Biological control of *Verticillium dahliae* by *Talaromyces flavus*

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Biological control of *Verticillium dahliae* by *Talaromyces flavus*

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Stellingen

1. Het is geen regel, dat antagonisten die gelijktijdig aan planten toegediend worden elkaar in hun werking remmen; het toedienen van een combinatie van antagonisten biedt dus perspectief de biologische bestrijding te verbeteren.

2. Biologische gewasbeschermingsmiddelen kunnen zeer persistent zijn en de werking er van kan in de tijd zelfs toenemen. De persistentie van chemische gewasbeschermingsmiddelen zal juist steeds minder worden door de eisen vanuit het oogpunt van de bescherming van het milieu. De markt voor persistente gewasbeschermingsmiddelen zal dus vooral op biologische middelen gericht worden.

3. Wachten kan voor sommige antagonisten een betere strategie zijn dan zich in aantal te vermeerderen of te groeien
(J.M. Hirst, 1965, pp. 69-81 in K.F. Baker & W.C. Snyder (eds.) Ecology of soil-borne plant pathogens, University of California Press, Berkeley).

4. Gewasbescherming door geïnduceerde resistentie kan worden toegepast met vrij beschikbare organismen. Dit versmalt de commerciële perspectieven van deze methode.

5. Het langdurig gebruik van zeer effectieve gewasbeschermingsmiddelen heeft in de agrarische sector geleid tot een houding en een organisatiestructuur die een remmende faktor is bij een grootschalige toepassing van geïntegreerde of biologische bestrijding.

6. Een ziekte moet niet steeds op dezelfde wijze bestreden worden om te voorkomen dat de ziektewekker de werking van de methode 'leert'.

7. De opvatting dat een biologisch gewasbeschermingsmiddel altijd veilig is, houdt risico's in voor de volksgezondheid en het milieu.

8. Het is onjuist aan te nemen dat het algemene belang van de agrarische sector parallel loopt met die van de individuele ondernemers en werknemers in deze sector.

9. *Phytophthora infestans* en varkenspest kunnen ook bij de mens ziekte veroorzaken.

10. Vermindering betekent in veel gevallen vooruitgang.

11. Het bestaan van wonderen en de voortgang van de wetenschap zijn als communicerende vaten te beschouwen.

12. De wetenschap heeft leugens nodig om de werkelijkheid te doorgronden.

13. Voordat Nederland het begrip '24-uurs-economie' kende, waren in België de winkels al op zondag open.

14. Een universiteit die op vrijdagavond de poorten sluit, is niet voorbereid op de toekomst.

15. Het is te eenvoudig politieke besluiten te nemen die vooral de generaties treffen die op dit moment nog geen kiesrecht hebben.

16. Veel Nederlanders menen dermate Europees te denken dat een Nederlandse opvatting veelal als de beste oplossing voor Europese vraagstukken wordt beschouwd.

17. Een man zonder buik heeft geen trek in het leven
(Salman Rushdie, 1995, De laatste zucht van de Moor, Uitgeverij Contact, Amsterdam/Antwerpen).

Stellingen behorend bij het proefschrift van Mario Nagtzaam 'Biocontrol of *Verticillium dahliae* by using *Talaromyces flavus*', te verdedigen op 14 oktober 1998 te Wageningen.

Venster op de utopie

Zij staat op de horizon, zegt Fernando Birri. Ik kom twee stappen dichterbij, zij gaat twee stappen achteruit. Ik zet tien stappen en de horizon wijkt tien stappen.
Hoe ver ik ook loop, ik zal haar nooit bereiken. Waartoe dient de utopie? Daartoe dient zij: om te lopen.

Eduardo Galeano in Dolende woorden

Voor mijn vader

Abstract

Nagtzaam, M.P.M., 1998. Biological control of *Verticillium dahliae* by *Talaromyces flavus*. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Verticillium dahliae causes vascular wilt in a wide range of host plants. Control of Verticillium wilt is by soil disinfestation and to a lesser extent by crop rotation or, for a few host plants, by growing resistant varieties. For environmental reasons, the development of alternatives to chemical soil disinfestation is being sought. Biocontrol by microbial agents is one of the options. The potential of *Talaromyces flavus* as a biocontrol agent in management of the disease is the subject of the thesis.

The effect of the pathogen on plants was studied to adequately interpret results of biocontrol experiments. Under controlled conditions, a linear relationship was demonstrated between pathogen inoculum density in soil and its population density on roots or in sap extracted from stems. In field experiments, incidence of stem infection by *V. dahliae* and yield of tubers showed a clear dose-response relation to the amount of pathogen inoculum applied to soil. Incidence of stem infection and density of stem colonisation by the pathogen gradually increased during the season. For studying the effects of antagonists on dynamics of *V. dahliae* a quantitative bioassay is recommended using eggplant as a test plant.

Recovery of viable propagules from old samples of seeds coated with ascospores of *T. flavus* in a clay formulation indicated that products containing the antagonist may have a long shelf life. On the pelleted seeds, a significant proportion of the ascospores had survived a storage period of 17 years.

In pot experiments with field soils, the fungus moved from seed tubers of potato and seeds of eggplant coated with ascospores to the developing roots, including the root tips, the site where *V. dahliae* infects the root, albeit at a low density. The population of *T. flavus* decreased log-linearly with distance from the seed. Results suggest that passive movement along the growing root is one of the main factors involved in colonisation by the fungus.

The potential of *T. flavus* to control *V. dahliae* was evaluated in several experiments. Its application to senescent stems collected from a field with a diseased potato crop reduced viability of microsclerotia. Incorporating an alginate wheat-bran preparation of *T. flavus* in soil (0.5% w/w) was followed by a decrease of >90% of the population of *V. dahliae* in soil. The microbial antagonist also reduced colonisation by *V. dahliae* of roots and infection of eggplants. Although to a lesser extent than with the antagonist, alginate wheat-bran without *T. flavus* also reduced pathogen colonisation. *T. flavus* was tested for efficacy to control wilt in two independent field experiments with potato. After application of a *T. flavus* preparation, stems were less densely colonised by *V. dahliae* in the treated plots than in the control plots in the first growing season of experiment 1 and

in the second growing season of experiment 2. A reduction in plant colonisation by the pathogen was not followed by higher yields of potato tubers. Although population density of *T. flavus* in soil had not increased during the growing season, it remained at a higher level in treated plots than in non-treated plots, also in the second year after introduction of the antagonist.

Treatment with combinations of *T. flavus* with *Bacillus subtilis*, *Fusarium oxysporum* or *Gliocadium roseum* gave similar control of root colonisation and stem infection by *V. dahliae* as application of the single antagonists. The results suggest that *T. flavus* is compatible with these antagonists.

The significance of propagule distribution in soil for the performance of mycoparasites was demonstrated using a simple model. Progress of infection of the host fungus was slower with a random distribution than a uniform distribution of mycoparasite propagules. With a random distribution, the average distance between propagules was 40-50% smaller than with a uniform distribution.

The results of the experiments presented in this thesis and in the literature, demonstrate that *T. flavus* has the potential for biocontrol of *V. dahliae*. However, thus far the results with this antagonist tend to be inconsistent. Control is often partial or even fails completely. This means that application of *T. flavus* as a single control method has little commercial potential. For the time being, biocontrol is considered as a method to be applied in combination with cultural methods, biological disinfestation, the use of tolerant cultivars or the selective application of chemical control. The next step in further research should focus on the nature of the inconsistency of biocontrol using this fungus.

Contents

Chapter 1

General introduction

Verticillium dahliae is a soil-borne pathogen that causes vascular wilt in a wide range of host plants. Among them are agricultural crops, vegetables, flower crops, annual and perennial ornamentals, and avenue trees. The pathogen occurs in the subtropical and temperate zones and its distribution is world-wide.

Control of Verticillium wilt is achieved by soil disinfestation and to a lesser extent by crop rotation and growing resistant varieties. For environmental reasons, the development of alternatives to chemical soil disinfestation is being sought. Biological control is one of the options. The potential of *Talaromyces flavus* as a biocontrol agent in management of Verticillium wilt is the subject of this thesis.

The first section of this chapter deals with the occurrence and importance of Verticillium wilt in The Netherlands. The second section presents the life cycle of *V. dahliae* and includes a description of the early dying syndrome. The third section provides details of the epidemiology of the disease. The fourth section is on control of the pathogen with emphasis on biocontrol as a potential alternative for chemical soil disinfestation.

Occurrence and importance of Verticillium wilt in The Netherlands

Verticillium wilt is a major problem in three important sectors of agriculture, *viz.* in production of potato and strawberry as field crops, in raising avenue trees in nurseries particularly lime tree, elm and maple, and in production of rose, chrysanthemum, eggplant and tomato in greenhouses. Moreover, the disease incidentally causes severe losses in various other host plants, e.g. sugarbeet, common ash and lilac.

