L. ('Verbeterde Rijnsburger')	onion	2, 3 (2 weeks)
Allium porrum L. ('Olifant reuzen')	leek	2, 3 (2 weeks)
Anethum graveolens L.	dill	1 (5 weeks)
Apium graveolens L. var. *rapaceum* (Mill.) DC. ('Roem van Zwijndrecht')	celeriac	1 (3 weeks), 2 (seed)
Asparagus officinalis L. ('Gynlim')	asparagus	1 (5 weeks), 2 (seed), 3 (2 weeks), 4 (4 weeks)
Beta vulgaris L. ('Bingo')	sugar beet	2, 3 (2 weeks)
Brassica oleracea L. convar. *capitata* L. ('Roem van Enkhuizen')	white cabbage	1 (5 weeks)
Chenopodium album L.	lamb's-quarters	3 (2 weeks)
Daucus carota L. ('Amsterdamse bak')	carrot	2 (seed), 3 (2 weeks)
Linum usitatissimum L.	flax	1 (5 weeks)
Lolium perenne L.	perennial rye-grass	3 (2 weeks)
Lupinus angustifolius L. ('Kubesa')	blue lupin	4 (2 weeks)
Lupinus luteus L. ('Ares')	yellow lupin	4 (2 weeks)
Lupinus luteus L. ('Gele bittere')	yellow lupin	1 (3 weeks), 2 (seed, 2 weeks), 3 (2 weeks)
Medicago sativa L.	alfalfa	2 (seed), 3 (2 weeks)
Phaseolus vulgaris L.	bean	3 (2 weeks)
Pisum sativum L. ('Kelvedon Wonder')	pea	2 (seed, 2 weeks), 4 (2 weeks)
Poa annua L.	Annual bluegrass	3 (2 weeks)
Stellaria media Vill.	chickweed	3 (2 weeks)
Triticum aestivum L. ('Okapi')	wheat	2 (seed), 3 (2 weeks)
Vicia faba L. ('Driemaal Wit')	broad bean	1 (3 weeks)
Zea mays L. ('Amador')	maize	1 (5 weeks)
Zea mays L. ('Anjou')	maize	2 (seed), 3 (2 weeks)

maize were planted in 1-l pots with two plants per pot and five replicates. Boxes and pots were arranged in five randomized complete blocks in a climate chamber at 20°C with a 16 h : 8 h light-dark photoperiod. After six weeks plants were uprooted and evaluated for disease symptoms, and root colonization was determined. Colonization of the roots by *F. oxysporum* was determined for alfalfa, asparagus, lupin, and perennial rye-grass with each treatment and for the other plant species with Foa CWB 1. For the species grown in pots, a sample of randomly cut root pieces was taken from each pot. For the species grown in boxes a composite sample was collected from each block, consisting of randomly collected root pieces of the three plants per block. Root samples weighing 2.0 g (fresh weight) were disinfested

in 1.0% sodium hypochlorite for 1.5 min (asparagus and maize) or 1 min (other species), rinsed three times in sterile distilled water and cut in 1-cm pieces. Roots were homogenized in 100 ml sterile distilled water in a blender for 3 min. The suspension was further diluted by transferring 10 ml of the suspension to 90 ml sterile distilled water. Samples of 0.5 ml of each dilution were placed onto three replicate plates with Komada's medium (Komada, 1975). The plates were incubated in the light on a laboratory bench at room temperature. After ten days the number of colonies of *F. oxysporum* were counted and a mean number of colony-forming units (cfu) of *F. oxysporum* per g fresh root tissue was calculated for each treatment x block combination. The numbers of cfu g^{-1} fresh root tissue were transformed to log(cfu+1) before analysis of variance was performed. Differences between treatments were evaluated with Tukey's multiple-comparisons procedure (Sokal and Rohlf, 1981). Six to 20 isolates of *F. oxysporum* were collected randomly from the Komada plates and tested for pathogenicity on asparagus under *in vitro* conditions, as described under general procedures.

Because results of series 1-3 were not conclusive for lupin and pea, susceptibility of these species together with asparagus was studied in an additional greenhouse experiment (series 4). Seedlings of asparagus (cv. Gynlim), two lupin species (*L. angustifolius* cv. Kubesa and *L. luteus* cv. Ares) and pea (cv. Kelvedon Wonder) were tested for their susceptibility to Foa CWB 1, 6, and 14. Pea and asparagus were also tested with *F. oxysporum* f.sp. *pisi* race 1 (CWB 200) and the lupin spp. and asparagus also with *F. oxysporum* f.sp. *lupini* (CWB 206). The seedlings were grown in a steamed mixture (1:1, v/v) of coarse sand and potting mix in seven replicate 1-l pots with two (pea and lupin) or three (asparagus) plants per pot. The pots were incubated in a greenhouse at 20-25°C and arranged per isolate to prevent cross-contamination. After five weeks, all pea plants inoculated with f.sp. *pisi* were dead and the pea plants of the other treatments were uprooted and evaluated for disease symptoms. The asparagus and lupin plants were uprooted and evaluated after 13 weeks.

Testing of lupin and pea under in vitro *conditions*. Seeds of pea ('Kelvedon Wonder'), *Lupinus angustifolius* ('Kubesa'), and *L. luteus* ('Ares') were disinfested by shaking for five min in 70% ethanol, rinsing in sterile distilled water, shaking for 25 min in 0.5% sodium hypochlorite, and finally rinsing five times in sterile distilled water. Seeds were placed in culture tubes (diameter 4.3 cm, height 22 cm) with 48 ml of Knop's agar and incubated at 27°C in the dark. After germination plants were incubated in a growth chamber at 20°C with a 16 h : 8 h light-dark photoperiod. When the first leaf was unfolded, plants were inoculated by placing two plugs of potato dextrose agar (PDA) with mycelium near the stem base. All species were inoculated with Foa isolates CWB 1, 6, 7, 10, and 14; pea was also inoculated with f.sp. *pisi* (CWB 200) and both lupin species with f.sp. *lupini* (CWB 206). Control plants received two plugs of PDA without fungus. After four weeks, final assessments for disease symptoms were made.

Susceptibility of asparagus to other formae speciales

Asparagus (cv. Gynlim) was tested for susceptibility to isolates of *F. oxysporum* f.sp. *asparagi* (CWB 1 and 14), f.sp. *pisi* (race 1, CWB 200, and race 2, CWB 201), f.sp. *lupini* (CWB 205 and 206), f.sp. *cepae* (CWB 208 and 209), f.sp. *lilii* (CWB 212), f.sp. *gladioli* (CWB 213), and *F. sacchari* var. *elongatum* (CWB 211), under *in vitro* conditions, as described under general procedures, and in soil. Field soil (loamy sand, pH-KCl 7.5, organic matter content 3.3%) was autoclaved and infested at a density of 10^4 cfu g^{-1} dry soil by incorporation of talcum inoculum. Uninfested soil served as a control. Soil of each treatment was divided over five 1-l pots and each pot received three pregerminated asparagus seeds. Pots were arranged by treatment in a greenhouse, grown at 20-25°C and treatments were separated by plastic sheets. After seven weeks, plants were uprooted and assessed for disease symptoms.

Effect of various crops on severity of Foa root rot

Soil was collected from a former asparagus field that was infested by Foa. The asparagus soil was mixed (1:1) with noninfested fresh soil to obtain a soil with an infestation level that would result in low disease levels. Both soils were loamy sands with an organic matter content of 3% and pH-KCl 6.8 and 7.5, respectively. The soil was divided into 4-l pots and grown with 11 plant species, whereas unplanted soil served as a control. The plant species were: alfalfa (*Medicago sativa* L.), asparagus (*Asparagus officinalis* L. 'Gynlim'), leek (*Allium porrum* L. 'Olifant reuzen'), lupin (*Lupinus luteus* L. 'Minarette'), maize (*Zea mays* L. 'Anjou'), pea (*Pisum sativum* L. 'Kelvedon Wonder'), perennial rye-grass (*Lolium perenne* L. 'Magella'), potato (*Solanum tuberosum* L. 'Eigenheimer'), sugar beet (*Beta vulgaris* L. 'Polykuhn'), wheat (*Triticum aestivum* L. 'Okapi'), and white mustard (*Sinapsis alba* L.). Plants were either directly seeded in the infested soil and later thinned, or planted as 7 to 14-day-old seedlings. The number of plants per pot was chosen in such a way that thorough rooting of soil was reached in a short period. There were six pots per treatment which were randomly arranged in six blocks in a greenhouse and grown at 20-25°C. After 13 weeks, plants were cut at soil level and the aboveground parts were discarded. After two weeks, soil was again seeded or planted as in the first cropping cycle. In the pots which had been cropped with potato the tubers from the first cycle were removed first. After 2, 5, and 7 weeks each pot received an application of 100 ml of the nutrient solution. After 11 weeks, the aboveground plant parts were again removed. After a fallow period of six weeks, the soil of each pot was mixed and crumbled by hand while seed tubers were removed and meristems cut to prevent regrowth. During all fallow periods the soil was kept moist. Five pregerminated asparagus ('Gynlim') seeds were then planted in each pot. Pots received nutrient solution at planting (200 ml) and after 2, 5, 7, 10, 11, and 12 weeks. After 15 weeks the plants were harvested and assessed for root rot. From all pots of two blocks a few root pieces with symptoms were collected, disinfested in 1% sodium hypochlorite for 1 min and

placed on PDA amended with oxytetracycline (50 μg ml^{-1}). Plates were assessed for growth of *F. oxysporum* after incubation at 23°C for one week.

Vegetative compatibility

Fungal isolates. Twenty-nine isolates of *F. oxysporum* collected from asparagus fields in the Netherlands in previous studies, were tested for their vegetative compatibility (Table 7). All isolates were tested for pathogenicity on aseptically grown asparagus plantlets, and several isolates were also tested on asparagus seedlings in soil in pot experiments. Results of both tests were consistent. Twenty-six isolates caused typical root lesions and were therefore assigned to Foa, three isolates (CWB 315, 316, and 317) were not pathogenic on asparagus. CWB 26-30, 34, and 36-39 were isolated from diseased one-year-old crowns collected from different fields within the asparagus growing area in the southeastern part of the Netherlands. The other isolates were isolated from diseased asparagus roots or soil collected from the experimental farm Meterikse Veld at Horst. All tests were made with cultures from single-spore isolates.

Selection and characterization of nit *mutants.* Mutants that could not reduce nitrate (*nit* mutants) were obtained using methods developed by Puhalla (1985) and Correll *et al.* (1987), and modified by Löffler and Rumine (1991). Mutants were selected by placing blocks of Czapek Dox agar (CDA) with growing mycelium on CDA supplemented with 5% potassium chlorate ($KClO_3$). Fast-growing sectors were transferred to CDA and those that grew as thin expansive colonies were considered *nit* mutants. An average of ten mutants were obtained for each isolate. The phenotypic class of the mutants was determined by growing the mutants on CDA supplemented with hypoxanthine (0.2 g l^{-1}) and on CDA supplemented with $NaNO_2$ (0.5 g l^{-1}).

Vegetative compatibility tests. Pairings were made by placing a NitM or a *nit3* mutant in the center of a CDA plate and three or four *nit1* mutants around it. Plates were incubated at 25°C and examined after one, two and three weeks for development of aerial mycelium, indicative of complementation. Intra-isolate pairings were performed for all combinations of *nit1* and NitM mutants to test for self-incompatibility of the isolate and to identify pairs of tester mutants which give strong heterokaryon growth. For CWB 27 and 37 NitM mutants were not obtained and *nit3* mutants were used instead. A *nit1* and a NitM (or *nit3*) mutant of each isolate was paired with a NitM (or *nit3*) and a *nit1*, respectively, of all other isolates. The inter-isolate pairings were conducted at least two times.

Results

Host range and root colonization

Among the 21 plant species tested, asparagus was the only host that showed typical root rot symptoms in all experiments. Occasionally, pea and lupin also showed mild symptoms of foot and root rot. In series 1, lupin plants showed foot rot symptoms in three of the five pots with infested soil, and died. Although the stem bases from these plants yielded *F. oxysporum*, we could not conclude that Foa caused the symptoms because these plants were also severely attacked by thrips. In series 2, brown to black lesions were found on the upper part of the main root of pea plants sown in soil infested with CWB 9 (eight of the 24 plants) and CWB 12 (seven of the 24 plants). All plants yielded *F. oxysporum*. Symptoms were not found when 2-wk-old plants were used instead of seeds. In the same series, emergence of lupin in soil infested with CWB 1, 7, 8, 9, and 12 was 33, 33, 100, 33, and 50% of emergence in the control, respectively. As with pea, the lupin plants stayed healthy when 2-wk-old seedlings were used instead of seeds. In series 3, large brown lesions developed on the stem base of one lupin plant infested with CWB 1 and two plants infested with CWB 14. From these plants Foa was recovered by isolation and subsequent testing of pathogenicity on asparagus.

In series 4, asparagus, pea and lupin were tested more extensively. Foa caused typical root rot symptoms on asparagus. Isolates of *F. oxysporum* f.sp. *pisi* (Fop) and f.sp. *lupini* (Fol) did not cause any disease symptoms on asparagus. Because the risk of cross-contamination was high in the greenhouse it was decided to arrange the pots per isolate. Therefore, conventional analysis of variance could not be performed and means with standard errors are given (Table 2). It seemed that the virulence of Foa CWB 14 on asparagus was lower than that of Foa CWB 1 and CWB 6. After five weeks, all pea plants grown in soil infested with Fop 200 were dead with the vascular system showing extensive browning. Pea plants of the other treatments stayed healthy without any vascular browning. Neither *L. luteus* nor *L. angustifolius* displayed typical wilt symptoms when infested with Fol. However, after 13 weeks all plants showed browning of the vessels extending to the top of the plants, in most plants accompanied by a brown discoloration of the stem base. The *L. luteus* plants infested with Fol had no root nodules and most roots had rotted. The *L. angustifolius* plants, however, had normal root nodules and no root rot was evident. The extensive vascular browning in stems was not found with any of the Foa isolates. However, brown discoloration of the external or internal stem base tissue was found in several plants, especially those of *L. luteus* inoculated with CWB 14 (Table 3). Five to ten stem bases per species-isolate combination were externally disinfested and placed onto Komada's medium. Stem bases of plants infested with Fol yielded consistently the same type of *Fusarium* colony which was easily recognizable as the introduced isolate of Fol because of its dark purple colour. Stem bases of plants infested with Foa CWB 14 also yielded consistently the same type of *Fusarium* colony, confirmed to belong to Foa by means of pathogenicity tests. Stem bases of plants infested with

Table 2. Root rot severity and root dry weight of asparagus plants grown in soil infested with *F. oxysporum* f.sp. *asparagi* (Foa), f.sp. *pisi* (Fop), or f.sp. *lupini* (Fol)(host range study, series 4).

Inoculum	Disease index (0-10)		Root dry weight (g/pot)	
	Mean	SEM[a]	Mean	SEM
Control	0.48	0.07	2.84	0.12
CWB 1 (Foa)	7.49	0.13	0.39	0.03
CWB 6 (Foa)	7.06	0.08	0.66	0.05
CWB 14 (Foa)	2.89	0.19	1.50	0.13
CWB 200 (Fop)	0.48	0.10	2.36	0.10
CWB 206 (Fol)	0.57	0.10	2.57	0.10

[a] Standard error of the mean.

Table 3. Symptoms on lupin plants grown in soil infested with Foa CWB 1, 6, or 14, or with Fol CWB 206 (host range study, series 4).

	Lupinus luteus				*Lupinus angustifolius*			
	External browning stem base	Internal browning stem base	Vascular browning in stem	No symptoms	External browning stem base	Internal browning stem base	Vascular browning in stem	No symptoms
Control	3 [1]	1		11	2	1	1	12
CWB 1	6	6		8				14
CWB 6	6			8				14
CWB 14	8	6		2	3	5		8
CWB 206	14	14	14	0	6	14	14	0

[1] Values in the table denote the number of plants with these symptoms. The maximum number is seven pots x two plants = 14 plants.

other Foa isolates or grown in uninfested soil yielded either no *Fusarium* colonies or *Fusarium* colonies of diverse morphology. Only a few of these isolates were found in pathogenicity tests to belong to Foa.

Pea and lupin plants grown in culture tubes under aseptic conditions developed very well and stayed healthy and green in the control treatments during the whole experimental period. Fop CWB 200 caused moderate to intense brown discoloration of the whole pea root system without, however, killing the plants or causing vacular discoloration. Fol CWB 206 caused severe foot rot resulting in plant death of one of the plants of *L. luteus*, the other three plants

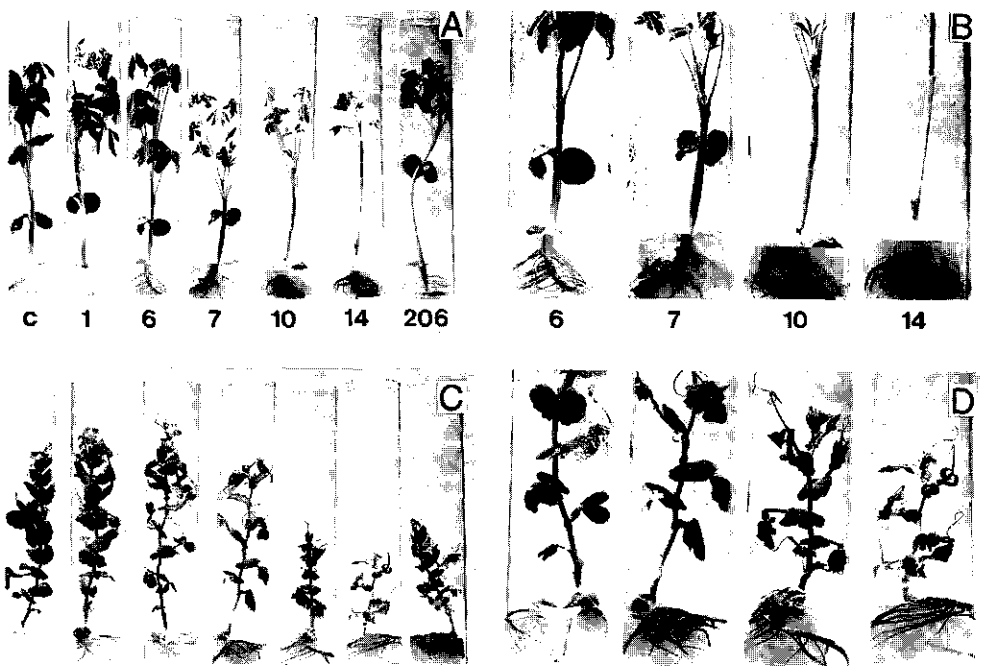


Figure 1. Pathogenicity tests on plants grown aseptically on Knop's agar.
A - *Lupinus luteus* plants not inoculated (C) or inoculated with Foa CWB 1, 6, 7, 10, or 14, or *F. oxysporum* f.sp. *lupini* CWB 206.
B - Detail of *L. luteus* plants inoculated with Foa CWB 6, 7, 10, or 14.
C - Pea plants not inoculated (C) or inoculated with Foa CWB 1, 6, 7, 10, or 14, or *F. oxysporum* f.sp. *lupini* CWB 200.
D - Detail of pea plants inoculated with Foa CWB 6, 7, 10, or 14.

of *L. luteus* and all four plants of *L. angustifolius* stayed healthy. Foa CWB 1 and 6 did not cause any symptoms on pea and the two lupin species, however CWB 7,10, and 14 caused intense brown discoloration and rot of the roots and stem base resulting in severe stunting and partial collapse of the three species (Fig. 1).

Although symptoms on roots were only found on asparagus, asymptomatic roots were infected and colonized by Foa in all plant species except onion and leek, when tested in artificially infested field soil (Table 4 and 5). By far the highest number of colony-forming units was found in roots of asparagus, followed by bean and chickweed. In a comparison of

Table 4. Colonization by Foa of externally disinfested roots of plants grown in a climate chamber in field soil artificially infested with Foa CWB 1 (host range study, series 3).

Plant species	Inoculum	Cfu of Fo g^{-1} air-dry roots		Fraction of path. isolates	Cfu of Foa g^{-1} air-dry roots
Alfalfa	1	174	bcd[1]	13 /16[2]	141[3]
Annual bluegrass	1	4	d	5 / 6	3
Asparagus	1	124881	a	17 / 20	106149
Bean	control	229		0 / 13	0
	1	2461	ab	8 / 20	984
Carrot	1	21	cd	7 / 9	16
Chickweed	1	1218	bc	13 / 20	792
Lamb's-quarters	1	112	bcd	20 / 20	112
Leek	1	0		-	0
Lupin	1	41	bcd	11 / 18	25
Maize	1	22		-	-
Onion	1	0		-	0
Perennial rye-grass	1	110	bcd	6 / 16	41
Sugar beet	1	475	bc	17 / 20	404
Wheat	7	508		1 / 10	51

[1] For statistical analysis, values were transformed to log (cfu+1). Means presented are back-transformed values. Means of treatments with CWB 1 were separated with Tukey's multiple-comparisons procedure. Means followed by the same letter are not statistically different ($P \leq 0.05$). Data for onion, leek, and maize were excluded from the analysis, as roots of onion and leek did not yield *Fusarium* colonies, and maize roots of the five replicates were combined into one composite sample.

[2] Number of pathogenic isolates / number of isolates tested.

[3] Fraction of isolates pathogenic to asparagus was determined for a composite sample per plant species * isolate combination, not for each replicate separately. Therefore, means for colony-forming units of Foa are only approximate values and mean separation by Tukey's procedure was not performed.

different isolates of Foa colonizing roots of alfalfa, asparagus, lupin, and perennial rye-grass (Table 5), analysis of variance showed a significant plant species x isolate interaction ($P \leq 0.05$). Therefore, differences among isolates were tested per plant species. Due to the large variation in numbers of colony-forming units, differences among isolates were not significant for any of the plant species; the only differences were between inoculated and non-inoculated plants (Table 5). Foa was also detected in low numbers in plants grown in the noninfested soil, indicating that either the soil has been naturally infested or that cross-contamination occured during the experiment.

Susceptibility of asparagus to other formae speciales

When asparagus was tested *in vitro* for susceptibility to seven formae speciales, typical root

Table 5. Colonization by Foa of roots of weed and crop plants grown in the climate chamber in field soil artificially infested with various isolates of Foa (host range study, series 3).

Plant species	Inoculum	Cfu of *F. oxysporum* g^{-1} airdry roots		Fraction of path. isolates	Cfu of Foa g^{-1} airdry roots
alfalfa	None	7	a[1]	1 / 9	1[2]
	CWB 1	174	ab	13 / 16	141
	CWB 5	715	b	15 / 20	536
	CWB 7	39	ab	18 / 18	39
	CWB 14	445	ab	19 / 20	423
asparagus	None	13	a	9 / 10	12
	CWB 1	124881	b	17 / 20	106149
	CWB 5	69071	b	14 / 20	48350
	CWB 7	13166	b	10 / 17	7745
	CWB 14	5628	b	19 / 20	5347
lupin	None	23	a	1 / 12	2
	CWB 1	41	ab	11 / 18	25
	CWB 5	291	ab	7 / 17	120
	CWB 7	130	ab	17 / 19	116
	CWB 14	1666	b	18 / 20	1499
perennial rye-grass	None	11	a	0 / 6	0
	CWB 1	110	a	6 / 16	41
	CWB 5	18	a	11 / 13	15
	CWB 7	59	a	17 / 17	59
	CWB 14	694	a	13 / 18	501

[1] Back-transformed values. Means within a plant species followed by the same letter are not statistically different according to Bonferroni's multiple comparisons procedure ($P \leq 0.05$).
[2] Fraction of isolates pathogenic to asparagus was determined for a composite sample per plant species * isolate combination, not for each replicate separately. Therefore, means for colony-forming units of Foa are only approximate values and mean separation by Tukey's procedure was not performed.

lesions and eventually plant death were caused only by Foa. The isolates from flower bulbs (*F. oxysporum* ff.sp. *lilii* and *gladioli*, and *F. sacchari* var. *elongatum*) caused superficial root browning, thickened secondary roots, and small brown lesions on the roots but never killed the plantlets. In the greenhouse experiment, typical root rot symptoms were caused by Foa (both CWB 1 and 14) but not by any of the other formae speciales. Severity of root rot caused by CWB 14 was significantly lower than that caused by CWB 1, the mean root rot rating for primary and secondary roots being 1.3 and 4.3, respectively, on a scale of 0 to 5. The lower virulence of CWB 14 compared to CWB 1 was also found in another experiment

Table 6. Root rot severity and root dry weights of asparagus plants grown in Foa-infested field soil previously cropped twice with different crops.

Preceding crop (0-10)	Disease index (g/pot)	Root dry weight
None (fallow)	2.14 a[1]	2.33 a[1]
Alfalfa	2.37 ab	1.98 a
Asparagus	9.11 c	0.17 b
Leek	3.16 b	1.82 a
Lupin	2.41 ab	1.68 a
Maize	2.77 ab	1.92 a
Pea	2.54 ab	1.93 a
Perennial rye-grass	2.91 ab	1.48 a
Potato	2.76 ab	1.95 a
Sugar beet	2.89 ab	1.72 a
Wheat	2.89 ab	1.62 a
White mustard	2.57 ab	1.82 a

[1] Means followed by the same letter are not statistically different according to Tukey's multiple comparisons procedure ($P \leq 0.05$).

(Table 2). A control of uninfested soil with uninfested talcum powder was not included in this experiment as it was found in earlier experiments that talcum powder did not cause any symptoms on asparagus plants (data not shown).

Effect of various crops on infestation of soil with Foa

Cropping artificially-infested field soil with asparagus greatly increased the severity of Foa root rot (Table 6). The other crops did not increase or only slightly increased root rot severity compared to the fallow treatment. *F. oxysporum* was isolated from the lesions on the roots of the asparagus plants in the bioassay. In pots with leek as a preceding crop a red discoloration of the secondary asparagus roots was found. From these roots *Phoma terrestris* was isolated. Occasionally, this fungus was also isolated from asparagus roots in most other treatments.

Vegetative compatibility

Nit mutants were readily recovered from all isolates on CDA amended with 50 g l^{-1} $KClO_3$, although in selecting mutants on CDA with 15 g l^{-1} $KClO_3$ as used by most researchers, it appeared that growth of several isolates was not restricted. The concentration was thus increased to 50 g l^{-1} $KClO_3$, following Roebroeck and Mes (1992). On average, 40% of the mutants selected were *nit1*, 22% were *nit3*, and 38% were NitM. All isolates were self-

compatible, as was concluded after heterokaryon formation in *nit1* x NitM (for two isolates *nit1* x *nit3*) pairings, and could therefore be assigned to a VCG. For several isolates it was found that not all *nit1* x NitM combinations yielded heterokaryons. The intensity of heterokaryon growth could also be different between *nit1* x NitM combinations of a given isolate. After intra-isolate pairings were performed, a pair of complementary *nit* mutants that gave a clear heterokaryon development was chosen to perform the inter-isolate pairings. All compatible inter-isolate pairings showed a clear line of aerial mycelium, but the time needed to form this and the intensity of heterokaryon growth was different.

Nine Foa isolates were assigned to three different VCGs. Eighteen isolates, including three isolates not pathogenic on asparagus, were not compatible with other isolates and were assigned to a unique VCG (Table 7). Two isolates had not yielded a NitM mutant and were therefore not assigned to a VCG.

Discussion

Foa was found to invade and colonize roots of numerous plant species and to cause mild disease symptoms on pea and lupin and severe symptoms on asparagus. Apparently, Foa has a broad parasitic host range (in terms of infecting without causing disease) but a limited pathogenic host range. This observation has also been made for several other, vascular and non-vascular, formae speciales of *F. oxysporum* (Armstrong and Armstrong, 1948; Hendrix and Nielsen, 1958; Katan, 1971; Elmer and Lacy, 1987; Menzies *et al.*, 1990). Menzies *et al.* (1990) inoculated 47 species and 11 cultivars within five of these species with four isolates of *F. oxysporum* f.sp. *radicis-lycopersici*. They identified noninfected species, symptomless hosts, susceptible species showing only mild disease symptoms, and highly susceptible species. For several formae speciales, the identification of symptomless and mildly susceptible hosts as well as the primary highly susceptible host indicates that the original forma-specialis concept as described by Snyder and Hansen (1940), based on selective pathogenicity, is not applicable in the strict sense for these formae speciales. However, we do not argue to dispense with the forma specialis designation as it is still a useful designation to describe those forms of *F. oxysporum* such as Foa which cause under field conditions severe disease on only a limited number of plant species.

In the short term, symptomless hosts apparently did not affect survival of Foa compared to soil from fallow regimes (Table 6). However, the invasion and colonization of symptomless hosts by Foa might contribute to extended long-term survival of the pathogen in the field and this could be a factor in the epidemiology of Foa root and crown rot. Production of chlamydospores and colonization of the highly persistent asparagus root residues (Blok and Bollen, 1996b) afford an efficient means for long-term survival.

Table 7. Isolates of *F. oxysporum* used in the study of vegetative compatibility groups in Dutch isolates of Foa, with the mutant types used for the complementation tests and the VCG grouping.

Isolates[1]	Origin[2]	Substrate	Mutant phenotype *nit1*	*nit3*	NitM	Intensity of heterokaryon[3]	VCG
CWB 1	Castenray	asparagus root	*		*	weak	1
CWB 2	Meterik	asparagus root	*		*	strong	2
CWB 3	Meterik	asparagus root	*		*	strong	3
CWB 4	Meterik	asparagus root	*		*	weak	4
CWB 6	Meterik	asparagus root	*		*	strong	1
CWB 7	Meterik	asparagus root	*		*	weak	5
CWB 11	Meterik	soil	*		*	strong (slow)	1
CWB 26	Mariaheide	asparagus root	*		*	strong	6
CWB 27	Afferden	asparagus root	*	*		strong	-[4]
CWB 28	Kessel	asparagus root	*		*	strong	7
CWB 29	Helden	asparagus root	*		*	strong	8
CWB 30	Helden	asparagus root	*		*	strong	9
CWB 31	Meterik	soil	*		*	strong	10
CWB 32	Meterik	soil	*		*	strong	11
CWB 33	Meterik	soil	*		*	strong (slow)	12
CWB 34	Horst	asparagus root	*		*	strong	9
CWB 35	Meterik	soil	*		*	strong	13
CWB 36	Horst	asparagus root	*		*	strong	14
CWB 37	Mierlo	asparagus root	*	*		strong	-[4]
CWB 38	Reusel	asparagus root	*		*	strong	15
CWB 39	Dinther	asparagus root	*		*	strong	16
CWB 40	Meterik	soil	*		*	strong (slow)	17
CWB 41	Meterik	soil	*		*	strong	4
CWB 42	Meterik	soil	*		*	strong (slow)	9
CWB 43	Meterik	soil	*		*	strong (slow)	9
CWB 44	Meterik	soil	*		*	strong	18
CWB 315	Meterik	asparagus root	*		*	strong	19
CWB 316	Meterik	asparagus root	*		*	strong	20
CWB 317	Meterik	soil	*		*	strong (slow)	21

[1] CWB 315, 316, and 317 are not pathogenic on asparagus, all other isolates belong to f.sp. *asparagi*
[2] Isolates from Meterik are from closely neighbouring fields on the experimental farm Meterikse Veld, all other origins are located in the asparagus-producing area in the southeastern part of the Netherlands
[3] Reaction of the mutant pair selected to represent the isolate in the inter-isolate pairings. With a strong reaction, an unbroken line of robust aerial mycelium is formed at the contact zone; with a weak reaction, a broken line of weak aerial mycelium is formed. Generally, heterokaryon growth in compatible interaction could be rated after one week, for some isolates it took two weeks (slow)
[4] Isolates from which no NitM mutant was obtained were not assigned to a VCG

Symptomless hosts afford the possibility of a constant renewal of part of the population, thus extending, possibly indefinitely, the survival period. Colonized root residues of symptomless hosts also add new reservoirs of inoculum. An additional epidemiological consequence of

symptomless hosts might be that the pathogen is spread by infected symptomless transplants of various vegetable species. This would be important especially for noninfested areas.

The *in vitro* pathogenicity tests clearly showed that Foa isolates can vary in their pathogenicity to secondary hosts, e.g., pea and lupin. Foa CWB 1 and 6 did not cause symptoms whereas CWB 7, 10, and 14 caused severe foot and root rot on pea and lupin under these conditions. Although only mild disease symptoms were caused on lupin when tested in soil (series 4), the results correspond with those of the *in vitro* tests because symptoms were present on most plants inoculated with isolate 14, whereas CWB 1 and 6 caused hardly any symptoms. Pea did not show symptoms with any of the Foa isolates in soil in series 4, which indicates that pea is less susceptible to our Foa isolates than lupin.

The number of colony-forming units of Foa was much lower in roots of symptomless colonized hosts than in those of asparagus. Other authors also found a higher colonization of susceptible hosts than of symptomless hosts (Katan, 1971; Elmer and Lacy, 1987). However, Smith and Snyder (1975) reported that populations of *F. oxysporum* f.sp. *vasinfectum*, the cotton wilt pathogen, may increase faster under barley as a symptomless host than under continuously planted cotton. In a greenhouse experiment with naturally infested field soil planted with various crop species, we did not obtain indications that planting of symptomless hosts will result in increased severity of Foa root rot. Asparagus strongly increased root rot severity but the disease severity of the other plant species was not significantly different from that of the fallow treatment (Table 6). Additional crop rotation experiments are needed to study long-term effects of symptomless hosts on disease severity. It would be interesting to include onion and leek in these experiments, as these were the only species not infected by Foa.

The low susceptibility of pea and lupin found in this study, and of gladiolus as reported by Graham (1955), implies that Foa will not normally be a problem in these crops. Only when these plants are growing under suboptimal conditions might Foa cause damage. This was obviously the case with the lupin crop from which Foa CWB 14 was obtained. Most of the lupin plants did not show any symptoms of foot and root rot but there was one strip of land in which all plants, probably as a result of a poor soil structure, showed severe foot and root rot symptoms.

The low susceptibility of lupin and pea and the influence of environmental conditions on symptom expression may explain discrepancies in results of host range studies. Grogan and Kimble (1959) found no symptoms on pea, and Armstrong and Armstrong (1969) observed none on lupin. However, Cohen and Heald (1941) found mild wilting of pea by Foa. Another reason for the discrepancies might be, as found here, that Foa isolates differ considerably in their pathogenicity to secondary hosts.

Elmer and Stephens (1989) found using an *in vitro* assay that isolates of f.sp. *apii*, *cepae*, and *gladioli* were pathogenic on asparagus. In a similar assay, our isolates of f.sp. *cepae* did not cause any symptoms on asparagus. The symptoms caused by *F. oxysporum* f.sp. *gladioli*

and *lilii* and *F. sacchari* var. *elongatum* were clearly different from the discrete, brown root lesions caused by Foa. When tested in soil, none of these isolates caused symptoms. Therefore, susceptibility of asparagus to the formae speciales of *F. oxysporum* tested in this study was not established. The results reported by Elmer and Stephens (1989) need confirmation through additional pathogenicity tests in soil.

In the introduction we reasoned that the genetic diversity of the Foa population in the Netherlands might be narrower than that of the population in the USA. However, this was not established in our analysis, as 24 Foa isolates were assigned to 18 VCGs. The majority of the isolates (18 of the 24) came from closely neighbouring fields of the same experimental farm, the remaining ones came from a limited area around this farm. Only three VCGs were found with more than one isolate. These findings concur with those of Elmer and Stephens (1989) and indicate that the Dutch Foa population is very genetically diverse. The latter conclusion corresponds with the finding that Foa isolates showed a differential pathogenicity to pea and lupin.

Puhalla (1985) put forward a micro-evolutionary model for the origin of VCGs in *F. oxysporum* and hypothesized that the relationships between formae speciales and VCGs are relatively simple, isolates in the same forma specialis belonging to one or a few VCGs only. Although examinations in many formae speciales have supported the latter (Leslie, 1993), the large number of VCGs within Foa identified in the USA and in the Netherlands clearly is an exception and raises the question of the origin of these VCGs. The Foa population resembles nonpathogenic, soil-borne populations of *F. oxysporum*, as these were found to be also very diverse with respect to vegetative compatibility (Correll *et al.*, 1986; Gordon and Okamoto, 1991). It might be speculated that pathogenicity to asparagus is a trait which is easily obtained by a simple mutation and could, therefore, arise in many different VCGs of originally nonpathogenic *F. oxysporum* resulting in the present situation with f.sp. *asparagi* divided in many VCGs. Another explanation may be that pathogenicity to asparagus is based on a gene useful in an undiscovered ecological niche, e.g. utilization of a specific substrate, and occurring in many strains of various VCGs. The hypothesis that pathogenicity to asparagus is easily obtained would correspond with the general opinion that the foot- and root-rotting forms of *F. oxysporum*, including Foa, are rather less specialized. However, for f.sp. *lycopersici*, a true vascular form, many VCGs were identified (Elias and Schneider, 1991), whereas for f.sp. *radicis-lycopersici*, causing foot and root rot in tomato, only a limited number of VCGs was identified (Katan *et al.*, 1991). Therefore, further research involving measures of genetic diversity is required to provide explanations for the VCG diversity found in f.sp. *asparagi*.

Chapter 8

Biological control of *Fusarium oxysporum* f.sp. *asparagi* by applying nonpathogenic isolates of *F. oxysporum*

Abstract

Root rot severity of asparagus plants grown in sterilized field soil inoculated with *F. oxysporum* f.sp. *asparagi* (Foa) was reduced by more than 50% when the soil was precolonized by each of 13 nonpathogenic (np) isolates of *F. oxysporum* originating from asparagus roots or field soils. In a greenhouse experiment, application of six np isolates to naturally infested field soil was followed by a 23 to 49% decrease of disease severity, depending on the isolate. One of them, Fo47 originating from *Fusarium* suppressive soil in France, was applied to field plots infested with Foa. Foa root rot was not suppressed in asparagus plants grown for one year in these plots.

Pathogenic and np isolates extensively colonized the root surface and isolates of both types infected the roots of asparagus plants grown in sterilized field soil, with significant differences among the np isolates. Inoculation of sterilized field soil with np isolates reduced germination of Foa chlamydospores by 43 to 64% depending on the isolate used.

It is concluded that np isolates of *F. oxysporum* can suppress asparagus root rot caused by Foa in naturally infested field soil. The differences for root colonization capacity among the np isolates imply that selection for this trait might reveal isolates that perform better under field conditions.

Introduction

In the Dutch provinces Limburg and Noord-Brabant, asparagus has been grown for decades and land where asparagus had not previously been grown and suitable for its production is limited. In replanted fields, early decline of the asparagus crop is generally encountered which limits commercial production to 6-7 years. In the Netherlands early decline is caused by crown and root rot incited by *Fusarium oxysporum* (Schlecht.) emend. Snyder & Hansen f.sp. *asparagi* Cohen & Heald (Foa). The fungus survives asparagus-free periods for at least 20 years and was found to be present in soil up to one meter deep (Blok and Bollen, 1996a). Control of Foa under field conditions is difficult to achieve due to the perennial nature of the crop, the persistence of Foa and its presence deep in the soil. Currently, farmers lack effective control measures. Resistant varieties are not available (Stephens *et al.*, 1989) and chemical disinfestation of soil or planting material is either ineffective or provides only short-term control (Manning and Vardaro, 1977; Lacy, 1979; Di Lenna *et al.*, 1988). Elmer (1995) succeeded in suppressing Fusarium crown and root rot under various field conditions by application of sodium chloride to asparagus beds. The author suggested that suppression results from a higher resistance of the roots due to higher manganese levels in the roots. The

latter is brought about by the action of manganese-reducing rhizobacteria whose numbers are increased after NaCl-application.

Soils suppressive to *Fusarium* diseases of various crops have been identified (Toussoun, 1975). Fluorescent pseudomonads and nonpathogenic *Fusarium* isolates were claimed to be the major organisms responsible for the suppression (Scher and Baker, 1982; Alabouvette, 1986). Organisms of both types have been tested, singly or in combination, as biological control agents against several pathogens in numerous crops. In a few cases the results showed good prospects for this form of control (Sneh *et al.*, 1984; Lemanceau and Alabouvette, 1991; Postma and Rattink, 1992; Leeman *et al.*, 1995). Damicone and Manning (1982) found a reduction of Fusarium crown and root rot in asparagus after treating seedlings with spore suspensions of a nonpathogenic strain of *F. oxysporum* and planting in a naturally infested field for eight weeks.

The aim of the present study was to explore the potential for exploiting nonpathogenic isolates of *F. oxysporum* as control agents against Foa root rot of asparagus. Their efficacy was first tested in sterilized soil in the climate chamber. The most effective isolates were further tested in naturally-infested field soil in greenhouse experiments. Subsequently, a preliminary field experiment was conducted to test the effect of one nonpathogenic strain on Foa root rot development in the production of one-year-old crowns used as planting material. As the ability to follow root growth seems a necessary trait for an antagonist to be effective against Foa in asparagus fields, root colonization and infection rates were estimated. The effect of nonpathogenic isolates on germination of Foa chlamydospores in the rhizosphere of asparagus was assessed also.

Materials and methods

General procedures

Isolation of F. oxysporum *from plant roots and pathogenicity testing*. Roots were collected from four different asparagus fields in late autumn when plants had started to turn yellow. In each field ten plants were selected which were still green, and one 5-cm part of each of five fleshy storage roots free of disease symptoms were collected from each plant. Root parts were washed five times in sterile distilled water, blotted dry, and rolled over a Petri dish with Komada's agar medium (Komada, 1975). Subsequently, the root parts were externally disinfested in 2% sodium hypochlorite for 5 min, rinsed three times in sterile distilled water and plated on Komada's agar medium. After incubation for 10 days in the light at 23°C, colonies which were identified as *F. oxysporum* based on colony morphology were subcultured onto oatmeal-agar slants, allowed to grow for one week and stored at 4°C until further use.

Pathogenicity of the isolates was tested on asparagus plantlets aseptically grown in culture

tubes on Knop's agar as described by Stephens and Elmer (1988) and modified by Blok and Bollen (1995). After four weeks, plants were assessed for the presence of disease symptoms. Isolates were tested on three plantlets and considered nonpathogenic when all three plantlets remained free of lesions. A total of 176 *F. oxysporum* isolates was tested for pathogenicity.

Production of fungal inoculum. Talcum inoculum was produced by growing a fungus in shaking cultures with malt extract broth. After seven days at 25°C, cultures were homogenized in a blender, centrifugated at 3400 x *g* for 30 min, and the precipitate resuspended in sterile distilled water and added to talcum powder (2:1, w/w). This mixture was dried with forced air for 14 days after which the talcum inoculum was passed through a 0.36-mm-mesh sieve.

Soil inoculum was produced in field soil (loamy sand; pH 6.2; organic-matter content 2.7%) amended with oat meal (7%, dw/dw). The soil was sieved through a 2-mm-mesh screen and put in double, autoclavable plastic bags (10 kg per bag) fitted with an inlet and an outlet that were closed with a cotton wool plug. The bags were autoclaved twice on consecutive days and inoculated by incorporation of 100 ml of a 1-wk-old shaking culture of Foa CWB 1 in malt extract broth. The bags were aerated with moistened forced air that had passed through a 0.45-μm filter, and incubated at room temperature. After 14 days, the soil was dried by placing it in open boxes covered with paper. When soil was air-dry, after a further 14 days, it was sieved through a 2-mm-mesh screen.

Population density of the fungi in both inocula was determined by dilution platings of three samples on malt extract agar amended with oxytetracycline (50 μg ml^{-1}).

Plant material. Asparagus seeds were disinfested in sodium hypochlorite and subsequently in a solution of benomyl in acetone according to Stephens and Elmer (1988). The seeds were either sown directly or pregerminated aseptically in Petri dishes on moist filter paper at 25-27°C. Asparagus cultivar Gynlim was used for all experiments except the field experiment where cv. Backlim was used. Seedlings for the field experiment were grown from disinfested seeds in potting-mixture balls (5 cm length, 2 cm diameter) in trays, in a commercial greenhouse nursery.

Screening of isolates for control of Foa root rot in sterilized field soil

Fifty-eight fungal isolates and one strain of a *Streptomyces* sp. were tested for their ability to control Foa root rot using a procedure similar to that of Tamietti and Pramotton (1987). All isolates were nonpathogenic to asparagus when tested *in vitro*. The *Streptomyces* strain was isolated from the commercial product Mycostop, kindly provided by Dr. J. Uoti, Kemira Oy, Helsinki, Finland. The fungal isolates belonged to *F. oxysporum* unless otherwise indicated and include: 39 isolates obtained from asparagus roots as described above; 12 isolates isolated in earlier experiments from soil from various asparagus fields (CWB 306-

317); 6 isolates with known biocontrol capacities, kindly provided by Dr. J. Postma (DLO-institute for Phytopathological Research, Wageningen, the Netherlands). The latter isolates (described in Postma and Luttikholt, 1993) were: Fo47 and a benomyl-resistant mutant Fo47-B10 (both originally provided by Dr. C. Alabouvette), 618-12 (originally provided by Dr. H. Rattink) and a benomyl-resistant mutant 618-12 B17, and Fmon, an isolate of *F. proliferatum* (originally from Dr. R. Tramier) and a benomyl-resistant mutant FmonB1. The isolates were single-spored before use and identified according to Nelson *et al.* (1983). Additionally, isolate M8 of *Talaromyces flavus* (kindly provided by M. Nagtzaam, Department of Phytopathology, Wageningen Agricultural University, Wageningen, the Netherlands) and a combination of *F. oxysporum* Fo47 and *Streptomyces* sp. were tested for their activity.

The isolates were first allowed to colonize sterilized field soil and subsequently, the colonized soil was infested with Foa and planted with pregerminated asparagus seeds. The number of isolates was too large to test in one series and, therefore, they were tested in two series, each with a control with sterilized soil only, a second control with only the pathogen, and a third one with Fo47.