In potato, the pathogen is associated with the early dying syndrome. Early dying results in yield losses up to 25% (Bollen *et al.*, 1989; Haverkort *et al.*, 1989). Incidence of the disease is increased when potato is grown in short rotations.

Problems with Verticillium wilt have been accelerated by changes in agricultural practices during the last decade. The success of the pathogen is due to an increased frequency of susceptible crops in the present rotation cycles, the polyphagous character of the pathogen, and the production of persistent resting structures by *V. dahliae* which survive in soil for many years. This combination of factors implies that problems arise not only in short rotations with the same susceptible crops, but also when cropping systems are changed from arable farming to arboriculture or floriculture. In The Netherlands, arboriculture and floriculture are economically flourishing and are expanding production sectors at the same time that arable farming is declining. Fields planted with lime tree, elm or maple often have a history of potato cultivation which implies that the soil has likely been infested with *V. dahliae*. Losses are heavy since symptom expression in

woody plants is rather slow and the disease is only noticed when considerable investments have already been made. The same applies to soils used for growing chrysanthemums or roses in greenhouses that have been built on former potato or strawberry fields. The problems with Verticillium wilt have led to the common practice of assessment for *V. dahliae* as a part of the analysis of soil to be used for nurseries or greenhouses. Verticillium wilt is also a common feature in forests and parks. The present governmental policies for nature conservation and outdoor recreation aim at an increase of the area with forests and parks. These are laid out on former arable land. Since this land has likely been infested with the pathogen, it is expected that the significance of Verticillium wilt will increase.

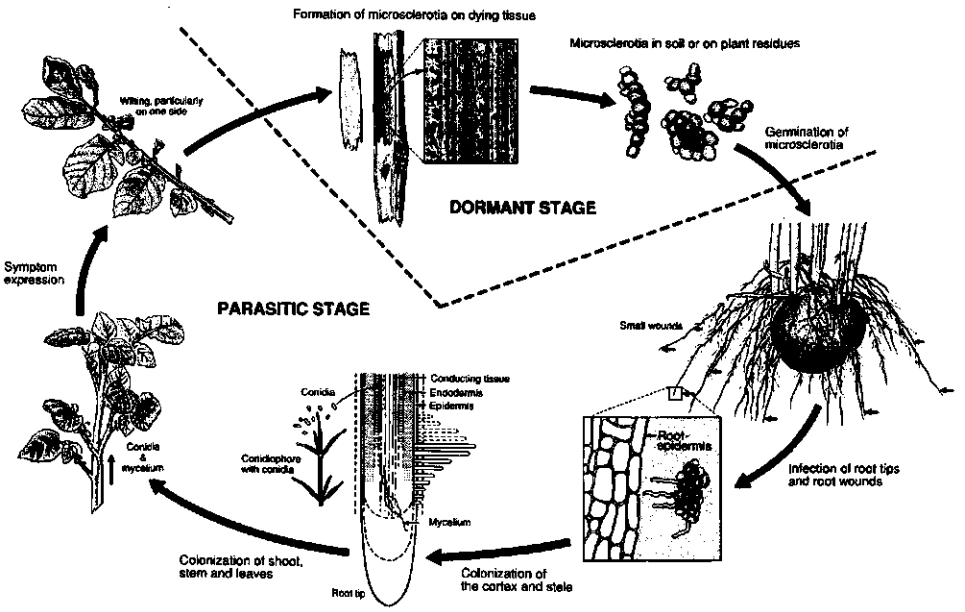

Fig. 1. Life cycle of *Verticillium dahliae* in potato.

The measures included in the governmental Multi Year Crop Protection Plan (MYCPP) are likely to increase incidence of Verticillium wilt. One of the goals in this plan is a substantial reduction in the use of soil fumigants for control of nematodes (Anonymous, 1990). Together with the potato cyst nematode and other major pathogens, fumigation reduces the population of free-living nematodes that predispose the roots to infection with *V. dahliae.* Replacement of fumigation in potato fields by cultural methods and growing varieties that are partially resistant to the potato cyst nematode will not, or only slightly, affect the populations of free-living nematodes and, therefore, leaving more opportunity for infection by *V. dahliae* than with fumigation (Bollen, 1993). This statement is not meant to detract from the efforts made in developing alternative methods for disease control by fumigation, but it indicates the need for developing alternative methods for control of Verticillium wilt.

Life cycle of *Verticillium dahliae* and disease symptoms in potato plants

Three stages can be distinguished in the life cycle of *V. dahliae:* a parasitic stage followed by a short saprophytic stage and a dormant stage. The resting structures in the dormant stage are microsclerotia that are formed in senescent and dead tissue of the host plant (Fig. 1). In a few host plants, such as cotton, microsclerotia occur in dead leaves; in woody host plants they occur in petioles (Tjamos and Tsougriani, 1990; Rijkers *et al.*, 1992); in potato in the superficial layers of the cortex of dead stems. In the field, microsclerotia on potato stems can be observed with the naked eye. Germination of the microsclerotia is highly sensitive to soil fungistasis and occurs only at high nutrition levels as induced by root exudates in the rhizosphere of host plants. However, exudates of non-hosts seem to induce germination as well (Schreiber and Green, 1963; Olsson and Nordbring-Hertz, 1985).

The parasitic stage starts when the germination hyphae infect the host plant at the root tips or at sites of lateral root emergence. Then the fungus advances inter- or intracellularly through the cortex tissue. Most of the infections seem to be superficial in the root cortex as most of the colonies are removed when the roots are surface-sterilised (Evans and Gleeson, 1973). Once the fungus has reached the vascular tissue, conidia are formed and transported by passive movement. In cotton, hyphae were observed to pass from one vessel to another through the pits (Schnathorst, 1981). The parenchyma cells adjacent to the infected vessels are often discoloured and filled with gum-like material. The vessels may become plugged by hyphae and products secreted by the parenchyma cells.

Symptom expression starts with flaccidity of the plant when the crop is exposed to dry conditions or solar radiation. Chlorosis first appears at the lower leaves. Unilateral chlorosis and wilting of the plant is observed when the vessels are infected at one side of the stem base only. Individual leaves often show the same pattern when one half becomes chlorotic and the other half remains green. Chlorosis is often, but not always, followed by necrosis of the leaf tissue due to toxigenic action by the fungus (Schnathorst, 1981). In general, the next stage is irreversible wilting of the potato plant. Severely infected crops are characterised by early dying that occurs a few weeks before senescence of a healthy crop. Early dying is not always preceded by wilting.
As soon as the tissue of infected plants senesces, the saprophytic stage of the fungus begins. From the xylem the mycelium of the pathogen invades the adjacent cells and colonises the dying tissue followed by the formation of a great number of microsclerotia. In and on dead stems of field-grown potatoes, $4x10^6$ microsclerotia g^{-1} dry weight were counted (Nagtzaam, unpublished). Potato cultivars differ considerably in the extent to which microsclerotia are produced (Slattery, 1981; Mol and Scholte, 1995). Together with the residues of the infected crop the microsclerotia are returned to the soil. During decomposition of the residues the microsclerotia are gradually released and the life cycle is completed. Part of the microsclerotia population survives for 12 to 14 years in soil (Wilhelm, 1955).

Epidemiology

Since pathogen inoculum rarely produces new inoculum that incites infections within the same growing season (Schnathorst, 1981), Verticillium wilt is considered to be a single cycle vascular wilt disease. Conidia or mycelia in soil do not contribute to long-term survival of the pathogen (Green, 1969). The fungus survives as microsclerotia, separately or in decayed plant tissue. Survival is affected by soil type, soil moisture and temperature (Green, 1980). Additionally, low populations survive on the roots of many symptomless crop and weed species, including monocotyledons (Martinson and Horner, 1962; Davis *et al.*, 1997b) and enhance survival of the pathogen in agro-ecosystems.