Loamy sand (pH-KCl 7.5, organic-matter content 3.3%) from an experimental field at Wageningen was sieved through a 4-mm-mesh screen, moistened to a soil water potential of pF 2.0 (-10 kPa), and autoclaved twice on consecutive days in 1-l Erlenmeyer flasks containing 750 g soil. For each isolate to be tested, one flask was inoculated with five plugs of growing mycelium on potato dextrose agar (PDA). The flasks were incubated at 23°C for three weeks and shaken once every three days. After colonization of the soil, a sample was taken from each flask and population density was determined by diluting 10.0 g dry-weight equivalent of soil in 90 ml sterile distilled water, shaking for 1 h, making serial dilutions in sterile distilled water, and plating on PDA amended with oxytetracycline (50 $\mu g\ l^{-1}$) for the fungi or on soil extract agar for *Streptomyces* sp.. The colonized soil was infested with the pathogen by mixing it with talcum inoculum of Foa isolate CWB 1 to reach a population density of 100 colony-forming units (cfu) g^{-1} dry soil. The soil was divided over six pvc tubes (height 20 cm, diameter 2.5 cm), planted with one pregerminated asparagus seed per tube. The tubes were placed completely randomized in a climate chamber at 20°C and 16 h light a day. During the first week, the tubes were closed with a lid to minimize evaporation. From the second week on, the tubes were weighed weekly and distilled water was added to maintain a soil water potential of pF 2.0. After six weeks, the plants were uprooted and the roots washed free of soil and rated for root rot on a 0-5 scale with 0= no symptoms; 1= few, small lesions, no rotted roots; 2= moderate number of lesions, no or slight restriction of root system; 3= many lesions, root system clearly restricted; 4= root system very severely restricted and largely brown; 5= plant death or nearly so. Dry weight of the roots was determined after drying at 105°C for 24 h.

Screening of isolates for control of Foa root rot in naturally infested field soil

Soil was collected on a former asparagus field at the experimental farm Meterikse Veld. Infestation with Foa was confirmed in a bio-assay. The soil was a loamy sand with pH-KCl 5.9 and organic-matter content 2.8%. A similar soil but without an asparagus history (fresh soil) was also collected on the same farm. Both soils were sieved through a 1-cm-mesh screen. In a concrete mixer the asparagus soil was mixed with talcum inoculum of one of six different isolates of *F. oxysporum* which had shown good control of Foa root rot in the primary screening in sterilized soil. The fresh soil was left uninfested. The amount of talcum inoculum added was sufficient to give a density of $1x10^6$ cfu g^{-1} soil. The isolates tested were: Fo47, 618-12, CWB 312, CWB 314, CWB 318, and CWB 319. Fo47 came from soil and 618-12 from the vascular tissue of a carnation plant. The isolates coded as CWB were isolated in this study; CWB 312 and 314 from soil, CWB 318 and 319 from disinfested asparagus roots. Isolates were selected that decreased root rot severity by >50% in the primary screening in sterilized soil. Additional reasons for further screening were the capacity of Fo47 and 618-12 in biocontrol of diseases in other crops. The strains are currently considered for commercialization (Dr C. Alabouvette, personal communication). The origin of an isolate (soil vs. roots) might be related to the biocontrol mechanism involved (e.g. competition for nutrients or infection sites vs. induced resistance) and, therefore, isolates from roots as well as from soil were included. A long shelf life is a desirable property of a biocontrol agent formulation and, therefore, only those isolates were included of which the viability in talcum inoculum had retained a level of more than 70% of the original population density after storage for eight months at 4°C. As a control treatment, the fungicide prochloraz (Sportak; 65 mg a.i. kg^{-1} dry soil) was applied to asparagus soil. The soil was divided over seven 1-l pots and planted with four 3-wk-old asparagus plants per pot. The plants were grown from disinfested seeds in a mixture of sterile coarse sand and sterile potting mix (1:1, v/v). The pots were placed in seven randomized blocks in a greenhouse at 23°C ± 2°. After nine weeks, each pot received 100 ml of a nutrient solution containing 1.67 g Nutriflora-t and 2.0 g calcium nitrate (Blok and Bollen, 1996a). After 14 weeks, plants were uprooted, the roots washed free of soil and evaluated for severity of Foa root rot. Loss of secondary roots (LSR) was rated on a 0-10 scale where 0= no loss of secondary roots and 10= total loss. The amount of lesions on primary (DPR) and secondary roots (DSR) were rated separately on a 0-5 scale where 0= no lesions, 1= 0-5% of the root length covered with lesions, 2= 6-20%, 3= 21-60%, 4= 61-95%, and 5= >95% or dead plants. Based on the ratings for DPR, DSR, and LSR, a disease index (DI) ranging from 0-10 was calculated (Blok and Bollen, 1996a). DI was calculated as: DI = (2*DPR + 2*DSR + LSR) / 3. Dry weight of roots was determined after drying for 24 h at 105°C.

Field experiment on control of Foa root rot by the nonpathogenic isolate Fo47

The potential of a nonpathogenic isolate of *F. oxysporum* to produce healthy crowns as

planting material in a field soil with low Foa infestation levels was assessed in a field experiment on reclaimed peatland (pH-KCl 5.1; organic-matter content 12.5%). Isolate Fo47 was chosen as this isolate is near commercialization and showed reasonable control of Foa root rot.

Inoculation procedures. The field was divided in three main plots measuring 3 x 50 m that were separated by grass strips of 3 m width. One main plot was left uninfested (Foa-control), the other two plots were infested by incorporating soil inoculum of Foa CWB 1 in April 1993. The soil inoculum was first thoroughly mixed with approx. 50 l of field soil, then spread by hand over the plot, and incorporated to a depth of 25 cm with a rotary spading cultivator. In the 0-25 cm layer of the two infested main plots, population densities applied were 2.5 (Foa-low) and 25 (Foa-high) cfu g^{-1} dry soil, respectively. These densities are very low but are in the same range of those in fields used for commercial production of asparagus planting material.

In early June 1994, 12 subplots (1.5 x 8 m) were laid out in each of the three main plots and three biocontrol treatments were applied in quadruplicate. The treatments included a control (no Fo47), Fo47-low (Fo47 supplied to the field soil only), and Fo47-high (Fo47 supplied to the field soil and to the potting soil in which the asparagus seedlings used as planting material were grown). Fo47 was supplied as a semi-commercial talcum inoculum, kindly provided by Dr C. Alabouvette (INRA, Dijon, France). Population density was 6.6 x 10^7 cfu g^{-1} talcum. The balls of potting soil in treatment Fo47-high were inoculated one day after seeding by immersing the trays in a suspension of talcum inoculum in tap water with 4.6 x 10^5 cfu ml^{-1}. The balls of the control and Fo47-low were immersed in a suspension of talcum powder without Fo47. Ten inoculated and ten noninoculated balls were collected and the population density of *F. oxysporum* was determined for each ball by plating soil dilutions on malt extract agar amended with oxytetracycline (50 μg ml^{-1}).

Each Fo47-low and Fo47-high subplot was inoculated by spreading a mixture of 94 g talcum inoculum in 5 l steamed coarse sand over the soil. Then, all subplots were rotary cultivated to a depth of 15 cm, and five days later, soil was mixed with a rotary spading cultivator to a depth of 25 cm. These operations were made in the order Foa-control, Foa-low, and Foa-high. Population density of Fo47 was 1500 cfu g^{-1} dry soil in the upper 25 cm. Ten days after supply of Fo47, seven-wk-old asparagus seedlings with rootball were planted. Per plot, seedlings were planted in three rows of 6 m with planting distance 30 cm between rows and 12 cm within rows.

Greenhouse bio-assay to assess soil infestation with Foa and effect of Fo47 on Foa infection of test plants. Immediately before supply of Fo47, soil samples were collected from each of the three main plots. For each plot, two composite samples were prepared by bulking 30 randomly collected samples, one composite sample from a depth of 0-30 cm, and one from

30-60 cm. Immediately after supply of Fo47, two composite samples were prepared from the layer 0-30 cm of each main plot by randomly collecting eight samples from each of the four replicate Fo47-low subplots and bulking these into composite samples per main plot. After thorough mixing, the soil of each sample was divided over five 1-l pots and planted with four pregerminated asparagus (cv. Gynlim) seeds. To check for cross-contamination during the bio-assay, two pots with autoclaved soil were included in each block. The pots were arranged in a randomized complete block design with five blocks. After ten weeks, plants were uprooted and evaluated for root rot. Because root rot severity was very low, an adapted rating scale was used. Root rot was rated separately for primary and secondary roots on a 0-3 scale with 0= no symptoms; 1= $\leq$3 lesions; 2= 3-10 lesions; 3= >10 lesions. Rating per pot was the mean of the ratings for the primary and secondary roots of the four plants per pot.

Rating of field-grown plants. In March 1995, the one-yr-old plants were harvested. From each subplot 10 plants were randomly collected from the middle 5 m of the middle row, the remaining plants were used in another experiment not described here. The collected plants were washed thoroughly with a high-pressure spray gun and rated for root rot severity on the storage roots on a 0-3 scale, where 0= no symptoms; 1= one or more brown lesions on < 10% of the storage roots; 2 = on 10-50% of the storage roots; 3 = on > 50% of the storage roots. A disease index (DI) per subplot was calculated as: $DI = (n_0 * 0 + n_1 * 1 + n_2 * 2 + n_3 * 3) / N$, with n_0, n_1, n_2, and n_3 being the number of plants in rating class 0, 1, 2, and 3, respectively, and N the total number of plants. From each main plot-subplot combination several root pieces with lesions were collected and plated on Komada's medium. Storage roots were externally disinfested in sodium hypochlorite (1% for 1 min), secondary roots were rinsed for 15 min in running tap water. Occasionally, colonies of *F. oxysporum* were tested for pathogenicity on asparagus *in vitro*.

Colonization of asparagus roots in sterilized field soil

The extent and rates of colonization of asparagus roots by *F. oxysporum* isolates was studied in an experiment with five nonpathogenic isolates (Fo47 and CWB 317 from soil and CWB 315, 316 and 319 from asparagus roots), and three pathogenic isolates (Foa 15, 24, and 89, all isolated from asparagus roots). Spore suspensions, obtained from colonies on PDA slants, were sieved through sterile glass wool to remove mycelial fragments, and adjusted to a density of 1×10^{-7} conidia ml^{-1}. More than 95% of the conidia were microconidia. Disinfested, pregerminated asparagus seeds with a radicle length of 2-3 mm, were coated by soaking them for 15 min in a spore suspension with 2% (w/v) of carboxy-methyl-cellulose as a sticker. The number of conidia per seed was determined for ten seeds by shaking the seeds in sterile distilled water and plating dilutions on Komada's medium. After drying on sterile filter paper, the seeds were planted at a depth of 2 cm in autoclaved field soil in pvc cylinders (height 20 cm, diameter 2.5 cm). The soil (loamy sand; pH-KCl 6.7; organic-matter

content 2.3%) was autoclaved twice on consecutive days and moistened to a water-potential value of pF 2.0 (-10 kPa). The cylinders were closed with a plastic cap to minimize evaporation. Ten cylinders per treatment were placed, completely randomized, in a climate chamber at 20°C. During the incubation period the plants were not watered.

After 18 days the first roots had reached the bottom of the cylinder and plants were harvested. The cylinders were placed horizontally and cut open, the main root was carefully removed, stripped from secondary roots, and cut in four segments of the same size. From each segment the middle 2 cm was used. For each treatment root segments of five plants were washed by vigourous shaking in sterile distilled water for 2 min, those of the remaining five plants were externally disinfested by shaking in 2% sodium hypochlorite for 2 min followed by three washings in sterile distilled water. The segments were blotted dry on sterile filter paper and cut in 20 1-mm fragments (scalpel was flamed between cuttings) that were placed on Komada's medium. After 10 days of incubation at 23°C the number of fragments colonized by *Fusarium* was determined for each segment.

Effect of nonpathogenic isolates of *F. oxysporum* on germination of Foa chlamydospores in rhizosphere soil

Field soil (loamy sand; pH-KCl 7.5; organic-matter content 3.0%) was inoculated with one of five nonpathogenic isolates of *F. oxysporum* i.e. Fo47, CWB 306, and 307 from soil and CWB 316 and 318 from asparagus roots. Inoculations were made by incorporating talc inoculum to a density of 5×10^7 cfu g^{-1} dry soil. Noninoculated soil served as a control. Subsequently, the soil was infested with stained soil inoculum of Foa CWB 1 to a density of 5×10^4 cfu g^{-1} dry soil. This inoculum was produced in the field soil mentioned above which was autoclaved twice on consecutive days and inoculated with talcum inoculum. After incubation for 12 days at 23°C, the colonized soil was dried for 30 days at room temperature. The dried inoculum contained 3.2×10^5 cfu g^{-1} dry soil. The inoculum was stained by mixing it with a solution of the fluorescent brightener Calcofluor white M2R (0.5 ml of a 0.3% solution per g of soil)(Couteaudier and Alabouvette, 1990). After 10 min the excess solution was removed.

The stained inoculum was mixed with the field soil with nonpathogenic isolates and the mixtures were moistened to pF 2.1 (- 12.6 kPa). The infested soil was spread as a 2-mm thick layer on the bottom of a Petri dish, three asparagus seedlings with roots of ca. 6 cm length were laid on it and overlaid with a 2-mm thick layer of the same infested soil. For each treatment, three dishes were prepared. The dishes were sealed with parafilm and incubated with the roots in their normal direction in an incubator at 25°C. After an incubation period of 24 h, the seed was removed and the root with adhering rhizosphere soil transferred to a tube with 2 ml sterile distilled water. The soil was separated from the root by vigorous vibration of the tube for 30 sec. One ml of the rhizosphere soil suspension was diluted in 9 ml sterile distilled water and filtered over a Millipore filter (SSWP, 0.3μm pore diameter)

that retained the spores. The filter was mounted on a microscope slide in immersion oil, covered with a cover slip and viewed under a Zeiss Axioskop equipped with an incident-light fluorescence illuminator, a 395-440 nm excitation filter, a 460-nm dichroic mirror and a 470-nm barrier filter. On each filter at least 100 propagules were examined. Propagules were considered germinated when the length of the germ tube equalled the diameter of the spore. On each of five consecutive days one dish with three seedlings was harvested for each treatment. For statistical analysis, each day was treated as a block.

Data analysis

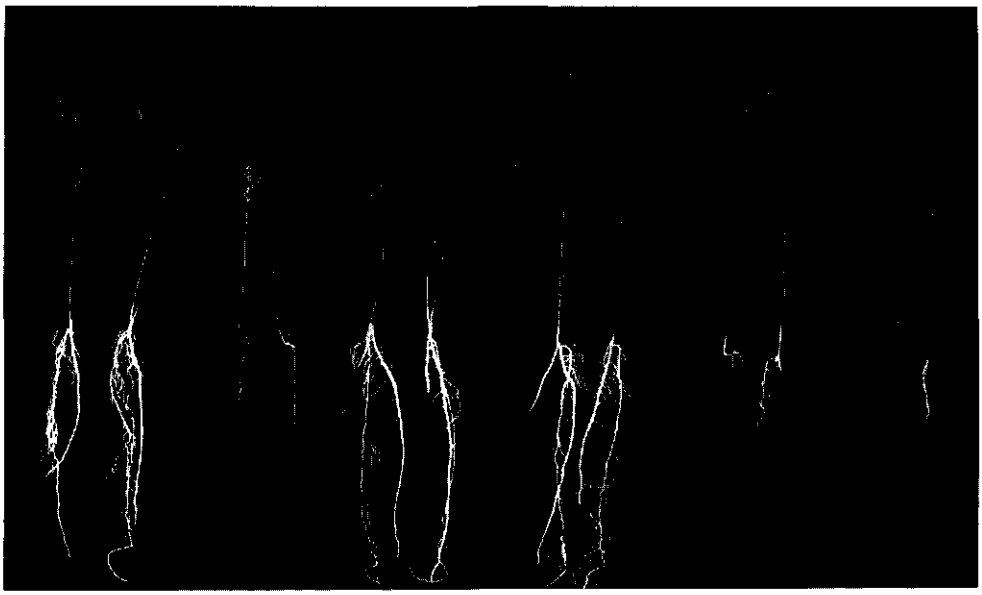
Data analysis was performed according to procedures described in Sokal and Rohlf (1981). The percentage of nonpathogenic *Fusarium* isolates recovered with two different isolation procedures was compared by a Chi-square test. Data for disease index and root weight were subjected to ANOVA after prior checks for normality and homogeneity of variances. When the treatment F-test was significant, means were separated by Tukey's multiple-comparisons procedure. The effect of Fo47 inoculation in the bio-assay with soil samples from the field experiment was evaluated by linear contrasts for each Foa-infestation level separately. Data for root colonization (proportions of colonized root fragments) and chlamydospore germination were transformed prior to analysis of variance and mean separation was made by Tukey's multiple-comparisons procedure. For the proportion of colonized root fragments and for the percentage germinated chlamydospores an angular and square-root transformation ($\sqrt{(x+0.5)}$) was applied respectively.

Results

Screening of isolates for control of Foa root rot in sterilized field soil

One hundred seventy-six isolates of *F. oxysporum* were obtained from symptomless asparagus roots and tested *in vitro* for pathogenicity on asparagus plantlets. Eighty-four and 92 isolates were recovered after washing in sterile distilled water only, and external disinfestation in sodium hypochlorite, respectively. Of both categories, 15 (17.9%) and 19 (20.7%) isolates were found to be nonpathogenic on asparagus, respectively. The percentage nonpathogenic isolates was not significantly different for the two isolation procedures according to the Chi-square test at $P \leq 0.05$.

The ability to reduce Foa root rot differed greatly between isolates (Fig. 1). Root rot severity, rated on a 0-5 scale, was 4.0 and 4.4 for series 1 and 2 in the treatment with Foa only and 1.7 and 2.0 in the treatment with Foa and Fo47. To allow for comparison of the results of both series, the effect of a treatment was calculated as the percentage reduction in disease index of the pathogen control. Nonpathogenic isolates of *F. oxysporum* and *F. proliferatum* reduced severity of root rot (Table 1). Isolates originating from soil suppressed



Bl Foa Fo 47 Fo 59 Fo 12 Fo 131

Figure 1. Root systems of test plants grown in sterilized field soil co-inoculated with Foa and nonpathogenic isolates of *F. oxysporum*.
Legend: Bl = blank, not inoculated; Foa = inoculated with pathogen only; Fo47, Fo59, Fo12, Fo131 = co-inoculated with the pathogen and nonpathogenic isolates of *F. oxysporum*.

the disease more than those from roots. Root dry weight was strongly correlated with root rot severity. The linear regression line describing the relation between the reduction in disease index (Y) and the reduction in root dry weight compared to the noninfested control (X) was calculated as: $Y = 80.55 - 1.02\ X$ with R^2(adjusted)=84.9%, $P<0.001$, and n=65.

The population density of the isolates of *Fusarium oxysporum* ranged from 0.6 to $9.6 * 10^6$ cfu g^{-1} dry soil. Population density of these isolates was not correlated ($P \leq 0.05$) with reduction of disease index.

Screening of isolates for control of Foa root rot in naturally infested field soil

Plants grown in untreated asparagus soil showed the symptoms characteristic of Foa root rot: a sparse root system and brown lesions on primary and secondary roots. Plants grown in fresh soil only occasionally had a few brown lesions. The antagonists significantly reduced

Table 1. Effect of inoculation of sterilized soil with nonpathogenic isolates of *Fusarium oxysporum*, *F. proliferatum*, *Talaromyces flavus*, and *Streptomyces* sp. on severity of Foa root rot.

		Number of isolates per disease reduction class				
strains	Code or origin	Average percentage reduction in disease index				Species[b]
	< 10%	10-30%	31-50%	51-70%	> 71%	
Streptomyces sp.	Mycostop			1		
T. flavus	M8		1			
F. proliferatum	Fmon			1		
F. proliferatum	Fmon B1			1		
F. oxysporum	Fo47				1 [1]	
F. oxysporum	Fo47 B10			1		
F. oxysporum	618-12				1	
F. oxysporum	618-12 B17			1		
F. oxysporum	Soil		1	1	6	4
F. oxysporum	Washed roots	16				
F. oxysporum	Disinfested roots	21		1	1	
F. oxysporum + *Streptomyces* sp.	Fo47 + Mycostop				1	

[1] Tested in two series with similar results.

root rot severity, with isolate CWB 318 decreasing the value of the disease index by almost 50% (Table 2). Inoculation of the asparagus soil with antagonists resulted in an increase of root dry weight of 9-24% compared to the untreated asparagus soil (Table 2). The prochloraz treatment was very effective against Foa as the disease index was even lower and the root dry weight higher than that for the fresh soil. The linear regression line for the relation between root dry weight (X) and disease index (Y) in the treatments with asparagus soil was calculated as: $Y = 2.37 - 0.29\ X$ with R^2(adjusted)=49.5%, $P<0.001$, and n=56.

Field experiment on control of Foa root rot by the nonpathogenic isolate Fo47

The root balls of planting material grown in potting mixture supplied with Fo47 contained $2.83 \pm 0.68 * 10^5$ cfu g^{-1} dry soil. In the balls of plants grown in non-supplied potting mixture the density of *Fusarium* propagules was below the detection limit of 15 cfu g^{-1} dry soil.

A greenhouse bio-assay was performed with soil samples from the field plots to assess soil infestation with Foa and effect of Fo47 on Foa infection of test plants (Fig. 2). Infestation level in samples from the noninfested main plot was low. The mean disease index of the samples from the plots with the low or the high level of artificial Foa infestation was significantly higher than that of the samples of the noninfested plot. The disease index of the

Table 2. Severity of Foa root rot and root dry weight of plants grown in fresh soil or naturally infested asparagus soil supplied with nonpathogenic isolates of *F. oxysporum* or treated with prochloraz.

	Disease index (0-10)		Root dry weight (g/pot)	
Treatment	Mean	% Reduction[1]	Mean	% Increase[2]
Fresh soil, untreated	1.57 de[3]	-	2.34 a[3]	-
Asparagus soil, untreated	4.81 a	-	1.17 b	-
Asparagus soil, Fo47	3.21 bc	33 (56)	1.45 b	24
Asparagus soil, 618-12	2.67 c	44 (55)	1.38 b	18
Asparagus soil, CWB 312	3.31 bc	31 (64)	1.29 b	10
Asparagus soil, CWB 314	2.90 bc	40 (66)	1.41 b	21
Asparagus soil, CWB 318	2.43 cd	49 (58)	1.40 b	20
Asparagus soil, CWB 319	3.69 b	23 (48)	1.27 b	9
Asparagus soil, prochloraz	0.69 e	86	2.63 a	125

[1] Percentage reduction of disease index compared to that of untreated asparagus soil. In brackets, the percentage reduction in the primary screening in sterilized soil.
[2] Percentage increase of root dry weight compared to that of untreated asparagus soil.
[3] Means followed by a common letter are not significantly different according to the multiple-comparisons procedure of Tukey ($P \leq 0.05$).

low and the high level of Foa infestation was not significantly different. Fo47 had not reduced root rot severity at any of the three Foa-infestation levels. The plants grown in the autoclaved soil stayed free of root lesions indicating that cross-contamination had not occurred in the bio-assay.