V. dahliae behaves as an obligate parasite as it infects only the living plant and does not colonise soil organic matter because it lacks competitive saprophytic ability. In non-sterile soil, its mycelium is readily lysed. Therefore, dispersal of the pathogen is mainly by passive transport. Transport of microsclerotia in soil particles and in diseased plant tissue by mechanical means (agricultural tools and machinery) or by the wind seem to be most important. Leaves of diseased trees containing microsclerotia spread Verticillium wilt in these crops. In addition to transport by mechanical means and by wind, water can

also spread microsclerotia (Easton *et al.*, 1969). Termorshuizen (1997) observed massive sporulation of *V. dahliae* on leaves and petioles of *Arabidopsis thaliana*. Under humid conditions, formation of conidia on microsclerotia present on the soil surface and on dead infected potato tissue was noticed. However, the significance of conidia in dispersal is considered to be limited because of their high sensitivity to drought. In wind tunnel experiments with sporulating microsclerotia as an inoculum source, the pathogen was not dispersed by conidia (Termorshuizen, 1997). Seed transmission has been demonstrated for safflower and sunflower (Klisiewicz, 1975; Sackston and Martens, 1959). In The Netherlands seed-potatoes do not play a role in pathogen dissemination because harvest is early in the growing season when microsclerotia have yet to form. Soil mesofauna can be important in dissemination of fungi. Williams and Whipps (1995) and Whipps and Budge (1993) showed that mesofauna can transmit viable conidia of *Coniothyrium minitans* in soil. The presence of mites and collembolans in and near Verticillium-infected plant debris in soil creates suitable conditions for spread in this way. In The Netherlands, compost made from crop residues and household waste is used to maintain or increase soil fertility. Spread of *V. dahliae* in certified compost to new fields would seem to be excluded as the conditions during the thermophilic phase of the composting process are lethal to Verticillium propagules when present in crop residues used as parent material for composting (Bollen and Volker, 1995). The conditions during composting are more severe than during exposure to 50 °C during 23 minutes which already kills 90% of the microsclerotia (Pullman *et al.*, 1981).

Severity of Verticillium wilt is related to the inoculum population at the start of the growing season (Ashworth *et al.*, 1979; Bollen *et al.*, 1989; Davis *et al.*, 1994). Verticillium populations vary in strain composition and density depending on the cropping history of the field, because the release of microsclerotia from colonised plant debris lasts at least one year following its incorporation into the soil. Slattery (1981) concluded that in potato fields most microsclerotia are incorporated in buried infected tissue. Data on the relationship between soil inoculum population and wilt severity are inconsistent (Termorshuizen and Mol, 1995). Yield loss in potato was observed at 10 up to 200 colony forming units per gram soil (Powelson, 1970; Nnodu and Harrison, 1979; Bollen *et al.*, 1989). Devay *et al.* (1974) were unable to demonstrate a significant correlation between inoculum density in field soils and percentage of diseased plants. Similar results were obtained by Ashworth *et al.* (1972), Bejarano-Alcázar *et al.* (1995) and Davis and Everson (1986). So, pathogen density in soil is not the only determinant of disease severity. One factor is the variation in germination rate of the microsclerotia and the number of germination hyphae (Hawke and Lazarovits, 1994). Another factor is the

pattern of pathogen distribution in soil. In commercial fields microsclerotia occur in clustered or aggregated patterns (Smith and Rowe, 1984; Xiao *et al.*, 1997) and the population density varies with soil depth (Ben-Yephet and Szumelewich, 1985). Up to now, no data are available on the effect of microsclerotial distribution on Verticillium disease development and severity. Variation in virulence of strains of Verticillium also affects disease development (Mol *et al.*, 1996). Virulence of an isolate depends on the host species, geographical origin and susceptibility of the host cultivar (Zilberstein, 1983b). Until 1980, intraspecific variation in *V. dahliae* had been considered the exception rather than the rule but recent studies showed the contrary (Jeger *et al.*, 1996). Other soil-borne pathogens can have a profound effect on disease progress and yield loss caused by *V. dahliae*. Migratory nematodes of the *Pratylenchus* group strongly enhance disease severity (Riedel *et al.*, 1985; Saeed *et al.*, 1997). Other nematodes, e.g. *Globodera*, *Heterodera* and *Meloidogyne*, are claimed also to predispose the plant to Verticillium wilt (Harrison, 1971; Jacobson *et al.*, 1979; Storey and Evans, 1987; MacGuidwin and Rouse, 1990). The literature on interactions with *V. dahliae* is less consistent for fungi than nematodes. Scholte *et al.* (1985) found that with potato cultivar Amethyst yield loss by *V. dahliae* was almost doubled in the presence of *Colletotrichum coccodes* whereas with cultivar Bintje no synergism was found between these fungi. Johnson and Miliczky (1993) reported that *C. coccodes* was not associated with *V. dahliae*.

Yield reduction is mainly the result of closure of the stomata, reduced photosynthesis, and early senescence of the canopy after blockage of the vascular system of the haulm (Haverkort *et al.*, 1990; Bowden and Rouse, 1991). High temperatures are considered to favour yield loss in potato (Francl *et al.*, 1990). The optimum temperature for the growth of *V. dahliae in vitro* ranges from 20 to 26 °C and the minimum temperature from 3 to 12 °C, depending on the tested Verticillium isolate (Brinkerhoff, 1973). During a cool summer in temperate zones, the level of pathogen inoculum can stay below the threshold necessary to cause disease symptoms and yield loss. Disease development is restricted at very high temperatures is (Pullman and DeVay, 1982). Germination of conidia and mycelial growth are inhibited at temperatures above 31-33 °C (Pullman *et al.*, 1981). Temperature may affect not only the pathogen but also the resistance level of the host plant (Brinkerhoff, 1973).

It is widely recognised that wet soil conditions enhance potato early dying in an arid atmosphere. These conditions favour rapid transpiration associated with a high flow of water and conidia through stems and leaves of the plant (Cook, 1973). Gaudreault *et al.* (1995) hypothesised that an increased disease severity under moist conditions is also

caused by slowing root growth and by indirectly increasing the rate of microsclerotial germination. The latter is due to an increased leakage of nutrients from roots under moist conditions (Kuan and Erwin, 1980) which enhances the likelihood that a microsclerotial germ tube encounters a root tip. Pullman and DeVay (1982) concluded that irrigation increased disease development as the root system increased rapidly and provided increased root surface for inoculum contact. Bollen *et al.* (1989) found that in wet summers potato stems were colonised by *V. dahliae* to about the same extent as in two years with a dry summer. Moreover, yields were reduced to a lower degree than in dry years. It is hypothesised that temperature is more critical than soil water potential in determining disease development in The Netherlands. At the same time, rain water decreases soil temperatures. In addition to temperature and soil water potential, fertilisation has been shown to influence disease incidence significantly (Davis and Everson, 1986; Cappaert *et al.*, 1992).

The major part of new inoculum is formed in the aerial parts of the host plants (Ben-Yephet and Szmulewich, 1985; Mol and Scholte, 1995). Various environmental factors influence formation of microsclerotia. In Israel, autumn crops had a higher microsclerotial production than spring-grown crops (Ben-Yephet and Szmulewich, 1985). This difference was attributed to the dry hot weather in spring and cool, moist conditions in autumn during senescence of the crop. Experiments *in vitro* corroborated this hypothesis as microsclerotial production was rapid and abundant from 18 to 28 °C , but was reduced between 5 and 15 °C and at 30 °C (Brinkerhoff, 1973). The results of Soesanto and Termorshuizen (1997) that the optimum for microsclerotia production on shoots and roots was at moderate temperatures (15 and 20), are in accordance with the findings in Israel. Mol and Scholte (1995) found that numbers of microsclerotia were higher in a late harvest than in an early one. They hypothesised that in a late harvest the pathogen already started to grow to the outer part of the potato tissue. In that case, competitors would develop too late to prevent pathogen reproduction. Another reason they suggested was that later in the season the vascular system of potato was more colonised by *V. dahliae*.

Present methods in control of Verticillium wilt

Thus far, the most effective method for controlling persistent soil-borne pathogens is soil disinfestation by steaming or by fumigation with eradicants. Recently, attempts have been made to reduce the amount of fumigants needed for Verticillium control, either by improving the techniques of application (Gamliel *et al.*, 1997) or by combinations with other control agents (Fravel, 1996). Fravel (1996) advocated integrated control by a

combination of treatment with metam sodium at low rates and application of biocontrol fungi. At a sublethal rate of the fumigant, growth of *V. dahliae* from microsclerotia was reduced but growth of the biocontrol fungus *Talaromyces flavus* was not. Infection of eggplant by *V. dahliae* was reduced by treatment with the combination, but not by the fumigant alone. Probably the pathogen is weakened by the fumigant, making it more vulnerable to its antagonist. In The Netherlands and in many other countries, fumigation, even at reduced rates, is discouraged due to the high costs, the health risk for workers, and the adverse effects on the environment. Solarization is an effective way of disinfestation of Verticillium infested soils, although it appears to be difficult to completely eliminate the pathogen (Melero-Vara *et al.*, 1995). However, its practice is not relevant for fields in temperate zones.

Various cultural methods are used in the control of Verticilium wilt. Crop rotation has received much attention (Schreiber and Green, 1963). Short rotations with non-hosts do not result in sufficient control because of the longevity of the microsclerotia (Easton *et al.*, 1992; Mol *et al.*, 1996). Since 1951, when Wilhelm published that inoculum of Verticillium can be reduced by soil amendments, much research has been done to suppress the disease by applying organic products. The results varied from no effects with straw and compost (e.g. Elmer and Stoner, 1993) to an appreciable reduction of survival by using alfalfa and oat residues (Green and Papavizas, 1968), chitin (Jordan *et al.*, 1972) or barley (Harrison, 1976). With these products questions can be raised about the practicability of the application since the effective rate of amendment was >0.5% w:w, except for chitin where it was 0.2%. Recently, the use of organic materials for reducing Verticillium has again become of topical interest. Lazarovits (1997) tested a wide spectrum of organic materials and obtained significant control of *V. dahliae*, also in the second crop year after application. Davis *et al.* (1996) evaluated several green manure treatments and found that Verticillium wilt was best controlled and yields were highest after treatments of either sudangrass or - to a lesser extent - maize. Koike *et al.* (1997) demonstrated that amendment with broccoli residues is very effective against Verticilium wilt in cauliflower. Plots treated with these residues had inoculum densities as low as the fumigated ones. Blok (1997) considerably improved the efficacy of disease control with green crop residues by inducing fermentative conditions in soil. Anaerobiosis was achieved by tarping the amended soil with plastic sheeting with a low permeability for oxygen. With this procedure a reduction to 0.08 or 15 % of the original *V. dahliae* population was obtained in grass-amended soil in two field experiments. It was suggested that toxic products are formed when green residues are decomposed under anaerobic conditions. Probably, the mechanism(s) of microsclerotia inactivation are to

some extent the same as those in flooded soil where conditions are also detrimental for survival of *V. dahliae* (Pullman and DeVay, 1981).
Since stems colonised with microsclerotia are the major source of soil infestation, removal of crop debris is often recommended as a cultural practice to prevent build-up of inoculum in soil. Mol *et al.* (1995) and Lamers and Termorshuizen (1997) found that it was not sufficiently effective in short rotations with host crops, but they suggest a role for it in rotations with a low incidence of susceptible crops or in combination with chemical and biological control measures.

Breeding for disease resistance and tolerance has been successful for a few host crops. Although resistant or tolerant cultivars are commercially available for tomato, hop, peppermint and potato, they are only widely used in tomato and cotton production (Allen, 1994). For other crops, performance is suboptimal and they are grown only in regions with acute wilt problems. The choice of potato varieties grown for industrial processing (e.g. for production of crisps and French fries) is not likely to change in the near future because of the long-term investments made in machinery required for specific varieties. Traditional variety preferences of consumers also delay changes.
Resistance against *V. dahliae* induced by other organisms has been reported in many hosts (Jorge et al, 1992; Melouk and Horner, 1975; Schnatthorst and Mathre, 1966). Research on stimulation of the plant defence system in this way has not yet led to commercial practice for control of *V. dahliae*. Induced resistance is dependent on environmental conditions. The effects are often temporal and inconsistent and there is a lack of field experiments. Moreover, it is difficult to make the method patentable for registration (Kuc and Strobel, 1992).

Biocontrol of soil-borne pathogens is approached in at least two ways. The first is by the search for soils that are naturally suppressive to the disease. These soils are a hostile environment either for pathogen growth and survival or for disease expression even if the soil contains high populations of pathogen inoculum (Baker and Cook, 1974). Thus far, soils suppressive to *V. dahliae* have not been found. It is not clear whether this is because specific searches for these soils have not been made or that these soils do not exist. Keinath and Fravel (1992) induced suppressiveness to Verticillium wilt by repeated cropping of the same soil with potato in the greenhouse. This suppressiveness was associated with specific types of soil.
The second method of biocontrol is by applying microbial antagonists. This approach has received more attention than the search for suppressive soils. More than 40 species of

fungi and bacteria have been tested for their *in vitro* or *in vivo* activity against *V. dahliae* (Table 1). About 25 species were tested in labour-intensive field experiments.

In field experiments, promising results were occasionally obtained by Marois *et al.* (1982), Fravel *et al.* (1986), Ordentlich *et al.*, (1990), Georgieva (1992), Johnston *et al.* (1994) and Tjamos *et al.* (1997). Up to now, biocontrol of *V. dahliae* is not commercially practiced. Research efforts on biocontrol of *V. dahliae* are characterised by separate and short-term projects. An exception is the work on *Talaromyces flavus* in the research programme of the Biocontrol Plant Diseases laboratory in Beltsville, USA. The work started with that of Marois *et al.* in 1982 and since then has been continued by Fravel and co-workers and by research workers in Israel, Germany and Greece.

It seems justified to conclude that up to now, no single method is available for a consistent and effective control that is compatible with the principles of sustainable agriculture. Therefore, there is a demand for an overall strategy built on partially effective methods that complement each other.

Objectives, approach and outline of the thesis

Anticipating the increased chance of infestation of potato and other crops by *V. dahliae* due to a reduction in soil fumigation in addition to existing problems with Verticillium wilt, two 4-years research projects were initiated and funded by the The Netherlands Multiyear Crop Protection Programme. One project geared towards control by cultural measures and the other to control by biological agents. Both projects were granted to the Wageningen Agricultural University.

The first project was performed at the Department of Agronomy from 1990 to 1994. The potential of cultural practices was studied either by growing non-host crops or by removal of crop debris and mechanical treatment of infested potato haulms. The project yielded a wealth of quantitative information on the ecology of *V. dahliae*, in particular on its dynamics. Mol (1995) demonstrated that the cultural practices mentioned did not provide effective control of Verticillium wilt under commercial conditions. This conclusion does not detract from the general merits of cultural practices for controlling other diseases.

The second project was performed at the Department of Phytopathology from 1992 to 1996. The results are presented in this thesis. The choice of *T. flavus* as an antagonist was based on its higher resistance to adverse conditions than other antagonists of the pathogen (Beuchat, 1992; Tjamos and Paplomatas, 1987), on its preference for the rhizosphere as a habitat (Marois *et al.*, 1984), and on its efficacy in biocontrol of *V.dahliae* in the USA (Marois *et al.*, 1982, 1984; Fravel *et al.*, 1986). It should be stated however that data on the efficacy of the antagonist are not consistent, for example Spink

and Rowe (1989) and Keinath *et al.* (1990) were not successful in their attempt to control Verticillium wilt.

T. flavus affects pathogen development in several ways. It is known as a mycoparasite on *Rhizoctonia solani* (Boosalis, 1956), on *Sclerotinia sclerotiorum* (McLaren *et al.*, 1986), on *Sclerotium rolfsii* (Madi *et al.*, 1992) and on *Verticillium dahliae* (Fahima and Henis, 1990; Fahima *et al.*, 1992). It produces antibiotics that inhibit growth of a wide range of fungi (Mizuno *et al.*, 1974). Kim *et al.* (1990), Fravel and Roberts (1991), and Madi *et al.* (1997) presented substantial evidence for production of glucose oxidase as the antifungal principle inhibiting development of *V. dahliae*.

Chapter 2 presents a quantitative bioassay to study the effects of various antagonists on dynamics of *V. dahliae*. It indicates that the level of internal colonisation of plants by *V. dahliae* is an useful additional parameter in biocontrol studies.

Chapter 3 deals with the persistence of the spores of *T. flavus* during storage of a product containing the antagonist. For commercialisation, a long shelf life is a strong point in favour.

Chapter 4 deals with dynamics of *T. flavus* on the roots of eggplant and potato. It demonstrates the potential to establish (itself) at locations on the root where *V. dahliae* infects the root.

Chapter 5 presents the results of *in vitro* and growth chamber experiments on biocontrol of *V. dahliae* in soil and on the roots of the host. *T. flavus* and other antagonists were tested. In an attempt to improve biocontrol efficacy of *T. flavus* combinations of antagonists were applied and their compatibilities were assessed.

Chapter 6 presents a theoretical study on the effect of spatial distribution of fungal antagonists on their performance as biocontrol agents.

Chapter 7 includes the presentation of the results of two field experiments. It is discussed whether application of *T. flavus* deserves a role in the strategy for biocontrol of Verticillium wilt in The Netherlands.

Chapter 8 contains a general and summarising discussion on (bio)control of Verticillium wilt.