One year after inoculation of the main plots with Foa, root rot of the crop was most severe in plots that had been inoculated at the high level (Table 3). On the control plot, that was not artificially infested but shown in the bio-assay to be naturally infested at the time the seedlings were planted, plants with Foa symptoms were found on two of the 12 subplots only with one and six symptomatic plants, respectively. The two Foa-infested main plots showed a regular distribution of plants with Foa symptoms. The Foa-high plot yielded plants with a level of Foa root rot similar to that regularly found in commercial plant lots. On all plots, plants were found that had brown root lesions, similar to the ones caused by Foa, on the secondary roots originating from the oldest, thin storage roots. Foa was not (in the uninfested plot) or only occasionally (in both infested plots) detected in these lesions; lesions on storage roots yielded Foa consistently. Therefore, only lesions on storage roots were considered when plants were rated for Foa root rot. On the Foa-low plot, the three Fo47 treatments had the same disease-index value. On the Foa-high plot, the Fo47 inoculation resulted in a slightly lower value for the disease index but differences were not significant. As root rot severity was low, fresh root weight did not differ between treatments (Table 3).

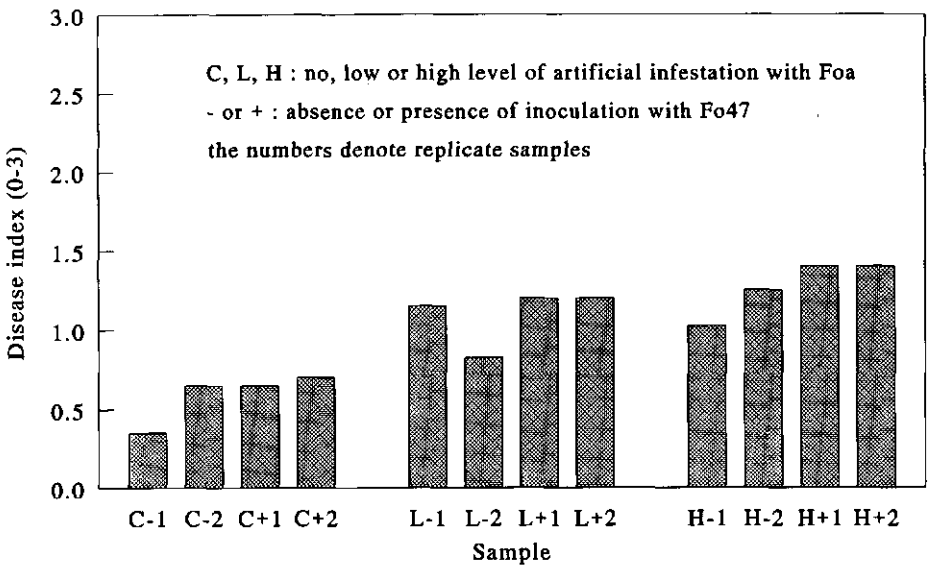


Figure 2. Severity of Foa root rot in a bio-assay with asparagus plants on soil samples from field plots infested or noninfested with Foa and later inoculated or noninoculated with Fo47.
Disease-index values of the samples from the main plot without Foa infestation (C) were significantly lower than those of the samples from either the main plot with the low (L) or the plot with the high (H) level of Foa infestation. The samples from the latter two main plots were not significantly different. The disease-index values of the samples from the subplots with or without Fo47 were not significantly different for either of the three main plots (linear contrasts; $P \leq 0.05$).

Table 3. Disease index (DI) for Foa root rot and fresh weight (FW) of one-yr-old asparagus plants grown in a field with three levels of Foa infestation and, within each Foa-level, three levels of Fo47 inoculation.

Foa-control			Foa-low			Foa-high		
Inoculum of Fo47	DI (0-3)	FW (g/plant)	Inoculum of Fo47	DI (0-3)	FW (g/plant)	Inoculum of Fo47	DI (0-3)	FW (g/plant)
None	0.0	89.6	none	0.2	74.2	none	1.4	81.4
Low	0.0	80.9	low	0.2	90.6	low	1.0	78.2
High	0.2	81.6	high	0.2	78.3	high	1.0	76.4
mean	0.1	84.0	mean	0.2	81.1	mean	1.1	78.7

Table 4. Colonization of asparagus roots by five nonpathogenic isolates of *F. oxysporum* and three pathogenic isolates (Foa) in sterilized field soil.

	Segment 1 (seed)		Segment 2		Segment 3		Segment 4 (tip)	
Isolate	Incidence[1]	CR[2]	Incidence	CR	Incidence	CR	Incidence	CR
Non-disinfested roots:								
Fo47	5/5	0.83 ab[3]	5/5	0.47 a	2/5	0.11	2/5	0.07
CWB 315	5/5	0.27 c	5/5	0.13 a	3/5	0.08	0/5	0.00
CWB 316	4/4	0.25 c	4/4	0.13 a	3/4	0.05	1/4	0.01
CWB 317	3/3	0.36 bc	2/3	0.31 a	2/3	0.17	1/3	0.02
CWB 319	3/3	0.18 c	3/3	0.14 a	0/3	0.00	2/3	0.12
Foa 15	4/4	0.83 ab	3/4	0.43 a	4/4	0.29	3/4	0.06
Foa 24	5/5	0.94 a	5/5	0.70 a	3/5	0.28	3/5	0.07
Foa 89	5/5	0.82 ab	3/5	0.44 a	3/5	0.26	2/5	0.04
Disinfested roots:								
Fo47	4/5	0.15 c	2/5	0.03 b	2/5	0.08	1/5	0.01
CWB 315	4/4	0.16 c	3/4	0.15 b	1/4	0.01	0/4	0.00
CWB 316	0/4	0.00	1/4	0.01 b	0/4	0.00	1/4	0.01
CWB 317	1/5	0.05 c	1/5	0.01 b	0/5	0.00	0/5	0.00
CWB 319	4/4	0.32 bc	4/4	0.15 b	1/4	0.01	0/4	0.00
Foa 15	-		-		-		-	
Foa 24	3/3	0.79 ab	2/3	0.42 ab	0/3	0.00	0/3	0.00
Foa 89	5/5	0.89 a	5/5	0.71 a	4/5	0.27	0/5	0.00

[1] Incidence: number of segments with outgrowth from at least one of the fragments / number of segments plated. Segments of five plants were plated except for treatments with plants with root lengths < 8 cm that were excluded and Foa treatments where some plants died.

[2] CR = colonization rate: fraction of 1-mm fragments colonized by *Fusarium*.

[3] Means within a column followed by a common letter are not significantly different according to Tukey's multiple-comparisons procedure at $P \leq 0.05$. Analysis of variance was performed on angular-transformed data. As the outcome was the same for transformed and nontransformed data, means of the original data are given to allow comparison with data of segments 3 and 4.

Colonization of asparagus roots in sterilized field soil

On inoculated seed 2.32 $\pm$ 0.70 * 10^5 cfu per seed were recovered on Komada's medium. All eight isolates tested did colonize the root surface over a considerable length (Table 4, non-disinfested roots). Most isolates were detected as far as the lowest segment near the root tip, although colonization rate was low at this site and *Fusarium* was not recovered from all roots. Statistical analyses of the data of the upper two segments showed that the isolates differed in their colonization of the first segment only. The three Foa isolates together with Fo47, showed a significantly higher colonization rate than the nonpathogenic isolates CWB 315, 316, and 319; isolate CWB 319 was intermediate. The data for the second segment showed the same pattern but differences were not significant. As shown by the data for disinfested roots, root tissue was less colonized than root surface (Table 4). However, all isolates were recovered from externally disinfested roots suggesting that infection of asparagus roots by isolates of *F. oxysporum* is common. The Foa isolates had considerably higher colonization rates than the nonpathogenic isolates. Isolate CWB 316 was only occasionally recovered.

Effect of nonpathogenic isolates of *F. oxysporum* on germination of Foa chlamydospores in rhizosphere soil

In a preliminary experiment at 25°C, germination percentage of Foa chlamydospores was found to reach a peak after 24 h and therefore, spores were incubated for 24 h. The germination percentage of Foa chlamydospores was strongly and significantly reduced in soil inoculated with all nonpathogenic isolates of *F. oxysporum* with differences between the latter only small and not significant (Table 5). In a separate experiment it was shown that talcum powder as the formulation product (incorporated at the maximum amount used for inoculation of the nonpathogenic isolates, i.e. 11.8 g talcum powder in 90 g soil) had no effect on the germination of chlamydospores.

Discussion

Three mechanisms have been proposed as involved in the suppression of pathogenic *Fusarium* spp. by nonpathogenic *F. oxysporum* strains: competition for nutrients in the rhizosphere, competition for infection sites on the root surface, and induced resistance (Mandeel and Baker, 1991). Preferably, a primary screening procedure should be followed in which all these mechanisms can be expressed to avoid that effective antagonists are missed. The screening procedure described by Tamietti and Pramotton (1987) meets this requirement and was, therefore, adopted in this study. It proved an easy and effective way to identify nonpathogenic isolates of *F. oxysporum* that significantly reduced severity of Foa root rot.

Table 5. Effect of five nonpathogenic isolates of *F. oxysporum* on germination of chlamydospores of Foa in rhizosphere soil.

Treatment	Germination of Foa chlamydospores (%)	Reduction compared to control (%)
Foa (control)	19.6 a[1]	
Foa + Fo47	10.2 b	48
Foa + CWB 306	7.0 b	64
Foa + CWB 307	11.0 b	43
Foa + CWB 316	9.5 b	52
Foa + CWB 318	8.4 b	57

[1] Data were transformed ($\sqrt{(x+0.5)}$) before analysis of variance, means in the table are the back-transformed values. Means followed by a common letter are not significantly different according to Tukey's multiple-comparisons procedure at $P \leq 0.05$.

All six reference strains (Fo47, 618-12, Fmon and their benomyl-resistant mutants) gave a significant reduction of root rot severity in sterilized soil indicating that strains selected for control of vascular forms of *F. oxysporum* can also be active against non-vascular forms. Obviously, it is not a prerequisite for biocontrol that an antagonist is associated with a specific host. This is further substantiated by the fact that both categories of root-derived isolates did hardly yield any effective isolates whereas the category of soil-derived isolates yielded several highly effective antagonists.

Three weeks after introduction into sterilized field soil, all *Fusarium* isolates had colonized the soil but large differences in population density were found. This is in agreement with the results of Nagao *et al.* (1990) and Amir and Mahdi (1993) who found that isolates of *F. oxysporum* can differ significantly in soil colonization capacity. Population density and reduction of root rot severity was not significantly correlated, perhaps because overall population density of the nonpathogenic isolates was high.

The six isolates that greatly reduced root rot severity in the sterilized soil and were further tested in naturally infested field soil all significantly reduced root rot severity in the latter soil. The reduced disease severity was significantly correlated with increased root weight although differences with the control were not significant for any of the isolates. These results are in line with those of Damicone and Manning (1982) who obtained a significant reduction of root and crown rot of asparagus in naturally infested soil by application of nonpathogenic isolates of *F. oxysporum*. However, it is not quite clear whether Foa or *F. moniliforme* was involved.

In the field experiment, Fo47 only slightly reduced root rot at the highest Foa level. Failure of Fo47 to control Foa in the field experiment might be explained by variable environmental

conditions that occur in the field and the long period during which the antagonist had to provide protection. Another factor might be that talcum inoculum is not a suitable formulation for field application. The dry talcum powder may form aggregates in moist soil resulting in an uneven distribution of the antagonist. This problem is not apparent when the talcum inoculum is applied in soil-less systems as practised by Alabouvette and co-workers (Alabouvette *et al.*, 1993). Moreover, the talcum powder does not provide the antagonist with a food source as starting energy that can be used to colonize the soil.

Colonization of asparagus roots by eight different *F. oxysporum* strains was assessed in sterilized soil. From the results of Garibaldi *et al.* (1990) it might be inferred that root colonization in sterilized soil is correlated to that in untreated field soil. They did not find difference in root colonization of radish and melon by *Fusarium* isolates between steamed and unsteamed soil. Furthermore, in this study, nonpathogenic isolates of *F. oxysporum* were isolated frequently from disinfested asparagus roots indicating that infection of living root tissue is not confined to plants growing in sterilized soil.

The significance of root colonizing capacity as a selection criterion for isolates for biocontrol depends on the major mechanism involved. If emphasis is on induced resistance, infection of the root is a crucial factor. In this study nonpathogenic isolates could still be recovered after disinfestation of the roots suggesting that they infected living roots. The level to which they did so might be a criterion for selection. If emphasis is on competition for nutrients in the rhizosphere or occupation of infection sites, colonization rate and density is the main factor in selection of isolates. Nonpathogenic isolates differed in colonization capacity, Fo47 colonizing the roots to the same extent as the Foa isolates and to a greater extent than the other isolates.

Nonpathogenic isolates of *F. oxysporum* reduced germination percentage of Foa chlamydospores in the rhizosphere by 43 to 57% indicating an effective competition for nutrients with the pathogen. The isolates originating from roots (CWB 316 and 318) did not differ significantly from the isolates originating from soil. Although only a small number of nonpathogenic isolates was tested, the fact that all isolates reduced germination to the same extent might indicate that competition for nutrients is a common mechanism of antagonism for nonpathogenic *Fusarium* isolates. Inhibition of chlamydospore germination by nonpathogenic *Fusarium* isolates was found for several other pathogens, e.g. *F. oxysporum* f.sp. *cucumerinum* (Mandeel and Baker, 1991), and f.sp. *lini* (Couteaudier, 1991).

In conclusion, nonpathogenic isolates of *F. oxysporum* were clearly shown to have potential to reduce severity of asparagus root rot caused by *F. oxysporum* f.sp. *asparagi* in naturally infested field soil. Although isolates with reasonable root colonization rates were selected, selection for isolates with even higher colonization rates would be advantageous. To obtain prolonged biological control in the field a delivery system has to be developed that enables the introduced antagonists to establish and maintain sufficiently high populations. The delivery system has to include an effective inoculum formulation and should also be combined with

cultural methods such as various means of partial soil sterilization (DeVay and Katan, 1991; Blok *et al.*, 1995) by which the soil ecosystem is disturbed thus opening up some 'window of opportunity' (Deacon, 1991) for the antagonist in the soil.

Chapter 9

Control of soilborne pathogens by inducing fermentative soil conditions

Abstract

A new method for reducing soil infestation with soilborne pathogens by induction of fermentative soil conditions was tested for its efficacy. Loamy sand infested with chlamydospores of *Fusarium oxysporum* f.sp. *asparagi* was amended with 5 different plant materials or left unamended, and incubated at 11 or 24°C in sealed glass bottles. Oxygen consumption rates were higher at 24 than at 11°C and differed between treatments. After 7 weeks, the fungus was not detected any more from alfalfa- and cabbage-amended soil irrespective of the incubation temperature. In 1994 and 1995, the method was tested in the field. In plots amended with fresh broccoli or grass and covered with plastic mulch (Hytileen, a 3-layered polyethylene sheeting of 0.135 mm thickness), fermentative soil conditions developed rapidly as was indicated by rapid depletion of oxygen and a decrease of the redox potential to values as low as -200 mV. After 15 weeks, survival of *F. oxysporum* f.sp.*asparagi*, *Rhizoctonia solani*, and *Verticillium dahliae* in inoculum samples buried at 15 cm depth was strongly reduced in amended mulched plots. Inactivation of *Globodera pallida* was not detected when the percentage living eggs and larvae was estimated under a dissecting microscope, but strong inactivation was apparent from hatching tests. The results clearly show the potential of this nonchemical approach to control soilborne pathogens.

Introduction

Soilborne pathogens are a potential threat for many agricultural and horticultural crops. Examples of notorious pathogens are *Rhizoctonia solani*, *Verticillium dahliae* and special forms of *Fusarium oxysporum*. Crucial factors to managing diseases caused by these pathogens are prevention of the build-up of inoculum densities to damaging levels or their reduction to nondamaging levels before a susceptible crop is sown or planted. Crop rotation with nonsusceptible crops has been used for these purposes successfully for centuries (Curl, 1963). In the last decades however, rotations have been narrowed because of mechanization and the use of cheap and easy-to-use chemical fertilizers, pesticides and soil fumigants. However, the growing awareness that pesticides and soil fumigants have effects that are incompatible with sustainable agriculture has led to severe restrictions in their use in recent years. As a consequence, the interest in old and new alternatives for chemical soil disinfestation has renewed.

Incorporation in soil of specific crop residues or other organic amendments can cause reductions in inoculum densities of soilborne pathogens and a concomitant decrease of disease severity (Baker and Cook, 1974; Cook and Baker, 1983; Huber and Watson, 1970; Zakaria

and Lockwood, 1980). In particular, cruciferous residues have been shown to possess the potential to suppress a number of soilborne diseases (Lewis and Papavizas, 1971; Muelchen *et al.*, 1990; Subbarao and Hubbard, 1996). The results are often specific for one or a few pathogens. Whereas the effects of amendment with crop residues can be spectacular, they are often inconsistent. This is particularly the case when the mechanism of disease suppression operates in an indirect way (Baker and Cook, 1974; Cook and Watson, 1969). The mechanisms involved include stimulation of microbial antagonism (Baker and Cook, 1974) and production of toxic volatiles during decomposition of the organic material in soil (Fries, 1973; Gilbert and Griebel, 1969; Lewis and Papavizas, 1974).

Soil solarization is a method of disease control by which soil is heated by solar energy. The soil is covered with transparent plastic mulch, leading under suitable climatic conditions to soil temperatures that are lethal to many plant pathogens. Besides the accumulation of heat, a number of other changes occur that contribute to the inactivation of fungi, bacteria, nematodes, weeds and insects (DeVay and Katan, 1991). The effect of solarization can be improved by incorporating crop residues or amendments before tarping the soil (Gamliel and Stapleton, 1993a; 1993b; Ramirez-Villapudua and Munnecke, 1987). Obviously, the application of solarization is confined to areas with an intense solar radiation.

Soil flooding is a third alternative to chemical disinfestation. Early reports describe the use of flooding to control *Sclerotinia sclerotiorum* in celery (Brooks, 1942), *Phytophthora parasitica* var. *nicotiana* in tobacco (Van Schreven, 1948), and *F. oxysporum* f.sp. *cubense* in banana plantations in Honduras (Stover *et al.*, 1953). Recent applications were reported for organic soils in Florida to control several soilborne diseases in vegetable production (Strandberg, 1987) and for flower bulb fields in the Netherlands to control *Rhizoctonia tuliparum* (Muller *et al.*, 1988). When a soil is flooded, oxygen is rapidly consumed by aerobic organisms resulting in anaerobic conditions leading to a shift from aerobic respiration (using molecular oxygen as electron acceptor) to fermentation (using organic compounds as electron acceptors) and anaerobic respiration (using inorganic compounds as electron acceptors)(Rowell, 1981). Anaerobic decomposition of organic matter can lead to the accumulation of compounds such as organic acids, alcohols, aldehydes, ammonium, mercaptans, sulfides, carbon dioxide, hydrogen and methane (Ponnamperuma, 1972; Yoshida, 1975). It is not exactly known how pathogens are inactivated during flooding. Possible factors are lack of oxygen, high levels of carbon dioxide, fungitoxic products of anaerobic decomposition of organic matter, and biological control by microorganisms that flourish during anaerobic conditions (Strandberg, 1987). Conditions that determine the applicability of soil flooding are the availability of an easily-manageable source of water and a suitable hydraulic conductivity of the soil.

In this study a new approach was developed that combines aspects of the three control methods discussed above. It aims at the control of a wide range of persistent soilborne pathogens in various soil types and under conditions of the temperate zone. The idea is to

induce fermentative soil conditions by incorporating relatively large amounts of green plant material into moist soil and by covering the soil with plastic mulch with a low permeability for oxygen. In fact, this approach is the elaboration of an idea launched by Menzies in 1968 (Menzies, 1970). The results of a pilot laboratory experiment and two field experiments are presented showing the prospects of this approach for controlling soilborne plant pathogens.

Materials and methods

Preparation of inocula and testing of pathogen survival

Fusarium oxysporum (Schlecht.) emend. Snyder & Hansen f.sp. *asparagi* (Cohen & Heald) strain CWB1 was originally isolated from asparagus roots in the Netherlands. For preparation of inoculum, the strain was grown in malt extract broth for 7 days at 25°C. After comminuting the cultures in a blender, the slurry was centrifugated at 3400 *g* for 30 min and the precipitate resuspended in sterile distilled water and added to talcum powder (2:1, w/w). This mixture was dried with forced air for 14 days after which the talcum powder was passed through a sieve with 0.36 mm-openings. Samples of *F. oxysporum* f.sp. *asparagi* consisted of approx. 15 g talcum inoculum in nylon bags. Survival was tested by plating 10-fold dilutions of the talcum inoculum in 0.1% water agar on Komada's medium (Komada, 1975) with 5 replicate 0.25-ml aliquots per dilution.

Rhizoctonia solani Kühn AG-3 strain 3R41 was originally isolated from a potato plant in the Netherlands. Inoculum was produced by growing the strain for 4 weeks at 20°C on sterilized perlite (2-5 mm diameter) soaked in a solution containing 1.5% malt extract and 0.1% pepton. Samples consisted of narrow nylon bags with a minimum of 100 perlite kernels colonized by *R. solani*. Survival was tested by placing 90 perlite kernels of each sample on 1.2% water agar amended with 50 μg ml^{-1} oxytetracyclin (16 kernels per 9-cm Petri dish). After 1 or 2 d at 20°C outgrowth of *R. solani* was rated on a 0-3 scale with 0 = no outgrowth; 1 = 1-7 hyphae per perlite kernel; 2 = 8-35 hyphae; 3 = >35 hyphae. A germination index (GI) was calculated as: GI = 100 x ((0 x # of kernels with rating 0) + (1 x # rating 1) + (2 x # rating 2) + (3 x # rating 3)) / (3 x total # of kernels).

Verticillium dahliae Kleb. inoculum for the 1994 field experiment was prepared by milling air-dried dead potato stems that were densely covered with microsclerotia of *V. dahliae*, to a powder. This powder was put at one end of a small pvc tube (diameter 1 cm, length 4 cm) filled with silver sand and at both ends closed with nylon gauze with 10-μm openings. In 1995, two different types of inoculum were used. The first type was similar to the one used in 1994 except that the stem tissue was mixed with soil from the experimental field and put in nylon bags (100 ml/bag). For the second inoculum type, soil was collected from an experimental field heavily infested with *V. dahliae* (clay, pH-KCl 7.2, organic-matter content 2.9%), air-dried, ground and mixed thoroughly and put in nylon bags (100 ml/bag). At the

end of the exposure period the samples were dug out, air-dried (2 weeks at room temperature), and ground to pass a 2-mm sieve. Survival was tested following the slightly modified protocol of Harris *et al.* (1983). Whole samples (1994) or 12.5 g of air-dry soil (1995) were wet-sieved over sieves with openings of 106 and 20 μm, respectively. The residue on the 20-μm sieve was collected with 25 ml (1994) or 50 ml (1995) 0.08% wateragar and 10 replicate 0.8 ml aliquots were plated on modified soil extract agar (MSEA; 15). The plates were left open for 15 min in a laminar airflow cabinet to dry. After 4 weeks in the dark at 22°C, the surfaces of the plates were washed under running tap water and the plates were assessed for the number of colonies of *V. dahliae* formed in the agar.

Globodera pallida Stone cysts were obtained from potato plants that had been inoculated with larvae of *G. pallida* (strain PAGV pa2). In 1994, samples consisted of 45 cysts placed at one end of a small pvc tube (diameter 1 cm, length 4 cm) filled with silver sand and at both ends closed with nylon gauze with 10-μm openings. In 1995, samples consisted of 100 cysts in small, narrow bags of nylon gauze with 10-μm openings. Survival was determined by extracting the cysts from the silver sand with acetone. After counting the number of cysts, these were crushed in tap water and the fraction of living eggs and juveniles was determined under a dissecting microscope. For the duplicate samples of four plots of the 1995-experiment (two nonmulched grass plots, and two mulched grass plots), the cysts were crushed and the egg suspensions were incubated in hatching fluid, consisting of potato root exudates, in the dark at 20°C. After 10 days the numbers of living and dead juveniles were determined.

Soil physical and chemical parameters

Oxygen concentration of the soil atmosphere was determined by extracting soil atmosphere from a 10-ml gas diffusion chamber with a syringe. After discarding the first 5 ml, the following 10 ml was injected into an oxygen analyzer (570A; Servomex, Crowborough, East Sussex, United Kingdom). The gas diffusion chamber consisted of a piece of pvc tubing (diameter 1.6 cm, length 5 cm) that was open at the bottom and closed with a butylrubber stopper at the top. The rubber stopper was pierced by copper tubing (internal diameter 1.0 mm) that stood out about 5 cm above the soil surface. The copper tube was fitted at the top with a small piece of polyethylene tubing in which a metal pin was pushed for airtight closure.

The redox potential of the soil (which gives a measure of the oxidation-reduction processes that are taking place in the soil) was measured with platinum (Pt) electrodes (TFDL, Wageningen, The Netherlands) and a glass calomel reference electrode (Schott Geräte, Hofheim, Germany). The Pt electrodes were inserted into soil to the desired depths at the start of the experiment and stayed in the soil during the whole experimental period. The reference electrode was inserted into moist soil prior to a measurement. Redox potentials were expressed as E_h-values by adding 247 mV to the measured potentials (Zausig, 1995).

Soil and air temperature data were collected with thermocouples connected to a datalogger

(SQ 32-16u, Grant, Cambridge, United Kingdom).

The soil physical and chemical parameters were determined at a depth of 15 cm and at least 1.5 m from the border of the plot to avoid border effects. The air temperature was recorded at 10 and 150 cm height. Temperature was recorded every hour, redox potential and oxygen concentration were determined 2 or 3 times a week.

Laboratory experiment

Loamy sand (pH-KCl 7.5; 3.3% organic matter) was mixed with talcum powder containing spores of *Fusarium oxysporum* f.sp. *asparagi* strain CWB1 to reach a density of 2.0×10^4 colony-forming units (cfu) g^{-1} dry soil. Soil samples plated onto Komada's medium immediately after infestation, yielded 1.5×10^4 cfu g^{-1} dry soil (recovery 75%). Water was added to a moisture content of 18% (wt/fresh wt) and the soil was amended at a rate of 0.5% (dw/dw) with one of five different plant materials and mixed thoroughly. The plant materials used were: grass (perennial rye-grass (*Lolium perenne* L.), roots and shoots of field-grown plants); cabbage (Brussels sprouts (*Brassica oleracea* L. convar. *oleracea* var. *gemmifera* DC.), roots and shoots of field-grown plants); compost (a mature compost of vegetable, fruit, and garden waste); wheat (*Triticum aestivum* L., air-dried and milled straw) and alfalfa (*Medicago sativa* L., air-dried and milled). The grass and cabbage was cut into small pieces (approx. 0.5 cm^2). Nonamended soil served as a control. For each treatment, twenty 100-ml glass bottles each received 70 g (dry weight equivalent) of the soil mixture. The bottles were sealed airtight with a butylrubber stopper with crimp cap and 10 bottles were incubated at 11°C and 10 bottles at 24°C.

After 1, 2, 4, 7, 11, 22, and 50 days a 200-μl sample was drawn from the head space of each bottle with a syringe and the oxygen concentration determined on a gas chromatograph. The gas chromatograph used was a Chrompack CP9001 (Chrompack, Raritan, NJ, USA) fitted with a thermal conductivity detector and a packed column MolSieve 13X. The carrier gas was argon and the oven temperature was 100°C.

After 52 days, 5 of the 10 bottles for each treatment-temperature combination were randomly chosen and survival of inoculum was assessed. Soil (10.0 g ovendry-weight equivalent) was suspended in Erlenmeyer flasks containing 90 ml 0.1% wateragar. After shaking the flasks for 30 min on an orbital shaker, the soil suspension was diluted in a 10-fold series by adding 1 ml suspension to 9 ml 0.1% wateragar in culture tubes. Aliquots of 0.5 ml were plated onto five replicate Petri dishes with Komada's medium. After incubation at 20°C for 7 to 10 days, the number of colonies of *F. oxysporum* f.sp. *asparagi* was counted. Colonies were identified as colonies of *F. oxysporum* f.sp. *asparagi* by comparing their morphology with that of colonies developing from talcum inoculum plated onto Komada's medium. Testing the pathogenicity of a number of randomly collected *F. oxysporum* colonies on asparagus seedlings grown under axenic conditions (Blok and Bollen, 1996a) revealed that this identification procedure gave correct results.

Field experiment 1994

In summer 1994 a field experiment was performed on a loamy sand (pH-KCl 6.1; 3.1% organic matter) at the experimental farm 'Meterikse Veld' at Horst, province of Limburg, the Netherlands. On 18 June, fertilizer was applied to amounts of 135 kg N, 240 kg K_2O, and 160 kg MgO ha^{-1}. The experiment had a randomized complete block design with the combinations of 2 mulching treatments and 3 plant material treatments in 3 blocks. The plots measured 4 m x 4.5 m of which the inner 1.5 m x 1.5 m was used to bury pathogen samples and determining soil physical and chemical characteristics, to avoid border effects.

The mulching treatments were: i) nonmulched, and ii) application of a plastic mulch. The mulch used was Hytileen (Klerks Plastic Industrie, Noordwijkerhout, the Netherlands), a 0.135-mm thick plastic film consisting of three layers of polyethylene strengthened with EVA (ethylene vinyl acetate) with a black and a white side, that is generally used for silaging because of its relatively low permeability for oxygen. The plastic was used with the black side up.

The plant material treatments were: i) nonamended (control), ii) broccoli (*Brassica oleracea* L. convar. *botrytis* (L.) Alef. var. *cymosa* Duch.), and iii) perennial rye-grass (*Lolium perenne* L.). For the broccoli treatment, 4-week-old plants (cv. Marathon) were planted on the experimental plots on 10 June and grown for 5 weeks. At the time of incorporation the biomass was estimated, by weighing randomly collected samples, at 3.8 kg fresh weight m^{-2} (0.5 kg ovendry weight m^{-2}). For the grass treatment, freshly mown grass was supplied from a production pasture at an amount of 4.0 kg fresh weight m^{-2} (0.8 kg ovendry weight m^{-2}).

On 11 July, the plant material was applied and all plots were rotary tilled to a depth of 20-25 cm. On 12 July, duplicate samples of *F. oxysporum* f.sp. *asparagi* and single samples of *R. solani*, *V. dahliae*, and *G. pallida* were buried in all plots at a depth of 15 cm. The field was sprinkler irrigated overnight (approx. 50 mm) and plots were mulched on 13 July (day 0). The edges of the plastic sheets were buried about 10 cm into the soil.

To test whether measurements of soil physical and chemical parameters would affect pathogen survival, 4 additional plots, each with a different treatment combination, were laid out in which all soil physical and chemical parameters were measured ('measurement plots'). The 4 plots were: a nonamended nonmulched plot, a nonamended mulched plot, a broccoli-amended nonmulched plot, and a broccoli-amended mulched plot. In each of these plots, 3 Pt electrodes, 3 gas diffusion chambers, and 2 thermocouples were installed at 15 cm depth. The holes in the plastic mulch around the Pt electrodes and the copper tubes of the gas diffusion chambers were closed with silicone sealant to minimise oxygen diffusion. In these measurement plots, samples with the same pathogens as in the other plots were buried at 15 cm depth.

During the experiment, the nonmulched plots were sprayed once with a herbicide (a mixture of simazine and diuron, both at 0.5 l ha^{-1}) to prevent weed growth, and were sprinkler irrigated when needed to prevent sand blowing. After 15 weeks, on 24 October, the

plastic was removed and the pathogen samples were dug out. Pathogen survival was determined as described above.

It can be envisaged that not only pathogens but a whole array of soil organisms will be affected by the combination of green amendments and plastic mulch. This can influence soil suppressiveness to pathogens. To test this for one of the pathogens, *F. oxysporum* f.sp. *asparagi*, composite soil samples, prepared by mixing 10 samples from the upper 15 cm, of each of the plots of the treatment combinations nonamended-nonmulched, nonamended-mulched, broccoli-nonmulched, and broccoli-mulched, were collected on the day the plastic was removed. The soil was mixed with talcum inoculum of *F. oxysporum* f.sp. *asparagi* strain CWB1 to a density of 100 cfu g^{-1} dry soil, a density known to result in a low root rot severity in nontreated soil. For each sample 5 pots with 500 ml soil were placed completely randomized in a glasshouse at 20°C. In each pot 50 rye plants (*Secale cereale* L. cv. Petkus) were grown from seeds to stimulate microbial recolonization of the soil. After 6 weeks, the rye was removed from the pots, the soil was screened through 2-mm openings and planted with pregerminated asparagus seeds (3 seeds per pot). After 16 weeks, root rot severity was assessed as described earlier (Blok and Bollen, 1996a).

Field experiment 1995

In summer 1995 an experiment was performed at the same location on a field situated closely to the 1994-field. The soil was again a loamy sand (pH-KCl 6.5; 3.4% organic matter). Most experimental details were the same as in 1994. Only now 5 blocks (replicates) instead of 3 were used and plot size was 6 m x 8 m (with inner 2 m x 4 m used for pathogen samples and measurements). As there were no indications that the measurements of soil physical and chemical parameters had an effect on pathogen survival in 1994, these measurements were done in all plots of 1 block in 1995. On the plots where broccoli had to be incorporated, 3-wk-old broccoli plants (cv. Marathon) were planted on 19 June. Plant material was incorporated by rotary tilling on 19 July, in amounts of 3.4 kg fresh weight m^{-2} (0.3 kg ovendry weight m^{-2}) and 4.0 kg fresh weight m^{-2} (0.5 kg ovendry weight m^{-2}), for broccoli and grass, respectively. In all plots, duplicate samples of *F. oxysporum* f.sp. *asparagi*, *R. solani*, *V. dahliae*, and *G. pallida* were buried at 15 cm depth. After burying of samples and installation of thermocouples, Pt electrodes and gas diffusion chambers, the field was sprinkler irrigated on 20 July. On 21 July the plastic mulch was applied. As in 1994, nonmulched plots were sprinkler irrigated when needed to prevent sand blowing. Weeds were controlled by one herbicide application (a mixture of simazine and diuron, both at 0.5 l ha^{-1}). After 15 weeks, on 3 November, the plastic was removed, the pathogen samples were dug out, and survival was determined.

Data analysis

Prior to statistical analyses data were checked for normality and homoscedasticity and

transformed when needed. The data for *F. oxysporum* f.sp. *asparagi* in the laboratory experiment were square-root transformed ($\sqrt{}$(cfu g^{-1} soil). For the pathogen samples from the field experiment the following transformations were used. A log-transformation ($\log_{10}(x + 1)$ was applied to the data of *F. oxysporum* f.sp. *asparagi* (cfu g^{-1} soil), a square root transformation ($\sqrt{}(x + 0.5)$ was applied to the data of *R. solani* (germination index), and of *V. dahliae* (cfu g^{-1}). The data of *G. pallida* were not transformed. Analysis of variance was applied to test for main effects and interactions. Preplanned comparisons between treatment combinations were tested with linear contrasts (Sokal and Rohlf, 1981). All analyses were performed using the Statistical Analysis System, version 6.04 (SAS Institute Inc., Cary, NC).

For presentation of the data on the survival of pathogens in the field experiments backtransformed means were used. For ease of comparison, means are presented as percentages of the value for the nonamended nonmulched control. For each pathogen the value for the latter treatment is given with the figures.

Results

Laboratory experiment

The oxygen concentration in soil decreased significantly with time and final concentrations were below 2% in all bottles (Fig. 1). The oxygen consumption rate was higher in the bottles incubated at 24°C than in those incubated at 11°C with clear differences among the treatments for both temperatures. Three pairs of treatments with similar oxygen consumption rates could be distinguished. Cabbage and alfalfa had the highest oxygen consumption rate, followed by grass and wheat, and by compost and the control treatment, respectively (Fig. 1).

After 7 weeks of incubation, *F. oxysporum* f.sp. *asparagi* was not detected any more in the bottles with alfalfa or cabbage, and was greatly reduced in the other four treatments (Table 1). Analysis of variance on the data of the latter four treatments showed a significant effect of both temperature and treatment (for both $P<0.001$) and the absence of a significant interaction effect ($P=0.106$).

Field experiments

Soil physical and chemical parameters. In both field experiments the oxygen concentration of the soil atmosphere at 15 cm depth decreased rapidly to less than 1% in the mulched plots (Fig. 2). In the nonamended, mulched plots the concentration started to increase gradually after 3 to 4 weeks. In the amended, mulched plots, in contrast, the concentration started to rise substantially after 7 to 8 weeks. In nonmulched plots concentrations were higher than in mulched plots. In the 1994-experiment, the concentration measured 1 day after application of the plastic was 2.9 and 12.0% in the broccoli-amended, nonmulched and the nonamended,

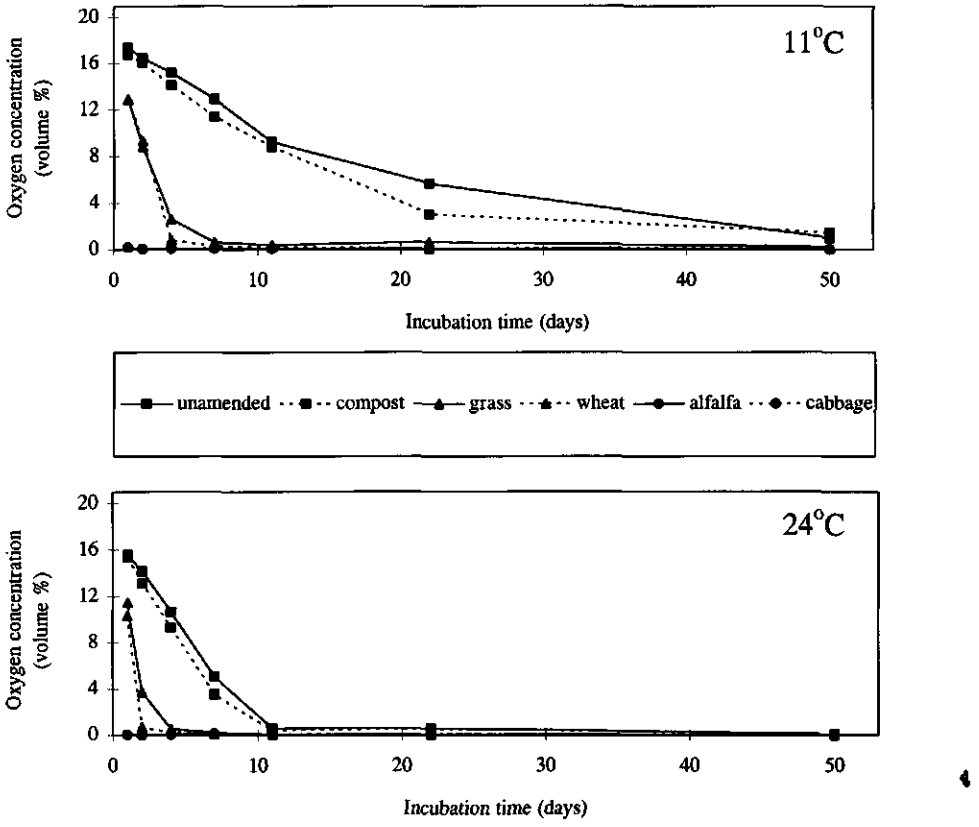


Figure 1. Oxygen concentration in the head space of sealed glass bottles containing field soil amended with one of five plant materials or left unamended, and incubated at 11 or 24°C.

nonmulched plots, respectively, and fluctuated between 8% and 21% during the rest of the experimental period with the mean concentration increasing towards the end. In the 1995-experiment the first measurements were carried out after 12 days. The concentrations fluctuated less than in 1994 and ranged between 18% and 21%.

In both experiments the value of the redox potential in the mulched, amended plots decreased immediately and reached levels indicative of strongly reduced soil conditions for shorter or longer periods of time (Fig. 3). In the 1994-experiment the redox potential in the broccoli-amended, mulched plot went down to a minimum of -193 mV on day 48. In the nonamended, mulched plot the redox potential did not decrease that much (the minimum was

Table 1. Population density of *F. oxysporum* f.sp. *asparagi* (cfu g^{-1} dry soil) in artificially infested soil amended with different plant materials after incubation at 11 or 24°C in closed bottles for 7 weeks.

Plant material	11°C	24°C	Mean
Control	1617 [1]	311	836 b [2]
Compost	1742	554	1066 a
Grass	798	79	345 d
Wheat	1071	248	588 c
Mean	1277 a	271 b	
Alfalfa	<4 [3]	<4	
Cabbage	<4	<4	

[1] For statistical analysis, data were transformed ($\sqrt{}$(cfu g^{-1} soil)) and, therefore, means are given as backtransformed values.
[2] Means followed by the same letter are not significantly different (Tukey, $P=0.05$).
[3] Lower limit of detection was 4 cfu g^{-1} soil.

110 mV) and increased again after 21 days. In the 1995-experiment the redox potential in the nonamended, mulched plot hardly decreased compared to the nonmulched plot. In the broccoli- and grass-amended plots the redox potential went down quickly to -13 and -196 mV, respectively, but also increased rather quickly after day 11. In the nonmulched plots the redox potential fluctuated only a little in both experiments and ranged from 500 to 700 mV (Fig. 3).

In both years the experiments started in mid-summer and lasted till well into autumn. In 1994, the daily maximum temperature of the soil at 15 cm depth in the mulched broccoli-amended plot ranged between 25.6 and 32.6°C in the first 4 weeks and decreased gradually to 11°C at the end of the experiment. In the nonmulched broccoli-amended plot this temperature was 6.6 to 0.4°C lower than in the mulched plot. In 1995, the daily maximum temperature of the soil at 15 cm depth in the mulched broccoli-amended plot ranged between 29.4 and 39.0°C in the first 4 weeks and decreased gradually to 10°C at the end of the experiment. In the nonmulched broccoli-amended plot this temperature was 12.6 to 0.6°C lower than in the mulched plot.

Survival of pathogens. Analysis of variance showed significant plant material x mulching interactions for *F. oxysporum* f.sp. *asparagi* in 1994 and for all three fungal pathogens in 1995 (Tables 2 and 3) indicating that the effect of mulching depended on the plant material treatment. Therefore, linear contrasts were calculated to test the effect of mulching for each

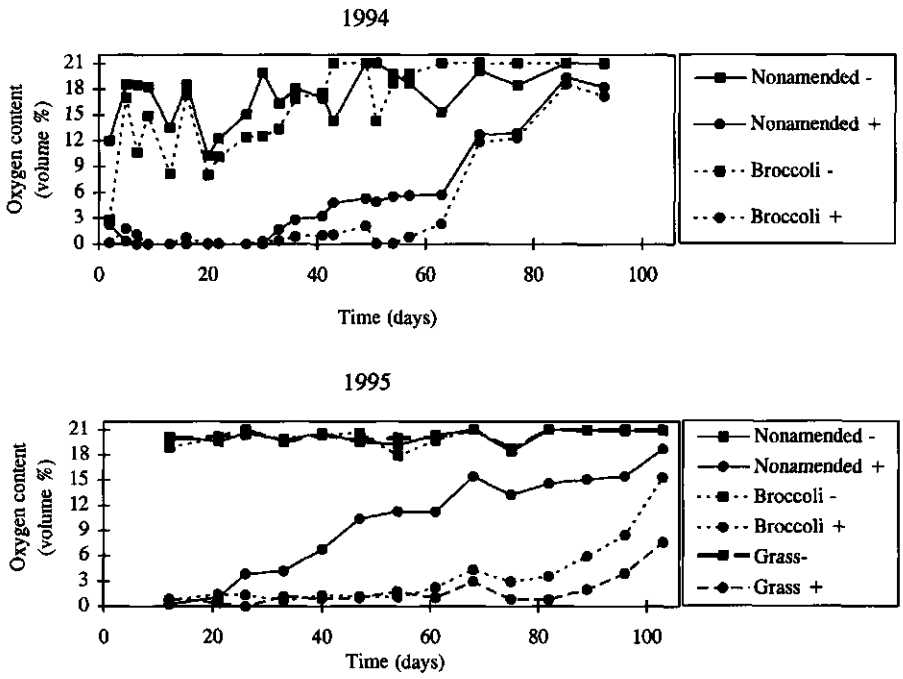


Figure 2. Oxygen concentration of the soil atmosphere at 15 cm depth in the 1994- and 1995-experiment. Nonmulched and mulched are indicated with - and +, respectively.

plant material treatment and the effect of plant material for both mulching treatments (Tables 2 and 3).

In general, good survival was found for samples from the nonmulched plots. Differences among the nonmulched plots, i.e. between nonamended control plots and the broccoli- or grass-amended plots and between the broccoli- and the grass-amended plots, were small and not significant in both experiments (Figs. 4 and 5, Tables 2 and 3). Large effects, however, were observed for the mulched plots. In the nonamended control plots of both experiments, application of plastic mulch resulted in a higher survival for *F. oxysporum* f.sp. *asparagi* and *V. dahliae* and in a lower survival for *G. pallida*. For *R. solani* a lower survival was found in 1994 but a higher survival in 1995. However, differences were significant only for both *R. solani* and *G. pallida* in 1995. Application of plastic mulch to the broccoli- and the grass-amended plots resulted in a strong inactivation of *F. oxysporum* f.sp. *asparagi*, *R. solani* and *V. dahliae*. When pathogen inactivation was not complete, it was usually stronger in the

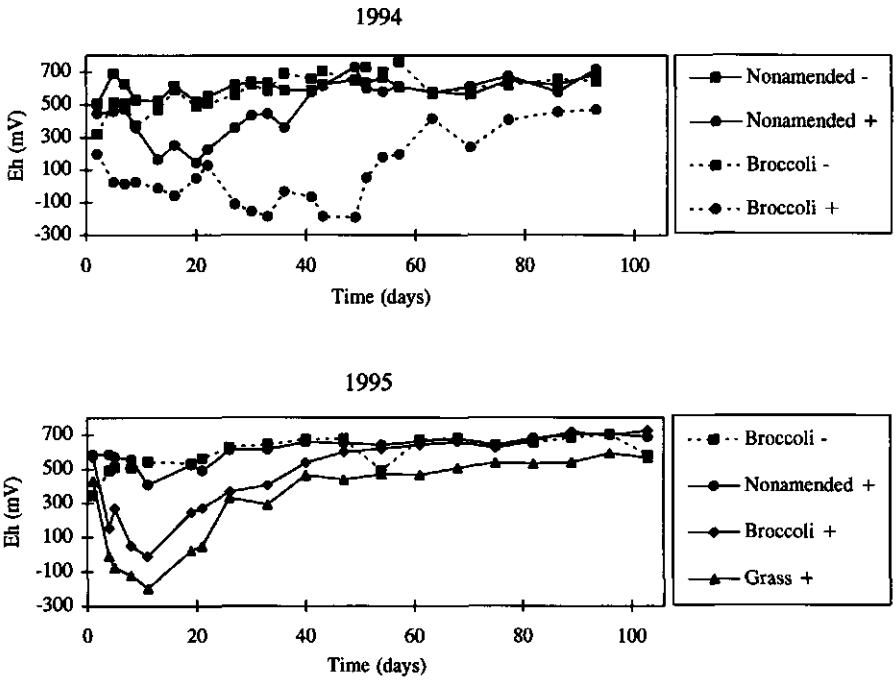


Figure 3. Redox potential (Eh) measured in field soil at 15 cm depth in the 1994- and 1995-experiment. Nonmulched and mulched are indicated with - and +, respectively.

grass-amended plots than in the broccoli-amended plots, but the difference was significant ($P<0.05$) only for *R. solani* in 1995.