Table 1. Literature on fungal and bacterial biocontrol agents against *V. dahliae*

Biocontrol agent	Host plant	Biocontrol effect[1]			Source
		In a controlled environment		Under field conditions	
		In vitro	Greenhouse		
Fungi					
Aspergillus sluteaceus	Eggplant	0	+	+	Marois *et al.*, 1982
A. fumigatus	Eggplant; mapple	+	+	+	Calarí & Peterson, 1967
Blastomyces luteus	Tomato; lion's mouth	+	+	0	Isaac, 1954
Cheatomium spp.	Eggplant	-	-	0	Calarí & Peterson, 1967; Marois *et al.*, 1982
Chloridium virescens	Eggplant	0	-	0	Marois *et al.*, 1982
Codinaea heterodermo	Eggplant	0	-	0	Marois *et al.*, 1982
Conlothyrium spp.	Strawberry	+	+	+/-	Jordan & Tarr, 1978
Doratomyces spp.	Strawberry	+	+	+	Jordan & Tarr, 1978
Eupenicillium javanicum	Eggplant	0	-	0	Marois *et al.*, 1982
Fusarium spp.	Eggplant	+	-/+	+	Jordan & Tarr, 1978; Marois *et al.*, 1982
F. equiseti	Potato	0	0	+	Davis *et al.*, 1997c
F. oxysporum	Eggplant; tomato	0	+	0	Jorge *et al.*, 1992; Yamaguchi *et al.*, 1992a,b
F. oxysporum f.sp. dianthi	Tomato	0	+	0	Matta & Garibaldi, 1977
Gliocladium roseum	Cotton; eggplant; potato; lucerne	0	+	+/-	Calarí & Peterson, 1967; Globus & Muromtsev, 1990; Johnston *et al.*, 1994; Keinath *et al.*, 1990, 1991; Millar *et al.*, 1984
G. virens	Eggplant	0	+/-	-	Keinath *et al.*, 1990, 1991a,b; Marois *et al.*, 1982
Gliomastix spp.	Strawberry	+	+	-/+	Jordan & Tarr, 1978
Hormodendron spp.	*Acer spp.*	+	0	0	Calarí & Peterson, 1967
Paecilomyces spp.	Mapple; eggplant	+/-	+	0	Calarí & Peterson, 1967; Marois *et al.*, 1982
P. lilacinus	Eggplant	0	0	+/-	Marois *et al.*, 1982
Penicillium spp.	Eggplant; *Acer spp.*	+/-	+/-	+	Calarí & Peterson, 1967; Jordan & Tarr, 1978; Keinath *et al.*, 1991a
P. funiculosum	Eggplant	+/-	-	0	Calarí & Peterson, 1967; Marois *et al.*, 1982
Pseudogymnoascus roseus	Eggplant	0	0	-	Marois *et al.*, 1982
Talaromyces flavus	Eggplant; potato; oilseed rape	+	+	+/-	Davis *et al.*, 1996; Fahima & Henis, 1990, 1995; Fahima *et al.*, 1992; Fravel & Roberts, 1991; Fravel *et al.*, 1996, 1995; Engelkes, 1993; Johnston *et al.*, 1994; Henis & Fahima,; 1990; Keinath *et al.*, 1991a, 1990; Kim *et al.*, 1988, 1990; Marois *et al.*, 1982; Marois *et al.*, 1984; Spink & Rowe, 1989; Zeise, 1997
T. trachyspermus	Eggplant	0	-	0	Marois *et al.*, 1982
Trichoderma hematum	Eggplant	0	-	0	Marois *et al.*, 1982
T. harzianum	Eggplant; potato	0	+	-/+	Elad *et al.*, 1980
T. koningii	Pepper	0	0	+	Georgieva, 1992
T. lignorum	Eggplant	0	+	+	Calarí & Peterson, 1967
T. viride	Strawberry	+	+	+	Jordan & Tarr, 1978; Marois *et al.*, 1982
Trichothecium roseum	Strawberry	+	0	-	Jordan & Tarr, 1978
Verticillium albo-atrum	Tomato	0	+	0	Matta & Garibaldi, 1977
V. dahliae	Sunflower; cotton	0	+	+	Price & Sackston, 1963, 1969; Schnathorst & Mathre, 1966; Zaki *et al.*, 1972
V. nigrescens	*Mentha piperita* L.; *Mentha cardiaca* L.	0	+	0	Melouk & Horner, 1975
V. tricorpus	Tomato	0	+	+	Matta & Garibaldi, 1977; Davis *et al.*, 1997a
Bacteria					
Bacillus uniflagellatus	Pumpkin; eggplant;	0	+	0	Gemrich & Vandestreek, 1980
B. subtilis	Maple	+	+	0	Berg & Ballin, 1994; Gregory *et al.*, 1985; Hall *et al.*, 1986; Podile *et al.*, 1985
Bacillus spp.	Eggplant	0	+	+	Tjamos *et al.*, 1997
Cellulomonas flavigena	Potato	+	+	0	Wadi & Easton, 1985
Erwinia carotovora	Cotton	+	-	0	Berg & Ballin, 1994
Pseudomonas fluorescens	Potato	+	+	+/-	Berg & Ballin, 1994; Leben *et al.*, 1987; Wadi & Easton, 1985
Streptomyces flavofungini	Potato	0	+	0	Wadi & Easton, 1985

1 +, +/-, -: Significant, moderate and no control, respectively; 0 = no data provided for.

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Chapter 2

The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*

Abstract

Using potato, eggplant and thorn apple as test plants, the relationship between soil inoculum density and plant infection was studied as a basis for the development of a quantitative bioassay of *Verticillium dahliae*. A linear relationship was demonstrated ($P<0.05$) between soil inoculum density and population density on roots for all three test plants and for soil inoculum density and population density in sap extracted from stems for eggplant. Correlation coefficients were higher with densities on or in roots (R^2 varying from 0.45 to 0.99) than with densities in stems (R^2 varying from 0.04 to 0.26). With eggplant, population densities on/in root and in sap extracted from stems were significantly correlated at 20 and 25 °C with Pearson's correlation coefficients of 0.41 and 0.53, respectively. For potato, root colonisation was higher at 15° than at 20 °C, whereas the reverse applied to eggplant. Stems of potato were less densely colonised than stems of eggplant. The pathozone sensu Gilligan (1985) was calculated to be <300 mm, indicating that infection was caused by microsclerotia which were located close to the roots. To assess the density of *V. dahliae* in plant tissue pipetting infested plant sap on solidified ethanol agar medium without salts yielded higher densities than using pectate medium or mixing sap with molten agar. A bioassay for determining effects of (a)biotic factors on development of *V. dahliae* in the plant is recommended with eggplants as a test plant, grown in soil infested with 300 single, viable microsclerotia g^{-1} soil at a matric potential of -6.2 kPa, and incubated at 20 °C for 8 weeks.

Introduction

Verticillium dahliae Kleb. causes early dying of potato resulting in considerable yield losses in The Netherlands (Bollen *et al.*, 1989; Scholte, 1989). Soil inoculum consists of microsclerotia that occur free or embedded in plant debris (Schreiber and Green, 1962) and may survive for many years (Wilhelm, 1955). In some studies disease incidence was shown to be related to soil inoculum density (e.g. Nicot and Rouse, 1987; Pullman and DeVay, 1982; Wheeler *et al.*, 1992), whereas in other studies such a relation could not be established (Ashworth *et al.*, 1972; Davis and Everson, 1986; DeVay *et al.*, 1974). The main reason for this discrepancy may be the effects of the environment on processes that occur over the long period between germination of microsclerotia in soil and the appearance of disease symptoms, usually late in the growing season (Termorshuizen and Mol, 1995).

Research on introduced biocontrol agents and other environmental factors on the dynamics of *V. dahliae* requires a reliable quantitative bioassay. Plant bioassays have been used in ecological studies on *V. dahliae* (e.g. Evans *et al.*, 1974) and for selection for resistance to the pathogen in host plants (Palloix *et al.*, 1990; Zeise, 1992). These

tests are generally based on symptom evaluation. Because symptom expression is strongly dependent on environmental factors, some of which are unknown, estimation of presence of the pathogen in the host plant provides absolute proof on infection of the plant. Evans *et al.* (1974) were the first to develop a quantitative bioassay based on root colonisation by *V. dahliae*. Estimation of inoculum density of *V. dahliae* in stem tissue in addition to that in or on the root would again add extra information. In studies where severity of symptom expression, yield and susceptibility of the host plant were shown to be associated with vascular colonisation, methods were described to quantify colonisation of the stem by *V. dahliae* (e.g. Pegg and Jonglaekha, 1981; Ordentlich *et al.*, 1990; Hoyos *et al.*, 1991; Johnson and Miliczky, 1993). However, thus far the degree of colonisation of the stem has not been used in a quantitative bioassay. Moreover, experimental evidence for a relation between numbers of microsclerotia in soil and colony forming units (cfu) in stem sap is lacking. In field experiments, Davis *et al.* (1983 and 1996) found a significant correlation between estimated inoculum density of *V. dahliae* in naturally infested soils and inoculum density in dried potato stems.

The objective of the present investigation was to evaluate the usefulness of the techniques that are used for estimating the population densities on or in plant roots and in stems for determining effects of biotic and abiotic factors on development of *V. dahliae* in the plant in growth chamber bioassays.

Materials and methods

Preparation of inoculum

Microsclerotia were obtained either from potato stems collected from an infested field or artificially produced on autoclaved rye seeds as described by Kotcon *et al.* (1984). The inoculum was air dried in a sterile flow cabinet to eliminate conidia and mycelial fragments (Green, 1969). After chopping the potato stems or grinding the rye seeds with pestle and mortar, the inoculum was blended in tap water. The resulting suspension was poured through nested 150, 75 and 20 μm mesh sieves. Material retained on the 20 mm sieve was rinsed with additional water and resuspended in tap water. A drop was pipetted on a microscope slide and the separation of microsclerotia from stem material was verified by examination under a binocular dissecting microscope (50x). The retained material was resuspended in 0.08% water agar (Slattery, 1981) to lessen sedimentation of the microsclerotia in the suspension. The density of the suspension was estimated by direct assessment of the number of microsclerotia in at least 10 drops of 10-20 μl.

Germination percentage of the microsclerotia was assessed by plating 0.2 ml of suspension on ethanol agar medium (EA)(Nadakavukaren and Horner, 1959), or by

transferring single microsclerotia to MSEA-medium (Harris *et al.*, 1993) as described by Hawke and Lazarovits (1994). Instead of streptomycin, chloramphenicol and chlortetracycline, the bacteriostatic antibiotic oxytetracycline (50 mg ml^{-1}) was added to these media.

Preparation of soil and seed

An autoclaved (4 h, 115 °C and 50 kPa) sandy soil (pH-KCl 7.0, 0.3% organic matter, fraction particles ≥16 µm 97.4%, density 1.23 g cm^{-3}) was used in all experiments. Recolonisation and microbial activity were standardised by growing the soil with wheat (*Triticum aestivum* L. cv. Okapi) before use. Ten days after sowing, the wheat was cut at soil level and the soil was air-dried and sieved through a screen of 2 mm mesh to remove roots.

Seeds of eggplant (*Solanum melongena* L.) of the susceptible cv. Black Beauty (Ferrandino and Elmer, 1993) and thorn apple (*Datura stramonium* L.) were surface-sterilised as described by Fahima and Henis (1990). Potato minitubers (Lommen and Struik, 1992), diameter 12-17 mm, of cv. Element, shown to be highly susceptible to *V. dahliae* (Scholte, 1990), were washed with tap water, surface-sterilised for 10 min in 1% NaOCl, washed twice with sterile water and dried on sterilised filter paper. The tubers were incubated in the dark at 20 °C until sprouts started to grow, and then used for the bioassay.

Bioassay

A suspension of microsclerotia was applied to air-dried soil to obtain the required inoculum density. Differences in germinability between inoculum preparations were adjusted for by applying different quantities of inoculum. In the experiments with eggplant grown at 15 and 20 °C, microsclerotia were used that originated from potato stems collected from an infested field. In other experiments artificially produced microsclerotia were applied. The level of colonisation of the roots can be affected by both the density and distribution pattern of microsclerotia in soil (Ashworth *et al.*, 1972a; Wheeler *et al.*, 1994). In the field, part of the microsclerotia population is associated with plant debris, especially in the year after cultivating a susceptible crop (Ashworth *et al.*, 1974, Slattery, 1981). The distribution pattern of microsclerotia depends on decay of the infected plant residues, which is strongly influenced by environmental conditions. For this reason we applied microsclerotia, rather than incorporating plant residues infected by *V. dahliae*. The soil was moistened to a 10% and 15% moisture level corresponding with a matric potential of -6.2 and -4.2 kPa, respectively. Soil matric potential was measured by mini-tensiometers. Pots (4 x 4 x 12 cm) with parallel sides and a bottom of 22.4 µm mesh

polyamide screen were filled with the soil. Three eggplant or thorn apple seeds or one potato minituber were placed in each pot. Pots were covered with plastic to maintain a high air humidity and incubated in growth chambers at a light intensity of 29 W m^{-2} 16 h day^{-1} and a air relative humidity of 80%. After emergence of the seedlings, the plastic was removed and the number of plants per pot was reduced to one. Soil temperatures at a depth of 5 cm below soil level were 1-2 °C higher than air temperatures. Soil moisture level was maintained by compensating for moisture losses and plant growth by adding half strength Hoagland's nutrient solution (about four times per week) after weighing the pots.

Detection in shoot tissue

Stems were cut at the soil line, defoliated, washed in tap water, surface-disinfected in 1% NaOCl for 1 min, washed twice in sterile water, and blotted dry on sterilised filter paper. In order to estimate the extent of penetration of the disinfectant, stems were soaked in a solution of equal volumes of fluorescein isothiocyanate and TRIS-buffer (pH 6.8) for 1 and 30 min, respectively. Fluorescence microscopy showed that the fluorescence suspension penetrated 1-2 mm into the stem ends for both soaking durations. Five mm long segments were cut off at both ends to remove tissue that had absorbed NaOCl.

Stem infection was assessed by plating one 5 mm portion of the stem base on EA. Plates were incubated at 20 °C and examined for growth of *V. dahliae* after 1 week. In a preliminary experiment, methods were compared to quantify colonisation of stem tissue. Sap was extracted from a 5 cm segment of the basal portion of a surface sterilised stem of potato using a plant press. If the first extraction yielded less than 0.1 ml of sap, the extraction procedure was repeated by further pressing after addition of a measured volume of sterile water. Extracted plant sap was mixed with molten agar medium according to Hoyos *et al.* (1991), or pipetted on solidified medium as described by Ordentlich *et al.* (1990). Between processing the samples, the press was cleaned with hot water, 95%-ethanol and cold water, respectively. Aliquots of 0.1 or 0.2 ml were pipetted into plastic Petri dishes (Greiner, diam. 8.5 cm, with ridges) and 20 ml of molten medium (45-50 °C) added while the dish was agitated gently. Alternatively, sap was spread over the solidified medium with a surface-sterilised glass rod. The plates were incubated in the dark at 20-22 °C. Zilberstein *et al.* (1983a) found that germination of microsclerotia was inhibited at lowered oxygen concentrations. Therefore, after 3 days the lids of the plates were moved to remove water barriers that possibly prevent inward oxygen diffusion. Starting at 7 days up to 3 weeks after preparing the plates, the number of cfu per ml sap was determined by counting the number of colonies that produced microsclerotia.

Three media were compared for their suitability to quantify *V. dahliae* in plant sap: pectate medium (PM) (Huisman and Ashworth, 1974; slightly modified by Bollen *et al.*, 1989), EA (Nadakavukaren and Horner, 1959) and EA amended with salts but without PCNB (EA+) (Ausher *et al.*, 1975). For all media, 50 mg l^{-1} oxytetracycline was added instead of streptomycin and chloramphenicol. All data were transformed to compensate for the possibility of overlapping colonies, using Gregory's (1948) multiple infection transformation. A mean colony size of 0.028 cm^2 at evaluation time was assumed, based on the ability to discriminate colonies on the agar plate. The transformed number of colonies $N_{S,t}$, with a Petri plate area of 56.7 cm^2 and N_S, the number of counted colonies per Petri plate, was calculated as $N_{S,t} = (-\ln(1- 0.028*N_S/56.7)*N_S)+N_S$.

Detection in root tissue.

To assess root colonisation, soil was washed off the roots under a gentle flow of tap water on a sieve with 0.25 mm pore size. Subsequently the roots were washed for 20 min in running tap water, rinsed twice in sterile water and blotted dry on sterile filter paper. The root system was cut into three parts, viz. the regions 0-3, 3-6 and >6 cm below the stem base, respectively. Each part was transferred to a plastic EA plate (diameter 8.5 cm). The roots were placed apart using preparation needles and pressed into the medium using a spoon. Root length was determined by automatic image analysis using a Quantimet 570 (Leica). Roots were then incubated for at least 7 days in the dark at 20-22 °C and regularly examined for growth of *V. dahliae*. The number of colonies obtained was adjusted for possibility of overlapping colonies assuming a mean colony length at evaluation of 2.5 mm. This length was based on observation of the plates and corresponds with the lengths of 2 and 2-3 mm reported by Evans and Gleeson (1973) and Huisman (1988b), respectively. The transformed number of colonies on the root, $N_{r,t}$, is given by $(L/0.0025)*(-\ln(1-0.0025*(N_r/L)))$, with root length L (m) and number of observed colonies of *V. dahliae* N_r on the root system.