Whereas in 1994 the variation between samples of the three fungal pathogens of the same treatment combination was small, it was large in 1995. In fact, strong reductions (similar to those in 1994) were observed in half of the samples and hardly any reduction was found in the other samples. As a result, the trends in the results of both experiments were similar but the mean reductions found for *F. oxysporum* f.sp. *asparagi*, *R. solani* and *V. dahliae* in the broccoli- and grass-amended mulched plots were larger in 1994 than in 1995.

The number of living juveniles and eggs of *G. pallida* as estimated under a dissecting microscope did not indicate large effects. In 1994 this number was lower in the amended plots compared to the nonamended plots, irrespective of mulching, but differences were not significant. In 1995 a significant main effect of plastic mulching was found ($P<0.001$). However, the 8 cyst samples (including duplicate samples from four plots) that were incu-

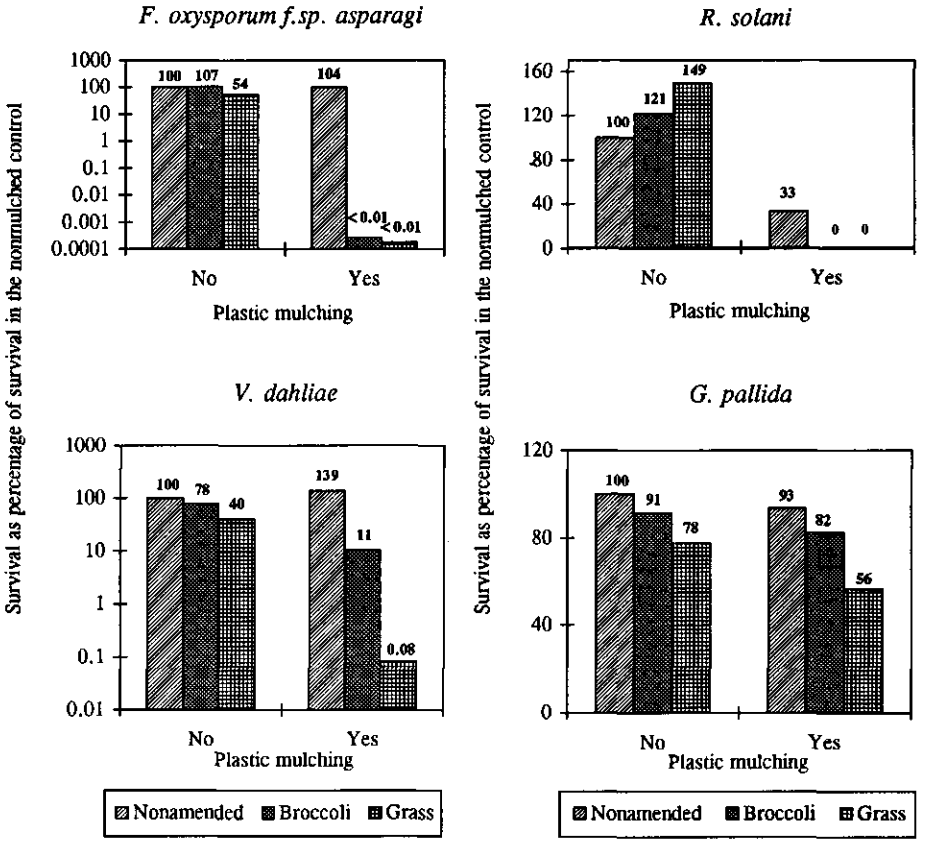


Figure 4. Survival of pathogens in samples buried at 15 cm depth in the 1994-field experiment. Backtransformed means are given as percentage of the value for the nonamended nonmulched control. The latter values were:
Fusarium oxysporum f.sp. *asparagi*: 1.1 x 10^6 cfu g^{-1} dry talcum; *Rhizoctonia solani*: germination index 16.2; *Verticillium dahliae*: 7.6 x 10^4 cfu g^{-1} dry stem tissue; *Globodera pallida*: 73.6% living eggs and juveniles.

bated in hatching fluid showed a large effect of mulching. The percentage living juveniles was 71 and 53% for the two grass-amended, nonmulched plots and only 7 and 3% for the two grass-amended, mulched plots.

Colonization of the soil by F. oxysporum *f.sp.* asparagi. In the bio-assay moderate disease severity levels were found, the disease index ranging from 3.3 to 3.8 on a 0-10 scale, with only slight differences between treatment combinations. Analysis of variance for the disease-

Table 2. Analysis of variance table and linear contrasts[1] for the survival of *Fusarium oxysporum* f.sp. *asparagi*, *R. solani*, *V. dahliae* and *G. pallida* after burial of inoculum samples at 15 cm soil depth in the 1994-field experiment.

		F. oxysporum f.sp. asparagi		*R. solani*		*V. dahliae*		*G. pallida*	
Source	Df[2]	MS[3]	$P>F$[4]	MS	$P>F$	MS	$P>F$	MS	$P>F$
Total (corrected)	17	7.106		4.032		16234		462	
Crop residue (A)	2	16.015	<0.001	0.671	0.582	66956	0.002	744	0.113
Plastic mulching (B)	1	57.894	<0.001	47.122	<0.001	36633	0.030	364	0.274
Blocks	2	0.278	0.128	1.293	0.370	2321	0.677	1593	0.021
A x B	2	14.616	<0.001	2.875	0.136	21788	0.059	50	0.835
Error	10	0.109		1.175		5722		272	
Linear contrasts:									
- Plastic: control vs crop residues	1	0.030	0.615	0.819	0.423	8869	0.242	261	0.351
- Plastic: cabbage vs grass	1	0.133	0.296	0.342	0.601	7233	0.287	153	0.470
+ Plastic: control vs crop residues	1	61.075	<0.001	5.931	0.048	151316	<0.001	625	0.161
+ Plastic: cabbage vs grass	1	0.024	0.648	0.000	1.000	10070	0.214	549	0.186
Control: - vs + plastic	1	<0.001	0.953	4.140	0.090	3565	0.448	35	0.728
Cabbage: - vs + plastic	1	44.941	<0.001	21.484	0.002	35328	0.032	65	0.636
Grass: - vs + plastic	1	42.184	<0.001	27.248	0.001	41315	0.028	365	0.274

[1] Analyses were performed on the following data: *F. oxysporum* f.sp. *asparagi*: log(cfu g^{-1} + 1); *R. solani*: $\sqrt{}$(germination index + 0.5); *V. dahliae*: $\sqrt{}$(cfu g^{-1} + 0.5); *G. pallida*: percentage living juveniles and eggs (not transformed).

[2] Degrees of freedom.

[3] Mean square.

[4] Probabilities associated with individual F tests.

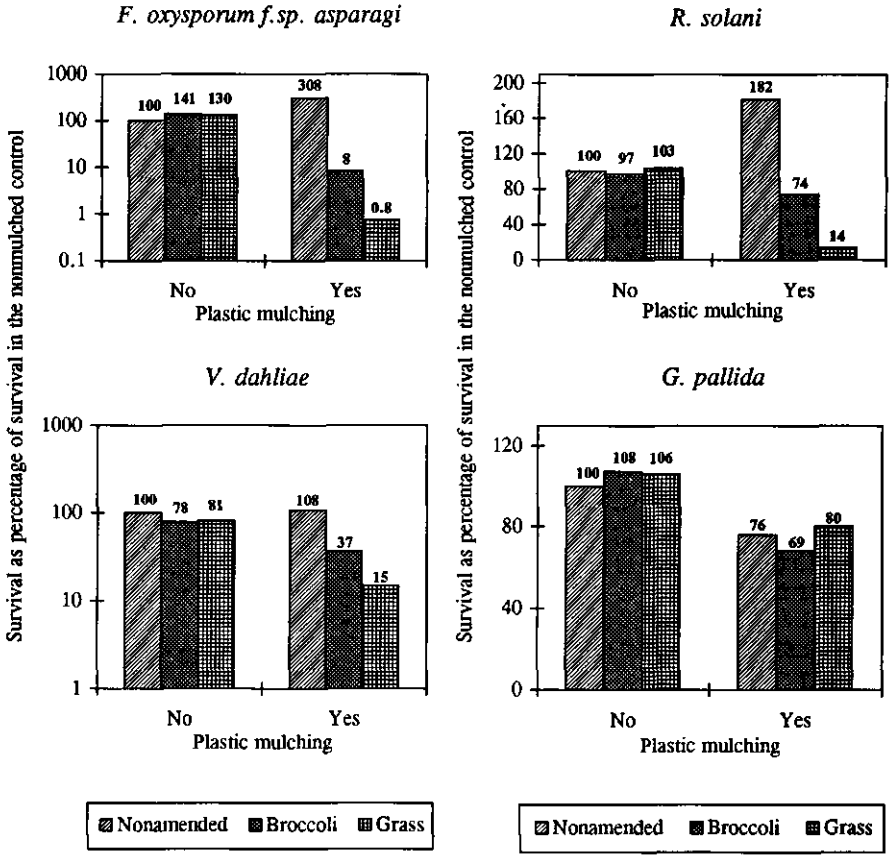


Figure 5. Survival of pathogens in samples buried at 15 cm depth in the 1995-field experiment. Backtransformed means are given as percentage of the value for the nonamended nonmulched control. The latter values were:
Fusarium oxysporum f.sp. *asparagi*: 5.1 x 10^5 cfu g^{-1} dry talcum; *Rhizoctonia solani*: Germination index 21.4; *Verticillium dahliae*: 9.7 cfu g^{-1} dry soil; *Globodera pallida*: 67.5% living eggs and juveniles.

index data did not indicate significant main effects or interactions (data not shown). Apparently, soil suppressiveness to *F. oxysporum* f.sp. *asparagi* was not affected by amendment with broccoli and mulching.

Discussion

The results of the laboratory experiment show that anaerobic soil conditions develop quickly when soil metabolic activity is stimulated by incorporation of readily decompo-

Table 3. Analysis of variance table and linear contrasts[1] for the survival of *Fusarium oxysporum* f.sp. *asparagi*, *R. solani*, *V. dahliae* and *G. pallida* after burial of inoculum samples at 15 cm soil depth in the 1995-field experiment.

		F. oxysporum f.sp. asparagi		*R. solani*		*V. dahliae*		*G. pallida*	
Source	Df[2]	MS[3]	*P*>F[4]	MS	*P*>F	MS	*P*>F	MS	*P*>F
Total (corrected)	29	1.707		2.989		0.673	464		
Crop residue (A)	2	3.924	0.040	11.723	<0.001	3.289	<0.001	0.53	0.996
Plastic mulching (B)	1	7.391	0.015	3.104	<0.107	4.047	<0.001	2444	<0.001
Blocks	4	1.027	0.434	2.959	0.060	0.079	0.876	1612	<0.001
A x B	2	4.742	0.023	12.545	<0.001	1.627	0.009	72	0.608
Error	20	1.033		1.085		0.266			
Linear contrasts:									
- Plastic: control vs crop residues	1	0.058	0.816	<0.001	0.989	0.351	0.264	29	0.658
- Plastic: cabbage vs grass	1	0.004	0.954	0.050	0.832	0.005	0.896	27	0.665
+ Plastic: control vs crop residues	1	14.557	0.001	36.085	<0.001	8.515	<0.001	19	0.717
+ Plastic: cabbage vs grass	1	2.712	0.121	9.222	0.009	0.962	0.072	43	0.589
Control: - vs + plastic	1	0.596	0.457	6.350	0.026	0.033	0.729	653	0.047
Cabbage: - vs + plastic	1	3.791	0.070	1.021	0.344	6.43	0.020	1727	0.003
Grass: - vs + plastic	1	12.488	0.002	20.826	<0.001	20.87	<0.001	455	0.091

[1] Analyses were performed on the following data: *F. oxysporum* f.sp. *asparagi*: log(cfu g^{-1} + 1); *R. solani*: $\sqrt{}$(germination index + 0.5); *V. dahliae*: $\sqrt{}$(cfu g^{-1} + 0.5); *G. pallida*: percentage living juveniles and eggs (not transformed).

[2] Degrees of freedom; due to missing values for *R. solani* the degrees of freedom for total and error was lowered by 1.

[3] Mean square.

[4] Probabilities associated with individual F tests.

sable organic matter and diffusion of oxygen from the atmosphere into the soil is prevented. Under these conditions chlamydospores of *F. oxysporum* f.sp. *asparagi* were strongly to completely inactivated after 7 weeks showing the potential of the approach for control of soilborne pathogens. Development of anaerobiosis was faster and pathogen inactivation stronger at 24°C than at 11°C indicating that field application should preferably take place in summer when temperatures are high. Faster inactivation of the pathogen at higher temperature was also found by Hagiwara & Takeuchi (1983) who anaerobically fermented radish residues infested with *F. oxysporum* f.sp. *raphani* by sealing the residues in plastic bags.

Although under field conditions the diffusion of oxygen into the soil was not prevented completely, as it was achieved in the laboratory experiment, anaerobic conditions developed nevertheless readily in both years in mulched plots, regardless of incorporation of plant material. The oxygen concentration of the soil atmosphere is the resultant of the diffusion from the adjacent nonmulched soil and through the plastic mulch and of the consumption by the soil microflora. The rapid development of anaerobic conditions in the nonamended, mulched plots indicates that the level of microbial respiration in these plots was relatively high. This was probably due to an increase of the normal soil respiration level by the soil tillage and irrigation prior to the mulching and by the increased soil temperature caused by the mulching. However, in these plots the amount of easily-decomposable organic material was small and could support the enhanced microbial activity for only a short time. As a result the oxygen concentration increased after 3 to 4 weeks. In the amended plots the amount of easily-decomposable organic matter was much larger resulting in longer periods of anaerobiosis. The different amounts of easily-decomposable matter may also explain the difference in the course of the redox potential between the nonamended and the amended plots.

The course of the oxygen concentration for the mulched plots was rather similar in both years. However, whereas in 1994 oxygen was hardly detected in the first period, a concentration of about 1% was still measured in the same period in 1995. This difference may explain that in 1995 the redox potential started to increase already after 11 days whereas in 1994 it started to increase only after 7 weeks.

Large and significant reductions in the number of viable survival structures were found for *F. oxysporum* f.sp. *asparagi*, *R. solani* and *V. dahliae* in the plots where plant material was incorporated and plastic mulch was applied. These results clearly show the potential of this method to control various persistent soilborne pathogens in a relatively easy way.

Although there was a significant effect of plastic mulch on the viability of juveniles and eggs of *G. pallida* in the 1995-experiment, the effects were small compared to those on the fungi when they were assessed by direct examination with the dissecting microscope. However, with the hatching test viability of cyst contents was shown to be strongly decreased in plots with plant material and plastic mulch. This result is in accordance with that of Turner

et al. (1983) who found complete inactivation of *G. pallida* after exposure to fermentation fluid for 10 days at 35°C in an anaerobic digester fed with different organic materials including grass. Because the number of samples in the hatching test was low, further experiments are needed to draw definite conclusions on inactivation of *G. pallida*. In addition to *G. pallida*, samples of other nematode species were buried in a few plots (Koot & Lamers, unpublished results). Strong reductions were found for *Pratylenchus penetrans* with the combination of plant material and plastic mulch, and for *Meloidogyne fallax* with plastic mulch regardless of the incorporation of plant material. Although the number of samples was small, this indicates that these nematodes may also be controlled by the method.

Control of various soilborne fungal pathogens after incorporation of cruciferous crops, including broccoli, has been reported by several authors (Gamliel and Stapleton, 1993a; Lewis and Papavizas, 1971; Mojtahedi *et al.*, 1991; Muelchen *et al.*, 1990; Ramirez-Villapudua and Munnecke, 1988; Subbarao and Hubbard, 1996). In many of these studies the soil was covered with plastic sheeting to solarize the soil or to retain the volatiles produced in soil. The suppression is attributed mainly to the toxic breakdown products of glucosinolates (Lewis and Papavizas, 1971; Mayton *et al.*, 1996; Smolinska *et al.*, 1997). However, in our experiments we did not obtain significant control of pathogens by incorporation of broccoli alone. There are four explanations for this failure. First, we added less organic material to the soil than has been done in most other studies. This implies that a lesser quantity of toxic break-down products could be produced. Second, species and cultivars of cruciferous crops differ greatly in their content of glucosinolates (Mayton *et al.*, 1996) and the broccoli cultivar used in this study may have contained very little. Third, the physical and chemical conditions under which hydrolysis of the glucosinolates takes place determine the type of decomposition products (Duncan, 1991). These conditions may have been different in this study. Fourth, it is possible that toxic volatiles were produced but did not remain in the soil long enough to have an effect. Gamliel & Stapleton (1993a) also obtained control in solarized plots with cabbage but not in those with cabbage alone. Our results with incorporation of plant material alone underline that although effects on pathogens can be substantial, they are characterized by inconsistency (Baker and Cook, 1974).

Application of the plastic mulch increased the daily maximum soil temperature at 15 cm depth by 0.4 to 6.6°C in 1994 and by 0.6 to 12.6°C in 1995 with largest increases in the first 4 weeks of the experiments. This increase did not result in lethal temperatures as is concluded from the fact that the survival in the mulched non-amended plots is generally not lower than in the nonmulched non-amended plots. However, sublethal temperatures in the mulched plots may have contributed to pathogen inactivation in the mulched plots amended with plant material by weakening the survival structures. Weakening of pathogen propagules by sublethal temperatures has been reported for various pathogens (DeVay and Katan, 1991).

The course of the oxygen concentration and of the redox potential in our amended mulched plots during the first weeks of the treatment showed a similar pattern as that commonly

observed in flooded soils (Reddy, 1987). It is reasonable to assume that analogous mechanisms of pathogen inactivation are involved. The mechanisms may include a direct effect of oxygen deficiency, fungitoxicity of compounds formed under the anaerobic conditions, biological control by anaerobic microorganisms or the germination-lysis mechanism as defined by Sneh *et al.* (1976). Studies on the effect of low oxygen concentrations on growth of fungi has extensively been reviewed by Tabak & Cooke (1968). From their review it is clear that many soil fungi, including *F. oxysporum* and *V. dahliae*, can survive and even grow at very low oxygen levels and at the elevated carbon dioxide levels that are almost invariably coupled to reduced oxygen levels. Thus, a low oxygen concentration alone will presumably not be an important factor. This conclusion is substantiated by the results of Mitchell & Alexander (1962) who studied the microbiology of flooded soils, and by our results showing that pathogens could survive well in nonamended, mulched plots where oxygen levels were low for several weeks. Strong indications for a role of anaerobic breakdown products in the inactivation of pathogens were reported by several authors. Mitchell & Alexander (1962) found that incorporation of readily metabolizable organic matter into flooded soil greatly accelerated the decline in numbers of *F. oxysporum* f.sp. *conglutinans* compared to nonamended flooded soil. The same was found by Menzies (1962) for *V. dahliae*, and by Watson (1964) for *Fusarium* spp., *V. dahliae* and *Pyrenochaeta terrestris*. Under reducing soil conditions carbon dioxide, ethylene, hydrogen, methane, organic acids, alcohols and aldehydes are among the compounds known to accumulate, at least temporarily (Adamson *et al.*, 1975; Ponnamperuma, 1972; Tsutsuki and Ponnamperuma, 1987; Yoshida, 1975). Several of these products are potentially fungitoxic and may, therefore, be implicated in the inactivation of pathogens. However, studies relating concentrations of these products in soil to the inactivation of fungi are scarce. Mitchell & Alexander (1962) suggested that acetic acid may be implicated in fungitoxicity in non-alkaline soils. Okazaki & Nose (1986) studied inactivation of *F. oxysporum* f.sp. *raphani* in glucose-amended flooded soil and found that the concentrations of acetic and n-butyric acid detected in soil were high enough to kill the chlamydospores of the fungus. Lynch (1977) found that in wet soils with wheat straw residues anaerobic conditions developed and acetic acid was produced in phytotoxic concentrations. A role for biological control by anaerobic microorganisms is suggested by the fact that various of these species, notably *Bacillus*, *Pseudomonas* and *Clostridium* spp., are well-known antibiotic-producers. Indications that these species may control pathogens were obtained by Taylor & Guy (1981) who inoculated soil infested with wood-destroying basidiomycetes with several *Bacillus* and *Clostridium* spp. Inactivation of pathogens was obtained with both broccoli and grass. This indicates that crop-specific compounds or their breakdown products, such as the glucosinolates specific for cruciferous crops, are not necessarily involved.

The inactivation of the fungal pathogens was greater in 1994 than in 1995. In 1994, a low redox potential occurred for several weeks in the amended mulched plots. In 1995, the redox

potential reached the same low level but increased again soon afterwards. This means that in 1995 lower amounts of toxic fermentation products can have accumulated resulting in less pathogen inactivation than in 1994. The explanation for the differences between the two experiments is most probably that higher amounts of plant material were incorporated in soil in 1994 than in 1995. In 1994, 0.8 and 0.5 kg dry matter m^{-2} were applied for grass and broccoli, respectively, in 1995 those amounts were 0.5 and 0.3 kg dry matter m^{-2}. Differences in amounts of plant material may also explain the slightly higher inactivation obtained with grass compared to broccoli.

The combination of plant material and plastic mulching greatly disturbs the soil ecosystem. It may be envisaged that this disturbance can result in an increased conduciveness of the soil to certain pathogens with the risk of a rapid recolonization when part of the population survives the treatment or when infested material is introduced. An example of the latter is found in banana cultivation where rapid recolonization by *F. oxysporum* f.sp. *cubense* is one of the reasons that flooding is no longer being used on a large scale for controlling this pathogen (Stover, 1979). However, there was no evidence for an increased risk for rapid recolonization of biologically-disinfested soil by *F. oxysporum* f.sp. *asparagi* in this study. Nevertheless, the experiences with banana and the expectations based on knowledge about the importance of soil fungistasis for the behaviour of pathogens in soil (Cook and Baker, 1983) warrants further evaluation of this aspect under field conditions. On the other hand, the disturbance of the soil ecosystem may also present opportunities for the introduction and establishment of populations of antagonistic organisms, as suggested by Katan (1981) for solarization.

The prospects for combining plant material and plastic mulching to control various persistent soilborne pathogens have clearly been demonstrated. Currently, laboratory and field experiments are being performed to study the mechanisms of pathogen inactivation and evaluate the effects of temperature, type and amount of plant material, mulch type and nutrient status of the soil on the efficacy of the treatment.

Chapter 10

General discussion

The results of the study on different aspects of early decline are summarized and an attempt is made to integrate them into a comprehensive account of the etiology and epidemiology of the disease in Dutch asparagus crops. The prospects for the development of an effective management strategy are discussed.

Etiology

The study of the etiology of early decline was focused on autotoxic compounds and pathogens. First, both factors were broadly characterized (Chapters 2 and 3). Subsequently, their contribution to the etiology of early decline was addressed (Chapters 4 and 5).

F. oxysporum f.sp. *asparagi* was identified as being by far the most important soilborne pathogen in Dutch asparagus fields based on its predominance in our inventory and its virulence (Chapter 3). Its dominant role in the etiology of early decline became clear from the results of the pot experiments described in Chapter 4. All plants grown in asparagus soil, showing the typical decline symptoms, yielded almost exclusively *F. oxysporum* f.sp. *asparagi*. Soil treatments that killed the pathogen (heat and fungicide treatments) prevented the appearance of decline symptoms.

Autotoxicity of asparagus root material was demonstrated both *in vitro* and in unsterilized field soil. The toxic products were very persistent; extracts of root material collected from fields where asparagus production was terminated 10 years before still inhibited plant growth. Three adverse effects of autotoxins on the asparagus plant were considered: i) direct inhibition of root growth, ii) predisposition to crown and root rot caused by *F. oxysporum* f.sp. *asparagi*, iii) decrease of soil fungistasis to *F. oxysporum* f.sp. *asparagi*. The role in the etiology of early decline of each of these effects will be evaluated below.

i) Direct growth inhibition. Incorporation of asparagus root fragments that were sterilized by gamma-irradiation in order to kill the pathogens did not result in inhibition of root growth when the amounts of residues were equal to the estimated maximum amount of root residues present in soil in a replant situation (Chapter 2). This suggests that the amount of inhibitory products left in soil after termination of the crop is too low to affect root growth. Root biomass of asparagus plants grown in asparagus soil was significantly lower than those grown in fresh soil. Heating the asparagus soil with its root residues for

30 min at 60°C or application of the fungicide prochloraz resulted in a root biomass of test plants equal to that in fresh soil (Chapter 4 and 5). As autotoxins are known to be resistant to both treatments, this result provides strong evidence that direct growth inhibition by autotoxins is not an important factor in early decline under field conditions. Moreover, diluting asparagus soil, containing autotoxins, with fresh soil to a proportion with only 20% asparagus soil, did not decrease disease severity or increase root weight. This observation provides additional evidence for the view that direct growth inhibition is negligible.

ii) Predisposition to crown and root rot caused by F. oxysporum *f.sp.* asparagi. Strong indications for this effect have been presented in the literature (Hartung and Stephens, 1983; Hartung *et al.*, 1989; Peirce and Colby, 1987). However, none of the three experiments in which this effect was studied provided any evidence for an effect on severity of root rot caused by *F. oxysporum* f.sp. *asparagi* (Chapter 5). The reasons for the discrepancy of my results with those of other authors have been discussed in Chapter 5 already; they are related to the microbial activity of the substrate used and the amount of asparagus root material incorporated into the substrate. Since the conditions in my experiments are more relevant to field conditions than those of the experiments described in literature, it is concluded that predisposition to crown and root rot due to autotoxins is not a major factor in early decline.

iii) A decrease of soil fungistasis to F. oxysporum *f.sp.* asparagi. Under *in vitro* conditions, asparagus root extract selectively inhibited soil fungi. Several isolates, including isolates of species known for their antagonistic activity against plant pathogenic fungi, were inhibited. Potentially, a selective effect can cause a shift in the composition of the soil microflora, resulting in a decreased fungistasis to *F. oxysporum* f.sp. *asparagi* and, consequently, in an enhanced development of the pathogen in soil. However, evidence for a decreased soil fungistasis to *F. oxysporum* f.sp. *asparagi* and an increase of soil colonization by the pathogen was not obtained in either of the five experiments in which these effects were studied.

In conclusion, based on the etiological data, *F. oxysporum* f.sp. *asparagi* can be considered as the major cause of early decline of asparagus crops in the Netherlands. Effects of autotoxins, either direct or indirect, via the host plant or the soil microflora, are not or only to a limited extent involved under field conditions.

F. oxysporum f.sp. *asparagi* has been identified as an important causal factor in early decline in many asparagus producing areas. In almost all studies outside of the Netherlands, *F. proliferatum* was also identified as a major pathogen. This is a remarkable difference with the situation in the Netherlands where *F. proliferatum* has yet to be isolated from asparagus (Van Bakel and Kerstens, 1970; Chapter 3). The absence of *F.*

proliferatum in Dutch asparagus plantings might result in less severe decline compared to situations were both pathogens are present and might largely explain why early decline is recognized as a problem by the farmer only in replant situations and not when asparagus is grown on virgin soil.

Epidemiology

Although the pathogen has been isolated from flowers and berries still on the plant (Manning *et al.*, 1980), the major source of contamination of Dutch seed lots was found to be infested soil adhering to berries that have fallen onto the ground. When these berries are mixed with berries picked from the plants, which is common practice, the whole seed lot becomes contaminated during the seed extraction process (Chapter 6). Seedlings from contaminated seed will develop root and crown rot and can act as inoculum sources for neighbouring seedlings. In addition, seedlings may become infected by soilborne inoculum that is present in the majority of virgin fields in the asparagus producing area, where also the majority of crowns is produced (Chapter 6). As a result of infection by seedborne or soilborne inoculum, the majority of crowns can be infected at the time of planting, although disease severity is low (Chapter 6).

In spite of the high percentage of infected crowns and the presence of soilborne inoculum in the upper layer of virgin soils, plants establish successfully and an economic life of about 10 years is generally realised. However, in the years following planting, the pathogen population gradually builds up and the whole rooted layer becomes infested (Chapter 4). As the majority of crowns planted is infested, it is expected that the field becomes infested rather homogeneously. The latter was confirmed indeed for several fields where soil infestation was tested by means of bio-assays (W.J. Blok and G.C.M. Coenen, unpublished).

At the end of the economic life of the crop, plants are destroyed by rotary tilling and crop residues become incorporated in soil. It may be envisaged that at this stage inoculum density increases further, due to colonization of the killed root and crown tissue from inoculum already present in the tissue.

After destruction of the host crop, the pathogen can survive in soil for very long periods without asparagus. The inoculum level decreases only gradually; in soils where asparagus production was stopped 5 or 11 years previously, asparagus seedlings still developed severe root rot (Chapter 4). Several factors contribute to this persistence. Firstly, the production of chlamydospores by the pathogen, which are known to survive in soil or plant residues for many years (McKeen and Wensley, 1961; Garrett, 1970). Secondly, the residues of asparagus roots, densely colonized by the pathogen, were shown to be very persistent in field soil (Chapter 6). Finally, the ability to symptomlessly

colonize roots of many crop and weed species affords the possibility that frequently part of the inoculum is renewed in the absence of asparagus (Chapter 7).

When, after a shorter or longer asparagus-free period, the field is replanted with asparagus crowns, the inoculum density of the pathogen is still high. Unlike the situation in the first planting, infested crowns are hardly important as an inoculum source because of the high infestation of the soil. A second difference is that the infestation is not restricted to the upper layer but that the whole rootable soil layer is infested. As a result, new roots that develop from the planted crowns cannot escape infection, as they can in virgin soils, but become continuously infected during growth. The early and extensive infection of the root system causes an early decrease in plant vigour and plant density and consequently a shortening of the economic life of the crop, in other words: early decline.

From the picture just drawn, *F. oxysporum* f.sp. *asparagi* appears to be a pathogen of moderate aggressiveness. On virgin soil an economic cropping life of about 10 years can be realised although at the time of planting the pathogen is already present in soil and on the majority of the crowns. Even in a replant situation with a high infestation of the soil in the whole rootable layer at the time of planting, plants become well established and give high yields in the first years. Kommedahl and Windels (1979) introduced the term 'host-dominant disease' for diseases in which the host primarily determines the outcome of the interaction between host and pathogen, in contrast to 'pathogen-dominant diseases' in which the pathogen is the primary determining factor. Crown and root rot caused by *F. oxysporum* f.sp. *asparagi* is an example of a host-dominant disease. When the plants are allowed to grow vigorously, infections by the pathogen will not cause much damage. However, any stress factor that reduces plant vigour will increase damage due to crown and root rot (Elmer *et al.*, 1996). A nice example of the latter is described in Chapter 4. When growth of asparagus seedlings was compared for fresh and asparagus soil, root dry weight was significantly reduced in the latter when the assay was performed in 4-l pots. In those pots, due to space limitation, roots could not grow vigorously during the whole experimental period, resulting in plant stress. When the assay was performed in long cylindrical 13-l pots allowing vigourous root development during the whole experimental period, the root dry weight in the asparagus soil was not lower than that in fresh soil although many lesions were present. Another illustration of the importance of plant stress for the development of crown and root rot was observed during one of the visits to asparagus nurseries. In one field, two low-lying sites were present where the plants frequently suffered from flooding. All plants sampled from these sites showed many large lesions caused by *F. oxysporum* f.sp. *asparagi*, whereas plants from outside these sites showed far less and smaller lesions.

Management

In the main asparagus-producing area most growers can no longer avoid early decline by planting on virgin soil as this is rarely available. To allow experienced and skillful growers to stay in asparagus, a strategy to manage crown and root rot should become available, thus making replanting more profitable. Measures should start already when asparagus is planted on virgin soil to restrict the build-up of inoculum that would cause early decline in the next crop. Although crop performance on virgin soils is generally satisfactory to the Dutch grower, it might be expected that taking measures for suppression of crown and root rot from the early start on will be repaid by an increase of yield and longevity of the first planting. Possible elements of a management strategy for *F. oxysporum* f.sp. *asparagi* are discussed below.

Durable genetic resistance of the high level found in *A. densiflorus* (Stephens *et al.*, 1989) would provide complete control of crown and root rot. Techniques for transformation of *Asparagus* spp. have become available recently (Delbreil *et al.*, 1993) and further research into the possibilities to transfer the resistance gene(s) of *A. densiflorus* to *A. officinalis* should be stimulated. However, even if transfer of the resistance gene(s) is successful, it is not to be expected that resistant cultivars of a satisfactory agronomic performance will be available within the next decade.

At present, a single measure that provides complete control of crown and root rot is not available. Therefore, a management strategy should combine various measures. As mentioned above, on virgin soil the main inoculum sources of *F. oxysporum* f.sp. *asparagi* are infected crowns and the soil. The latter can be ignored when the field has no proximity to other (former) asparagus fields. Planting of pathogen-free crowns is an important means to delay build-up of inoculum when a field is first planted with asparagus. Possibilities for production of healthy planting stock are mentioned in Chapter 6 and include the use of a procedure to avoid seed infestation and production of planting stock in noninfested fields outside production centres. Chemical disinfestation of seed is possible with a high concentration of benomyl in acetone (Damicone *et al.*, 1981). A bio-assay with asparagus seedlings can be performed to test whether a soil is noninfested. In France, the bio-assay has been commercially available for several years already (Nourisseau *et al.*, 1992). In the Netherlands, it will shortly become available to the farmer through the Plant Inspection Service (H. Koenraadt, personal communication).

At the end of the economic life of the crop, plants generally are destroyed and the crop residues incorporated into soil by rotary tilling. It might be argued that removing the crowns with part of the root system will decrease soil infestation. This was tested in a field experiment where crowns were either removed or destroyed in four replicate blocks. A difference in mean infestation level was not detected when soil collected in the same

year, was tested in a bio-assay with asparagus seedlings (W.J. Blok, unpublished results). This might be explained by the fact that the amount of crop residues removed was too small to cause a significant decrease in infestation level. However, another explanation might be that short-term survival of the pathogen is mainly through the population in soil outside the crop residues. As this population is not affected by removal of plants there will be no effect of this measure on the soil infestation level in the short term. But, as it seems reasonable to assume that long-term survival of the pathogen is mainly through the population in crop residues, it might be envisaged that removal of plants will decrease soil infestation in later years.

When a former asparagus field is replanted, the soil is by far the most important inoculum source for the new crop. For the management of crown and root rot in these fields it will be crucial to reduce the level of soil infestation prior to planting. A nonchemical way of soil disinfestation was sought by testing the combination of plastic mulching and incorporation of easily decomposable organic matter for its efficacy (Chapter 9). This new combination of well-known agricultural measures resulted in fermentative soil conditions and was clearly shown to have the potential to inactivate *F. oxysporum* f.sp. *asparagi* and other soilborne pathogens. Research aimed at further development of the method for application in the production of various crops and at unravelling the mechanism of pathogen inactivation is in progress. For the production of asparagus as a deep-rooting, perennial crop, it is important that the pathogen is inactivated not only in the upper layer, as was found in the present study; but also in deeper layers. Results of recent field experiments (not described in this thesis) indicate that by incorporating grass to a depth of 40 or 80 cm into soil naturally infested with *F. oxysporum* f.sp. *asparagi*, the infestation level is indeed significantly decreased to these depths. Data on the relationship between inoculum density or inoculum potential of *F. oxysporum* f.sp. *asparagi* and damage to the crop are currently lacking. Therefore, long-term experiments are needed to test the effect of this method on annual yields and longevity of the asparagus crop.

Another interesting field for further research regards the introduction in soil of antagonists of *F. oxysporum* f.sp. *asparagi* after biological soil disinfestation. Candidates are nonpathogenic isolates of *F. oxysporum*. As was shown in Chapter 9, these antagonists have potential to reduce severity of crown and root rot. However, when applied to untreated soil in the field, reduction of root rot severity was not obtained. The crucial step where most antagonists fail when applied in the field is the establishment and maintenance of sufficiently high populations in existing ecosystems with a high level of microbial competition (Deacon, 1991). Biological soil disinfestation greatly disturbs the soil ecosystem: when anaerobic conditions prevail most of the aerobic organisms will be killed or kept dormant and populations of anaerobic organisms will increase; upon return to aerobic conditions the soil will gradually be recolonized by aerobic organisms. It is

tempting to study the ways in which antagonists introduced during early stages of the recolonization might be able to establish high population densities. These populations might then protect the plants during the first cropping years, which is the most critical period, and restrict colonization of the soil by the pathogen.

In the section on the epidemiology of crown and root rot it was pointed out that plant vigour determines largely the damage due to crown and root rot. Therefore, it is important to restrict plant stress as much as possible. Sources of plant stress that can be avoided include poor soil structure, insufficient crop fertilization, pests, diseases other than crown and root rot, and weeds. Good crop management ensuring vigourous growth is especially important during the first years after planting when the plants have to build up their crown and root system. In addition, application of rock salt might be useful to further suppress crown and root rot. An evaluation of efficacy and feasibility to incorporate this measure into a management strategy for *F. oxysporum* f.sp. *asparagi* is recommended (Elmer *et al.*, 1996).

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Summary

In the Netherlands, asparagus is mainly produced in the southeastern part of the country. This area has been the production centre for decades and currently virgin soil suitable for asparagus production is scarce. Therefore, most growers are forced to replant in former asparagus land. When former asparagus land is replanted, crop establishment is generally successful but after a few years with normal yields, plant vigour and density start to decline and as a result the economic life of the crop is only five to six years instead of 10 to 11 years for crops on virgin soil. This phenomenon, termed replant disease or replant-bound early decline, is the main agronomic problem in Dutch asparagus production. The causal factor of the disorder is very persistent in soil: even after a 25 year break in asparagus production, crop performance is not as good as on virgin soil.

The thesis deals successively with the etiology of early decline (Chapters 2-5), aspects of the epidemiology of the disease relevant to the development of control means (Chapters 6 and 7), and attempts to manage the disease by biological control (Chapter 8) and by a newly developed method combining incorporation of green plant material and application of plastic mulch (Chapter 9).

Etiology

It was hypothesized that soilborne fungal pathogens and autotoxic compounds present in asparagus roots are the main causal factors in early decline. In addition to direct effect of these factors on the asparagus plant, indirect effects of the autotoxins, by predisposing the plants to infection by pathogens or by decreasing soil fungistasis, were studied.

In Chapter 2, growth inhibition by autotoxins was considered. Asparagus roots were found to decompose very slowly. Biomass of root residues was 4180 and 11060 kg dry weight ha^{-1} in two fields, respectively, one year after the crop was abandoned, and still 420 and 1140 kg dry weight ha^{-1} after 10 years. These old root residues still contained autotoxins as was found in bioassays with asparagus and garden cress. The potential of autotoxins to inhibit asparagus root growth in field soil was demonstrated by growing asparagus seedlings in natural soil supplied with gamma-irradiated roots. However, a significant degree of inhibition was demonstrated only at an amount that was 10 times higher than the amount of root residues present when a field is replanted, which is 2 g dry weight l^{-1} soil. It was concluded that the concentration of autotoxins in soil is too low to consider growth inhibition by these compounds as an important factor in the etiology of replant-bound early decline.

In Chapter 3, results are given of a survey made to identify the most important soilborne fungal pathogens in Dutch asparagus crops grown on virgin or asparagus soils.

Fungi were isolated from roots and stem bases of plants showing symptoms of foot and root rot or showing growth decline without specific disease symptoms. After identification, isolates of each species were tested for pathogenicity to asparagus on aseptically grown seedlings on Knop's agar. Species that caused symptoms, and that were isolated from more than 5% of the plants, were further tested for their pathogenicity in pot experiments. In the latter experiments, symptoms were caused by *F. oxysporum* f.sp. *asparagi*, *F. culmorum*, *Phoma terrestris*, and *Phialophora malorum*. The first species was identified as by far the most important soilborne pathogen of asparagus in the Netherlands based on its predominance on plants with decline symptoms and its high virulence. A remarkable result of the survey was the absence of *F. proliferatum* (syn. *F. moniliforme)* since this species, together with *F. oxysporum* f.sp. *asparagi*, is considered to be a major cause of early decline in most asparagus-producing areas outside the Netherlands.

Chapter 4 describes the characterization of replant-bound early decline in pot experiments and a further study of its etiology. When asparagus seedlings were grown in virgin or asparagus soil for 12 to 15 weeks, root systems from plants grown in the asparagus soil had many brown lesions, most of the secondary roots had rotted, and root dry weights were significantly lower than those of plants grown in virgin soil. From the lesions *F. oxysporum* f.sp. *asparagi* was isolated almost exclusively. In experiments performed under greenhouse and field conditions similar symptoms were obtained which indicated that the results obtained under greenhouse conditions are representative of phenomena in commercial plantings. In four different asparagus soils, disease severity was similar for plants grown in samples from depths of 0-30, 30-60, or 60-90 cm, indicating that the whole rootable layer is infested to about the same extent. Plants grown in soil from a field where asparagus production was terminated 11 years previously were severely diseased which confirms earlier reports on the persistence of the causal factor of replant-bound early decline. When asparagus soil was diluted with virgin soil to give mixtures with 100%, 80%, 50%, and 20% asparagus soil, disease severity did not decrease with increasing dilution of the asparagus soil but was significantly higher in all mixtures than in the virgin soil. These results imply that the disease is caused by a pathogen colonizing the soil rather than inhibition by autotoxins emanated from asparagus root residues of the preceding crop. This conclusion was further substantiated by the results of greenhouse and outdoor experiments with heat and fungicide treatments of soil. Replant-bound early decline was nullified by heat treatments for 30 min at 55 or 60°C but not 45 or 50°C. Eliminating the causal agent by heat rules out autotoxins as an important cause of early decline as these were shown to be extremely heat-stable. *F. oxysporum* f.sp. *asparagi* is eliminated by a 30-min treatment at 55-60°C but not 50°C. Prochloraz, known for its toxicity to *F. oxysporum*, also nullified early decline. Moreover, in these experiments, disease severity was related to the density of *F. oxysporum* f.sp. *asparagi* in soil. It was concluded that *F. oxysporum* f.sp. *asparagi* is the main cause of replant-bound early decline of asparagus

crops in the Netherlands.

The possibility that autotoxins might predispose the plants to infection and increase severity of crown and root rot caused by *F. oxysporum* f.sp. *asparagi* was considered in Chapter 5. Sterilized asparagus root material, either chopped or milled, added to field soil in amounts up to 20 g dry weight kg^{-1}, did not influence severity of crown and root rot. When asparagus soil was pasteurized for 30 min at 65°C, at which pathogens were killed but autotoxins were left intact, and then inoculated with *F. oxysporum* f.sp. *asparagi*, disease severity was not higher than in similarly treated virgin soil. Thus, evidence for an increased crown and root rot severity due to autotoxins was not obtained.

Effects of autotoxins on the soil microflora that might affect disease severity, were also studied (Chapter 5). In *in vitro* experiments, 31 out of 112 fungal isolates were inhibited by an extract of asparagus roots but not by extracts of chicory or strawberry roots. Sensitive species included well-known antagonists of pathogens like *Gliocladium* spp. and *Trichoderma harzianum*, but growth of *F. oxysporum* was not inhibited. Colonization by *Fusarium* spp. of soil artificially infested with the pathogen was greatly enhanced by incorporation of pathogen-free root material of asparagus, Brussels sprouts and chicory, but not by that of strawberry or perennial rye-grass. As the fraction of pathogenic isolates among the *Fusarium* population was small and had much decreased in the period after incorporation of the root materials, it was concluded that the competitive saprophytic ability of *F. oxysporum* f.sp. *asparagi* is far less than that of the nonpathogenic *Fusarium* isolates. Soil fungistasis to the pathogen was not or only slightly reduced in field soil amended with root material of the various crops.

In conclusion, *F. oxysporum* f.sp. *asparagi* was identified as the main cause of replant-bound early decline. Evidence that autotoxic compounds, acting either directly or indirectly, are involved in the etiology of the disease was not obtained.

Epidemiology

In a survey to identify the inoculum sources of *F. oxysporum* f.sp. *asparagi* in the Netherlands, the pathogen was found on seeds, in soils without an asparagus history and on crowns used as planting material (Chapter 6). Seed infestation occurs mainly during the seed extraction process when infested soil adhering to fallen berries is mixed through the whole seed lot.

A high correlation was found between proximity of virgin fields to asparagus fields and the incidence of infestation with the pathogen. Infestation was detected in 69% of virgin soils located in an asparagus production centre but in only 6% of virgin soils at a distance of more than 1 km from asparagus fields and outside a production centre.

Forty-nine lots of planting stock (one-year-old crowns) from 23 nurseries were

collected and rated for symptoms of crown and root rot. Only two lots were completely free of symptoms and most of the lots had more than 75% of symptomatic plants although the disease severity was generally very low.

The infestation of asparagus root residues by *F. oxysporum* f.sp. *asparagi* was studied to evaluate its role in long-term survival of the pathogen (Chapter 6). Asparagus root residues were retrieved from eight former asparagus fields and from three fields with a standing asparagus crop. The density of the total *Fusarium* population in the residues and the percentage of isolates belonging to *F. oxysporum* f.sp. *asparagi* was determined. Population density of *Fusarium* spp. declined from 2 x 10^6 to 1 x 10^5 colony-forming units g^{-1} air-dry root tissue during the first 10 years and were still > 10^4 cfu g^{-1} 20 to 25 years after asparagus production ended. The *Fusarium* population in the residues was dominated by *F. oxysporum* (75% of the isolates) and 74% of the isolates of this species belonged to f.sp. *asparagi*.