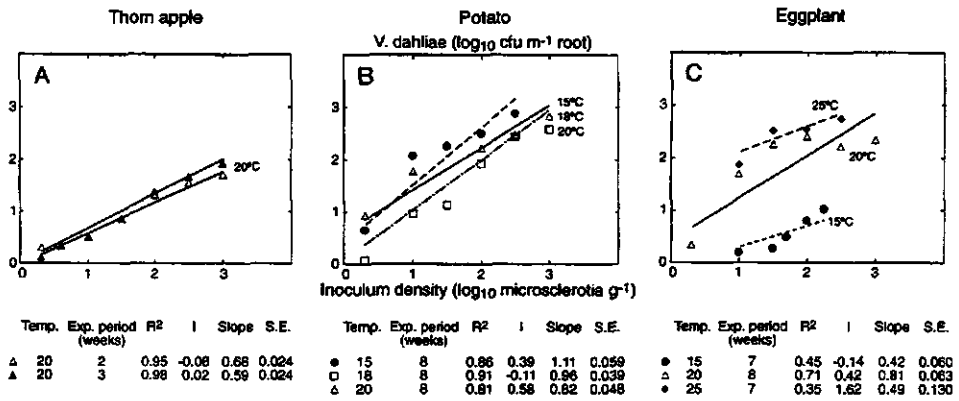
Statistical design and data analysis

All experiments were designed as randomised blocks with 10 to 12 replicates and each pot representing an experimental unit. Data of stem and root colonisation were analysed after l $\log_{10}(N_t+1)$ transformation. Root and stem colonisation were regressed on $\log_{10}$(inoculum density g^{-1} soil + 1) and homogeneity of regression slopes was tested. Stem colonisation was also regressed on root colonisation and Pearson correlation coefficients calculated between root and stem colonisation. In all analyses zero's were included.

Results

Assays for V. dahliae in plant tissue

Two methods and three media were evaluated to assess the density of *V. dahliae* in stems. Colonies developed on all media. Pipetting infested plant sap on EA (solidified alcohol agar medium) without salts, on EA with salts, on pectate medium or mixing the sap with molten EA yielded 67, 7, 14 and 5 cfu ml^{-1} sap, respectively (N=10). With pipetting sap on EA without salts the highest density ($P<0.05$) was obtained. This method was used in all further experiments.



	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
△	20	2	0.95	-0.08	0.68	0.024
▲	20	3	0.98	0.02	0.59	0.024

	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	8	0.86	0.39	1.11	0.059
□	18	8	0.91	-0.11	0.96	0.039
△	20	8	0.81	0.58	0.82	0.048

	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	7	0.45	-0.14	0.42	0.060
△	20	8	0.71	0.42	0.81	0.063
♦	25	7	0.35	1.62	0.49	0.130

Fig 1. Log-log relationships between inoculum densities of *Verticillium dahliae* in soil and colony forming units per unit root length at different temperatures (°C) and experimental periods (weeks) for thorn apple (A), potato (B) and eggplant (C). Data were analysed by regression analysis which was statistically significant in all experiments ($P<0.001$). R^2, intercept (I), slope and standard error of slope (SE) are presented.

Relationship between inoculum density and colonisation *of stems and roots*

The relationship between inoculum density in soil inoculated with viable microsclerotia and root colonisation is shown in Figure 1. In all experiments, a significant linear relationship was found between I log_{10}-transformed numbers of microsclerotia g^{-1} soil and log_{10}-transformed numbers of cfu m^{-1} root, for all test plant species and all temperature regimes. Slopes of the lines varied between 0.42 and 1.11 (Fig. 1). At densities □ 1 microsclerotium g^{-1} soil root systems were colonised in all pots. For potato, slopes of the lines were higher at higher at 15 °C than at 20 °C ($P<0.05$).

The relation between soil inoculum density and stem colonisation is shown in Figure 2. Log_{10}-transformed numbers of *V. dahliae* ml^{-1} sap showed a linear relation with

log_{10}-transformed numbers of microsclerotia g^{-1} soil ($P<0.01$) except at 15 °C with potato. The coefficient of determination increased with increasing temperature.

With eggplant, root and stem colonisation were significantly correlated at 20°C and 25 °C ($P<0.01$) with Pearson's correlation coefficients of 0.41 and 0.53, respectively, but not at 15 °C. Regression analysis of density in the stem on soil inoculum density resulted in lower coefficients of determination than regression of root colonisation on soil inoculum density (Figures 1 and 2).

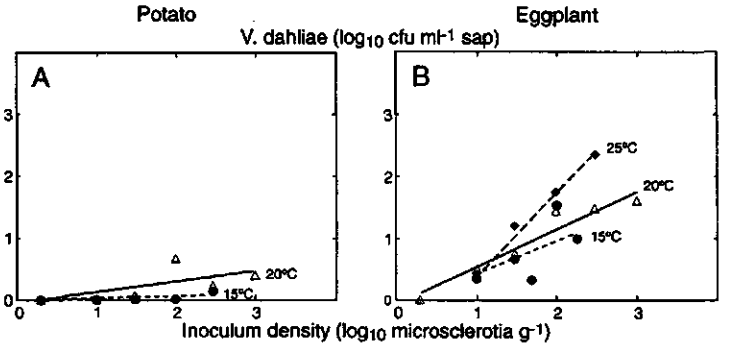
Stem colonisation generally increased with increasing soil inoculum density (Table 1). Occasionally, typical symptoms of disease developed as unilateral chlorosis and/or wilting of leaves. In the series with eggplant at 25 °C, contamination with *V. dahliae* resulted in a low level of infection in the noninoculated control. Most probably this occurred by dispersal of Verticillium propagules present in water or soil during handling processes in the growth chambers.

The pathozone width was calculated for eggplant at 15 and 20 °C on the basis of the data on inoculum densities and root colonisation, using the displacement model (Gilligan, 1985), excluding the mean radius of the microsclerotia and assuming an average root diameter of 0.34 mm. The width ranged between 0.1 mm and 0.3 mm and did not differ between inoculum densities and temperature ($P<0.05$).

Table 1. Proportion of stems of eggplants and potatoes infected by *Verticillium dahliae* in relation to inoculum density in soil.

Experimental conditions				Inoculum density in soil (number of microsclerotia g^{-1} soil)						
Test plant	Temperature	Experimental period (weeks)	Detection method	0	1	10	30	100	300	1000
Eggplant	15°C	8	sap	0[1]	-[2]	0.20	0.20	0.56	-	-
			segment	0	-	0	0	0.40	-	-
	20°C	8	sap	0	0	0.20	0.50	0.60	0.60	0.60
			segment	0	0	0.70	0.70	0.40	0.70	1.00
	25°C	7	sap	0.11	-	0.18	0.50	0.58	0.83	-
			segment	0.11	-	0.10	0.40	0.66	0.92	-
Potato	15°C	8	sap	0	0	0	0	0	0.10	-
			segment	0	0	0	0	0.10	0.30	-
	18°C	8	sap	0	0	0	0	0	0	0
			segment	0	0	0	0	0	0	0.10
	20°C	8	sap	0	0	0	0.10	0.30	0.20	0.33
			segment	0	0	0	0	0.50	0.50	0.80

[1] Each number represents the proportion of infected stem portions found among 10-15 plants examined.
[2] Inoculum density not included.



	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	8	0.04	-0.02	0.04	0.027
△	20	8	0.07	-0.04	0.17	0.071

	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	7	0.11	-0.11	0.53	0.19
△	20	8	0.26	-0.07	0.61	0.13
◆	25	7	0.26	-1.00	1.37	0.34

Fig 2. Log-log relationships between inoculum densities of *Verticillium dahliae* in soil and colony forming units per ml plant sap at different temperatures (°C) and experimental periods (weeks) for potato (A) and eggplant (B). Data were analysed by regression analysis which was significant in all experiments ($P<0.01$) except at 15 °C with potato. R^2, intercept (I), slope (SI) and standard error of slope (SE) are presented.

Influence of soil temperature and moisture level on colonisation *of roots and stems*

At 20 °C and a soil moisture level of 10%, the basal part of the stem was colonised by *V. dahliae* in 100% of the plants examined 8 weeks after sowing (Table 2). Infection tended to be faster at the low soil moisture level. Root colonisation remained at the same level during the experimental period at 20 °C, but decreased from 7 weeks after sowing at 25 °C (Fig. 3).