Chapter 7 deals with host specificity and vegetative compatibility of isolates of *F. oxysporum* f.sp. *asparagi*. The fungus was found to have a narrow host range as a pathogen, but a broad host range as a parasite. Severe root rot was caused only on asparagus, mild symptoms were occasionally found on lupin and pea. However, the pathogen was detected in externally disinfested roots of 12 out of 14 species tested. Asparagus was not susceptible to isolates of *F. oxysporum* ff.sp. *pisi, lupini, cepae, lilii,* and *gladioli* and *F. sacchari* var. *elongatum*. When naturally infested asparagus soil was cropped twice for 11-13 weeks with 11 plant species including asparagus and several symptomless hosts, and finally with asparagus as a biotest plant, root rot severity was increased only in the soil cropped with asparagus.

It was concluded that the population of *F. oxysporum* f.sp. *asparagi* in the Netherlands is very diverse genetically. Twenty-four isolates of the pathogen were assigned to 18 different vegetative compatibility groups.

Management

In Chapter 8 results are presented of a study aimed at exploring the potential of biocontrol by inoculation with nonpathogenic isolates of *F. oxysporum*. Thirteen isolates were found to reduce severity of crown and root rot by more than 50% when they were allowed to colonize sterilized field soil before inoculation with the pathogen. In a greenhouse test with naturally infested field soil, six nonpathogenic isolates reduced disease severity by 23 to 49%. One of these isolates was applied to field plots infested with the pathogen and to seedlings planted in these plots. Root rot was not reduced in asparagus plants grown for one year in these plots.

Pathogenic and nonpathogenic isolates extensively colonized the root surface and isolates of both types infected the roots of asparagus seedlings grown in sterilized soil, with significant differences among the nonpathogenic isolates. Inoculation of sterilized field soil with nonpathogenic isolates reduced germination of chlamydospores of the pathogen in the rhizosphere of asparagus seedlings by 43% to 65% depending on the isolate used. It was concluded that nonpathogenic isolates of *F. oxysporum* have the potential to suppress crown and root rot in naturally infested field soil. To obtain prolonged biological control in the field, a delivery system has to be developed that enables the introduced antagonists to establish and maintain sufficiently high populations.

A new method for reducing soil infestation with soilborne pathogens by induction of fermentative soil conditions was evaluated for its efficacy (Chapter 9). Loamy sand infested with chlamydospores of *Fusarium oxysporum* f.sp. *asparagi* was amended with five different plant materials or left unamended, and incubated at 11 or 24°C in sealed glass bottles. Oxygen consumption rates were higher at 24 than at 11°C and differed according to plant material treatment. After 7 weeks, the fungus was not detected any more from alfalfa- and cabbage-amended soil, irrespective of the incubation temperature. In 1994 and 1995, the method was tested in the field. In plots amended with fresh broccoli or grass and covered with plastic mulch (Hytileen, a 3-layered polyethylene sheeting of 0.135 mm thickness), fermentative soil conditions developed rapidly as was indicated by rapid depletion of oxygen and a decrease of the redox potential (Eh) to values as low as -200 mV. After 15 weeks, survival of *F. oxysporum* f.sp.*asparagi*, *Rhizoctonia solani*, and *Verticillium dahliae* in inoculum samples buried at 15 cm depth was strongly reduced in amended mulched plots. Inactivation of *Globodera pallida* was not established when it was based on assessment of the percentage living eggs and larvae under a dissecting microscope, but was clearly demonstrated when it was based on hatching tests. In these tests, most of the eggs and larvae were found to be dead. The results clearly show the potential of this nonchemical approach to control a range of soilborne pathogens.

Finally, in the general discussion (Chapter 10), the results of the study on different aspects of early decline are summarized and an attempt is made to integrate them into a comprehensive account of the etiology and epidemiology of the disease in Dutch asparagus crops. In addition, the prospects for the development of an effective management strategy are discussed.

Samenvatting

In Nederland wordt asperge voornamelijk geteeld in het noorden van de provincie Limburg en het oosten van de provincie Brabant. De teelt is al tientallen jaren in dit gebied geconcentreerd en nog niet eerder met asperge beteelde grond die geschikt is voor de teelt van asperge is er schaars geworden. Daarom zijn veel telers in dit gebied genoodzaakt om nieuwe percelen aan te leggen op grond waar al eerder asperge heeft gestaan. Herinplant levert echter altijd minder goede resultaten op dan teelt op verse grond. De eerste jaren na het planten verlopen meestal zonder problemen maar al na enkele jaren met normale opbrengsten neemt de groeikracht van het gewas af en gaan planten dood. Ten gevolge hiervan is de economische levensduur van een gewas op aspergegrond slechts vijf tot zes jaar, terwijl die van een gewas op verse grond 10 tot 11 jaar bedraagt. Dit verschijnsel staat bekend als herinplantziekte. Deze term kan verwarring geven omdat de problemen niet direct na het planten optreden, maar pas in een later stadium. De engelse term 'early decline', versnelde veroudering, beschrijft het verschijnsel beter. De term herinplantziekte is echter zodanig ingeburgerd dat deze hier toch zal worden aangehouden. Herinplantziekte is momenteel het belangrijkste teeltkundige probleem in asperge. De veroorzakende factor is bijzonder persistent: zelfs na 25 jaar blijven de opbrengsten nog achter bij die van een teelt op verse grond. In dit proefschrift wordt achtereenvolgens aandacht gegeven aan de oorzaak van de herinplantziekte (Hoofdstuk 2-5), aan aspecten van de epidemiologie van de ziekte die relevant zijn voor de ontwikkeling van bestrijdingsmethoden (Hoofdstuk 6 en 7) en aan de bestrijding van de ziekte langs biologische weg, zowel door beënting van grond en plantgoed met microbiële antagonisten (Hoofdstuk 8) als door toepassing van een in dit onderzoek ontwikkelde methode van biologische grondontsmetting (Hoofdstuk 9).

Oorzaak

Als hypothese werd gesteld dat herinplantziekte veroorzaakt kan worden door pathogene bodemschimmels en autotoxische stoffen uit aspergewortels. Naast directe effecten van beide factoren op de aspergeplant werd ook onderzocht of autotoxinen de plant indirect kunnen beïnvloeden door een verhoging van de aantasting door pathogene bodemschimmels.

Hoofdstuk 2 handelt over groeiremming van asperge door autotoxinen. Aspergewortels blijken zeer langzaam te worden afgebroken in de grond. De hoeveelheid wortelresten in de grond van twee percelen bedroeg één jaar na het beëindigen van de teelt respectievelijk 4180 en 11060 kg drooggewicht ha^{-1}, na 10 jaar was dit 420 en 1140 kg drooggewicht ha^{-1}. Uit biotoetsen met asperge- en tuinkerszaden bleek dat deze oude, grotendeels

verteerde wortelresten nog steeds autotoxinen bevatten. Aspergewortels waarin ziekteverwekkers en andere organismen waren gedood middels gamma-bestraling, veroorzaakten na toediening aan de grond een significante remming van de wortelgroei van aspergeplanten. De remming trad echter alleen op bij een hoeveelheid wortelresten die tienmaal hoger is dan de hoeveelheid die in de grond aanwezig is bij herinplant. Er werd geconcludeerd dat de concentratie van de autotoxinen in de grond te laag is om groeiremming door deze stoffen als een belangrijke oorzaak van herinplantziekte te beschouwen.

In Hoofdstuk 3 worden de resultaten beschreven van een inventarisatie die tot doel had de belangrijkste pathogene voet- en wortelschimmels in aspergegewassen op verse en herinplantgrond te identificeren. Schimmels werden geïsoleerd uit wortels en stengelbases van planten met symptomen van voet- en wortelrot en van planten met een zwakke groeikracht zonder specifieke ziektesymptomen. Na identificatie werden één of meer isolaten van iedere soort getoetst op pathogeniteit voor asperge op aseptisch gekweekte plantjes op Knop's agar. Soorten die symptomen veroorzaakten en die bovendien uit meer dan 5% van de planten waren geïsoleerd, werden verder getoetst op pathogeniteit in potproeven. In deze proeven werden symptomen veroorzaakt door *F. oxysporum* f.sp. *asparagi, F. culmorum*, *Phoma terrestris* en *Phialophora malorum*. Op basis van de hoge isolatiefrequentie en de sterke virulentie werd geconcludeerd dat *F. oxysporum* f.sp. *asparagi* verreweg de belangrijkste pathogene bodemschimmel is in de Nederlandse aspergeteelt. Opvallend was dat *F. proliferatum* (syn. *F. moniliforme)* niet werd aangetroffen. Deze schimmel is naast *F. oxysporum* f.sp. *asparagi*, in andere aspergeteeltgebieden een belangrijke veroorzaker van herinplantziekte.

In Hoofdstuk 4 wordt nader ingegaan op de oorzaak en op een aantal andere aspecten van de herinplantziekte die werden onderzocht in potproeven. Kiemplanten die gedurende 12 tot 15 weken werden gekweekt in aspergegrond hadden veel bruine lesies op de hoofdwortels, veel zijwortels waren afgerot en het wortelgewicht was significant lager dan dat van planten gekweekt in verse grond. Uit de lesies werd vrijwel uitsluitend *F. oxysporum* f.sp. *asparagi* geïsoleerd. In kasproeven werden dezelfde symptomen waargenomen als in veldproeven, waaruit werd geconcludeerd dat de resultaten die werden verkregen onder kasomstandigheden representatief zijn voor de verschijnselen in praktijkpercelen. Voor grond afkomstig van vier voormalige aspergepercelen werd gevonden dat de ziekte-index van planten opgekweekt in grond uit de lagen 0-30, 30-60 of 60-90 cm vrijwel gelijk was. Hieruit blijkt dat de grond homogeen besmet is in de gehele bewortelde laag. Wortels van planten opgekweekt in grond van een perceel waar 11 jaar geleden de teelt werd beëindigd, waren flink aangetast. Dit bevestigt eerdere meldingen over de grote persistentie van de veroorzakende factor. Wanneer aspergegrond werd verdund met verse grond tot mengsels met 100%, 80%, 50% en 20% aspergegrond nam de ziekte-index niet af met toenemende verdunning. Hieruit wordt afgeleid dat de

herinplantziekte in eerste instantie wordt veroorzaakt door een pathogeen dat de bewortelde grond kan koloniseren en niet door autotoxinen die vrijkomen uit wortelresten van het vorige aspergegewas. Deze conclusie wordt verder onderbouwd door de resultaten van potproeven onder kas- en veldomstandigheden met selectieve warmte- en fungicidebehandelingen van de grond. Herinplantziekte werd voorkomen door de grond gedurende 30 minuten te verhitten bij 55 of 60°C, maar niet door verhitting bij 45 of 50°C. Dit sluit autotoxinen als oorzaak uit omdat deze zeer hitte-stabiel zijn. *F. oxysporum* f.sp. *asparagi* wordt gedood bij 55-60°C, maar niet bij 50°C. Prochloraz, dat bekend is om zijn toxische werking tegen *F. oxysporum* f.sp. *asparagi*, voorkwam herinplantziekte eveneens. In deze experimenten was de aantastingsgraad gerelateerd aan de dichtheid van *F. oxysporum* f.sp. *asparagi* in de grond. Er werd geconcludeerd dat het optreden van *F. oxysporum* f.sp. *asparagi* de belangrijkste oorzaak is van herinplantziekte van asperge in Nederland.

In Hoofdstuk 5 wordt nagegaan of autotoxinen kunnen leiden tot een verhoogde aantasting van voet- en wortelrot, veroorzaakt door *F. oxysporum* f.sp. *asparagi*. Als aspergewortelmateriaal, in stukjes gesneden of vermalen, in hoeveelheden tot 20 g drooggewicht kg^{-1} grond, werd gemengd door grond die kunstmatige was besmet met het pathogeen, werd geen verhoogde aantasting gevonden. Ook als aspergegrond gedurende 30 minuten werd verhit bij 65°C, waarbij pathogenen werden gedood maar autotoxinen intact bleven, en vervolgens werd besmet met het pathogeen, was de aantasting niet hoger dan die in verse grond die op dezelfde wijze was behandeld. Er werd in dit onderzoek dus geen enkele aanwijzing verkregen voor een verhoogde aantasting van voet- en wortelrot ten gevolge van autotoxinen.

In Hoofdstuk 5 worden ook de resultaten gegeven van onderzoek naar effecten van autotoxinen op de bodemmicroflora die zouden kunnen leiden tot een verhoogde aantasting van voet- en wortelrot. *In vitro* werden 31 van de 112 getoetste schimmelisolaten geremd door een waterig extract van aspergewortels, maar niet door extracten van aardbei- of witlofwortels. Gevoelige soorten waren onder andere *Gliocladium* spp. en *Trichoderma harzianum*, maar niet *F. oxysporum*. Kolonisatie door *Fusarium* soorten van grond besmet met *F. oxysporum* f.sp. *asparagi* werd sterk gestimuleerd door het inwerken van wortelmateriaal van asperge, spruitkool en witlof maar niet door dat van aardbei of Engels raaigras. Aan het eind van deze experimenten was het aandeel van het pathogeen in de *Fusarium*populatie slechts klein en sterk afgenomen vergeleken met de beginsituatie. Hieruit werd geconcludeerd dat het competitief saprofytisch vermogen van het pathogeen geringer is dan dat van de niet-pathogene *Fusarium*isolaten. De bodemfungistase ten aanzien van *F. oxysporum* f.sp. *asparagi* bleek niet of nauwelijks gereduceerd te zijn in grond met wortelmateriaal van verschillende gewassen.

Samengevat luidt de conclusie dat *F. oxysporum* f.sp. *asparagi* de belangrijkste oorzaak is

van herinplantziekte van asperge in Nederland. Aanwijzingen dat autotoxische stoffen eveneens een rol spelen, hetzij direct door groeiremming van de aspergeplant, hetzij indirect door verhoging van de aantasting van voet- en wortelrot, werden niet gevonden.

Epidemiologie

In een inventarisatie van de belangrijkste inoculumbronnen van *F. oxysporum* f.sp. *asparagi* in Nederland, werd het pathogeen aangetroffen op het zaad, in grond zonder aspergeteelthistorie en op éénjarige planten gebruikt als plantmateriaal (Hoofdstuk 6). Zaadbesmetting treedt vooral op tijdens het zaadextractieproces. In de praktijk worden op de grond gevallen bessen met aanhangende, besmette grond gemengd met geplukte bessen, waardoor de hele partij besmet raakt.

Er werd een hoge correlatie gevonden tussen de kans op besmetting van een perceel verse grond en de afstand tot aspergepercelen. Besmetting werd aangetroffen in 69% van de percelen verse grond gelegen in het aspergeteeltgebied maar slechts in 5% van de percelen gelegen buiten het aspergeteeltgebied en met een afstand van meer dan 1 km tot het dichtstbijzijnde aspergeperceel.

Plantgoedbesmetting kwam veel voor. Negenenveertig partijen éénjarige planten verzameld by 23 plantenkwekers, werden beoordeeld op voet- en wortelrot veroorzaakt door *F. oxysporum* f.sp. *asparagi*. In slechts twee partijen werden geen planten met symptomen aangetroffen, in de meeste partijen bleken meer dan 75% van de planten symptomen te vertonen, hoewel de mate van aantasting gemiddeld erg laag was.

Om een indruk van de overleving op de lange termijn te krijgen werd de besmetting van wortelresten op verschillende tijdstippen na beëindiging van de aspergeteelt bepaald. (Hoofdstuk 6). Wortelresten werden opgegraven op acht voormalige aspergepercelen en op drie percelen met een staand aspergegewas. De dichtheid van de *Fusarium*populatie in deze wortelresten werd bepaald en tevens het percentage isolaten behorend tot *F. oxysporum* f.sp. *asparagi*. De populatiedichtheid van *Fusarium* spp. nam gedurende de eerste 10 jaar af van 2×10^6 tot 1×10^5 kolonievormende eenheden (kve) g^{-1} luchtdroog wortelmateriaal en bedroeg 20 tot 25 jaar na het beëindigen van de teelt nog steeds $> 10^4$ kve g^{-1}. De *Fusarium*populatie in de wortelresten werd gedomineerd door *F. oxysporum* (75% van de isolaten) terwijl 74% van de isolaten van deze soort behoorden tot f.sp. *asparagi*.

Het onderzoek naar de waardplantspecificiteit en vegetatieve compatibiliteit in *F. oxysporum* f.sp. *asparagi* is beschreven in Hoofdstuk 7. De schimmel bleek een nauwe waardplantenreeks te hebben als pathogeen, maar een brede waardplantenreeks als parasiet. Zware aantasting door wortelrot werd alleen in asperge waargenomen, erwt en lupine werden af en toe licht aangetast. Het pathogeen werd echter geïsoleerd uit

uitwendig ontsmette wortels van 12 van de 14 getoetste soorten. Asperge bleek niet vatbaar te zijn voor isolaten van *F. oxysporum* ff.sp. *pisi, lupini, cepae, lilii* en *gladioli* en *F, sacchari* var. *elongatum*. Wanneer natuurlijk besmette grond tweemaal gedurende 11-13 weken werd beteeld met 11 plantesoorten, waaronder asperge, en vervolgens met asperge als toetsplant, bleek de aantasting door wortelrot alleen te zijn toegenomen in de grond waarop asperge had gestaan.

Er werd geconcludeerd dat de populatie van *F. oxysporum* f.sp. *asparagi* in Nederland genetisch zeer divers is. Vierentwintig isolaten van het pathogeen behoorden tot 18 verschillende vegetatieve-compatibiliteitsgroepen.

Bestrijding

In Hoofdstuk 8 worden de resultaten gegeven van een onderzoek naar de mogelijkheden voor onderdrukking van voet- en wortelrot veroorzaakt door *F. oxysporum* f.sp. *asparagi* met niet-pathogene isolaten van *F. oxysporum*. Dertien van de 50 getoetste isolaten bleken de aantasting met meer dan 50% te reduceren als gesteriliseerde grond vóór de besmetting met het pathogeen werd gekoloniseerd door deze isolaten. In een kasproef met natuurlijk besmette grond reduceerden de zes getoetste isolaten de aantasting met 23 tot 49%. Eén van deze isolaten werd toegediend aan plantgoed en aan grond van veldjes besmet met het pathogeen. Na één jaar bleek de aantasting door wortelrot niet te zijn gereduceerd.

Zowel pathogene als niet-pathogene isolaten koloniseerden uitgebreid het worteloppervlak van aspergekiemplanten in gesteriliseerde grond. Daarnaast infecteerden isolaten van beide typen de wortels van de kiemplanten, met significante verschillen tussen niet-pathogene isolaten.

Inoculatie van gesteriliseerde grond met niet-pathogene isolaten reduceerde de kieming van chlamydosporen van het pathogeen in de rhizosfeer van aspergekiemplanten met 43 tot 65%, afhankelijk van het gebruikte isolaat. Er werd geconcludeerd dat niet-pathogene isolaten van *F. oxysporum* de potentie bezitten om voet- en wortelrot te onderdrukken in natuurlijk besmette veldgrond. Om ook in het veld biologische bestrijding gedurende langere tijd te verkrijgen, dient er een toedieningsmethode te worden ontwikkeld die het de geïntroduceerde isolaten mogelijk maakt voldoende hoge populaties op te bouwen en in stand te houden.

In Hoofdstuk 9 wordt een nieuwe methode voor het verlagen van de bodembesmetting met pathogenen geïntroduceerd. De werking berust op het induceren van anaerobe bodemomstandigheden waaronder gisting optreedt. Lemige zandgrond besmet met chlamydosporen van *F. oxysporum* f.sp. *asparagi* werd direct of na menging met materiaal van vijf verschillende gewassen geïncubeerd bij 11 of 24°C in afgesloten glazen flesjes. De zuurstofconsumptie was bij 24°C hoger dan bij 11°C en was afhankelijk van de aard

van het toegediende plantemateriaal. Na 7 weken werd de schimmel niet meer teruggevonden in de behandelingen met lucerne en kool, onafhankelijk van de incubatietemperatuur. In 1994 en 1995 werd de methode getoetst in het veld. In veldjes waar vers gras of broccoli was ondergewerkt en de grond met plastic (Hytileen, een 3-lagige plastic van 0.135 mm dikte) was bedekt, ontwikkelden zich snel bodemomstandigheden waaronder gisting optreedt, wat bleek uit een sterke daling van het zuurstofgehalte en een daling van de redox potentiaal (Eh) tot waarden van -200 mV. Na 15 weken was de overleving van *F. oxysporum* f.sp. *asparagi*, *Rhizoctonia solani*, en *Verticillium dahliae* in monsters op 15 cm diepte sterk gereduceerd in veldjes met gras of kool en afgedekt met plastic. Bestrijding van *Globodera pallida* werd niet aangetoond wanneer deze werd gebaseerd op schatting van het percentage levende eieren en larven onder de prepareermicroscoop, maar wel als dit gebeurde met loktoetsen. Daarbij bleek een groot deel van de eieren en larven gedood te zijn. De resultaten van deze proeven tonen duidelijk aan dat deze niet-chemische ontsmettingsmethode grote mogelijkheden biedt voor de bestrijding van bodempathogenen.

Tenslotte wordt in de algemene discussie (Hoofdstuk 10) een samenvattend beeld gegeven van de oorzaak en de epidemiologie van herinplantziekte in Nederland. Tevens worden de vooruitzichten voor de ontwikkeling van een effectieve strategie ter beheersing van de ziekte besproken.

List of publications

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* The indicated publications are included in this thesis

Curriculum vitae

Willem Jacobus Blok werd geboren op 22 januari 1963 te Middelharnis. Na het behalen van het VWO-diploma aan de reformatorische scholengemeenschap 'Guido de Brès' te Rotterdam in 1981, begon hij met de studie Planteziektenkunde aan de Landbouwuniversiteit (toen nog Landbouwhogeschool geheten) te Wageningen. In september 1984 werd het kandidaatsdiploma behaald. De doctoraalstudie omvatte een verzwaard hoofdvak Fytopathologie met twee onderzoeksonderwerpen, een hoofdvak Plantenveredeling en een bijvak Nematologie. Stages werden verricht op het Proefstation voor Tuinbouw onder Glas te Naaldwijk en op het Long Ashton Research Station te Long Ashton, Engeland. Het doctoraaldiploma werd in januari 1988 met lof behaald. Van mei 1988 tot september 1995 was hij als toegevoegd onderzoeker in dienst van de Landbouwuniversiteit en verrichtte hij op de vakgroep Fytopathologie onderzoek naar de oorzaak, epidemiologie en bestrijding van herinplantziekte van asperge. Het grootste deel van dit onderzoek is beschreven in dit proefschrift. Momenteel is hij, in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), werkzaam als post-doc onderzoeker op de vakgroep Fytopathologie waar verder onderzoek wordt verricht naar de methode van biologische grondontsmetting beschreven in hoofdstuk 9 van dit proefschrift.