Discussion

Differences in root colonisation among plant species observed in the present research and elsewhere (Huisman and Gerik, 1989; Evans *et al.*, 1974) can be due to various factors, e.g. root morphology, growth rate and growth pattern of roots, phytoalexin production and the amount and composition of root exudates. The latter is probably the most important factor. Exudation depends not only on plant species but also on environmental conditions and on microbial activity in the rhizosphere (Schreiber and Green, 1963). We aimed to standardise the recolonisation and microbial activity of soil by cropping the steamed soil with wheat. However, random deposition of airborne microbes on the freshly steamed soil can then still affect the process of recolonisation. In most experiments (15°C and 20°C with potato and 20°C with eggplant), the same batch of recolonised soil was used and consequently the factors mentioned above could not have been major sources of variance. The densities for root colonisation in our experi-

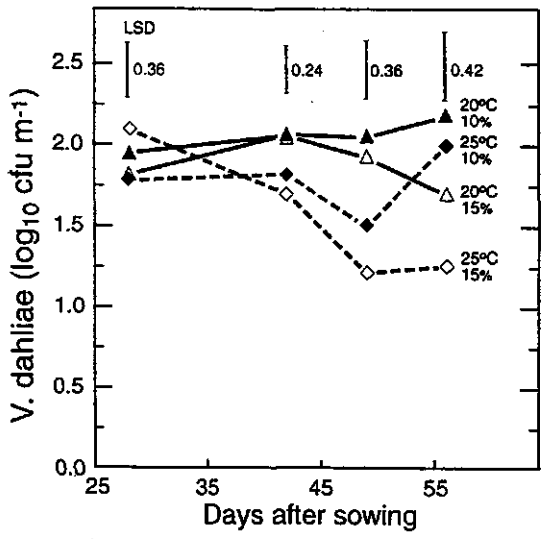


Fig 3. Colony density of *Verticillium dahliae* per unit root length at two temperatures (°C) and two soil moisture levels (%). Least significant difference ($P<0.05$) is shown.

ments were in the same range as those reported by Evans *et al.* (1974) and Gaudreault *et al.* (1995) who performed their experiments also under controlled conditions. Root colonisation densities in the field are often lower (Huisman, 1988b, Nagtzaam, 1995). This may be related to the interruption of the germination and infection processes due to different temporary biotic and abiotic conditions in the field.

Soil inoculum density and the population density in or on roots showed a $\log_{10}$-$\log_{10}$ relationship. This is in agreement with the observations of Evans *et al.* (1974) and Huisman (1988a) who found that the number of colonies on the root was directly proportional to inoculum density in soil. The linear relationship on $\log_{10}$-$\log_{10}$ scales does not support occurrence of extensive multiplication of *V. dahliae* neither on or in roots or in soil, nor does it support systemic colonisation of a large part of the root system in soil or extensive growth in the root cortex, as this would lead to deviations from a linear relation on $\log_{10}$-$\log_{10}$ scale (Huisman and Gerik, 1989).

Several authors demonstrated a correlation between symptom expression and stem colonisation by *V. dahliae* (Busch and Schooley, 1970; Hoyos *et al.*, 1991; Ordentlich *et al.*, 1990; Schreiber, 1992). Results of field experiments conducted by Ordentlich *et al.* (1990) indicated a correlation between stem colonisation and yield of potato tubers. In the present study, the relationship between inoculum density in soil and population density of *V. dahliae* in stem sap was linear on a $\log_{10}$-$\log_{10}$ scale. Also significant correlations were obtained between population density on/in roots and in sap

extracted from stems. We emphasise that our results are based on only one type of soil and with inoculum added under controlled conditions. Therefore, the significance of our results for other conditions is difficult to infer. Estimating population densities of *V. dahliae* in stem sap in addition to densities on the root provides extra information in studies on effects of environmental factors on the dynamics of *V. dahliae* in the soil or in the plant. The population densities in stem sap of the test plants were similar to those reported for potato by Hoyos *et al.* (1991). Although colonisation of the roots was higher with potato than with eggplants, the stems were not more colonised and infected. Apparently, in potato the ascent and proliferation of the pathogen in stems is more limited by physical or chemical barriers than in eggplant.

Table 2. Proportion of eggplant stem portions infected by *Verticillium dahliae* at four experimental periods, two temperatures and two soil moisture levels at an inoculum density of 300 microsclerotia g^{-1} soil.

Experimental period (weeks)	Temperature and soil moisture level			
	20 °C		25 °C	
	10% -6.2	15% -4.2	10% -6.2	15% moisture content -4.2 kPa
6	0.7[1]	0.5	0.8	0.5
7	0.8	0.6	1.0	0.6
8	1.0	0.9	0.9	0.9

[1] Each number represents the proportion of infected stems among 10 plants examined.

The frequency of stem colonisation never reached 100%, and in some cases remained lower than expected at an inoculum density of 300 microsclerotia g^{-1} soil. In field soil, disease incidence of >90% has been reported from 5 ms g^{-1} soil onwards (e.g. Nicot and Rouse, 1987). The absence of nematodes in the soil used for our bio-assays might have played a role. Moreover, we applied in our experiments individual microsclerotia instead of aggregates of microsclerotia in plant residues which probably have a higher infection capacity.

Correlations of soil inoculum density were higher with root colonisation than with stem colonisation. This is easily explained as root colonisation is one of the processes involved between germination of microsclerotia and stem colonisation. It could be argued

that the lower correlation coefficients observed at suboptimal temperatures for *V. dahliae* (Figures 1 and 2) reflect the lesser activity of *V. dahliae* at these temperatures.

Among the plant species, eggplant had the highest infection level of stems. Therefore, we recommend a bioassay with eggplant at a temperature of 20 °C, at a matric potential of -6.2 kPA and an experimental period of 8 weeks. A temperature of 20°C is preferred as colonisation of the root system was constant over time (Fig. 3), whereas variation in stem and root colonisation increased at lower temperatures (Fig. 1 and 2). An experimental period of at least 8 weeks and a soil inoculum density of 300 microsclerotia g^{-1} soil is recommended to assure a sufficiently high infection percentage (Tables 1 and 2). At first sight this density seems rather high compared with population densities in infested soils. For example, Bollen *et al.* (1989), Davis *et al.* (1996), Melero-Vara *et al.* (1995) and Tjamos and Paplomatas (1987) reported soil inoculum densities of 17-27, 40-70, 2-85, 1-28 microsclerotia g^{-1} field soil, respectively. However, under a highly susceptible crop, populations can mount up to 1500 propagules g^{-1} soil (Jordan, 1971). In cotton fields in California with an incidence of Verticillium wilt, densities usually range between 100 and 200 viable propagules g^{-1} soil (Schnatthorst, 1981). Moreover, estimates of numbers of propagules based on recovery from soil should be considered with caution. They often lead to underestimation of the actual population since recovery ranges from 1-60% dependent on the isolation methods (Nicot and Rouse, 1987; Termorshuizen, 1995).

Root colonisation by *V. dahliae* depends on the ability of the microsclerotia to germinate, grow and infect. These processes are affected by the secretion of exudates by the host plant. Our calculations of the pathozone width, indicating that microsclerotia must be present within a distance of 0.1-0.3 mm from the root surface in order to infect the root, support the stimulatory role of root exudates for germination of microsclerotia. The estimated pathozone width corresponds with data available in the literature for *V. dahliae*. Huisman and Gerik (1989) calculated a pathozone width of 0.13-0.43 mm for different host plants. Olsson and Nordbring-Hertz (1985) and Mol and Van Riesen (1995) found that microsclerotia germination percentages decreased rapidly to background levels at distances of 5 mm and 1 mm from the root, respectively.

In summary, a bioassay for determining effects of (a)biotic factors on development of *V. dahliae* in the plant is recommended with eggplants as a test plant grown in soil infested with 300 single, viable microsclerotia g^{-1} soil at a matric potential of -6.2 kPa and incubated at 20 °C for 8 weeks.

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Chapter 3

Long shelf life of *Talaromyces flavus* in coating material of pelleted seed

Abstract

Spores of the biocontrol agent *Talaromyces flavus* were recovered from coating material of Chinese aster and tomato seeds in which they were incorporated 17 years before. The seeds had been stored at room temperature. About 20% of the ascospores had retained their heat resistance and survived treatments in aqueous suspension at 60 °C for 30 min. None of the Chinese aster seeds and 90% of tomato seeds germinated after the storage period. Presence of *T. flavus* during storage had not affected germinability of the seeds.

For commercialisation of a biological control agent, a long shelf life is a strong point in favour (Bowers, 1982; Powell and Faull, 1989; Rodgers, 1993; Taylor and Harman, 1990). According to Rodgers (1993), convential distribution chains for agrochemicals dictate that shelf lives of one to two years are requested for products stored under ambient conditions. Data on the shelf life of biocontrol agents applied to seeds in order to protect the seed and the young plant against seed- or soil-borne pathogens are scant. Gordon-Lennox *et al.* (1987) found that *Pseudomonas* sp. retained its viability for 120 days on sugar beet seeds that were treated with a suspension of the bacteria and that *Chaetomium globosum* survived for 2.5 years on seeds that were treated with ascospores in a methyl cellulose formulation. Suslow and Schroth (1982) otained viable populations of rhizobacteria from sugar beet seeds treated with the bacteria in methyl cellulose and stored for one year. This paper presents an observation on the longevity of propagules of *Talaromyces flavus* (Kloecker) Stolk and Samson in the pellet material of Chinese aster and tomato seeds.

T. flavus is a potential antagonist for the biocontrol of a range of soil-borne plant pathogens, e.g. *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* (Adams, 1990; Fravel *et al.*, 1986; Marois *et al.*, 1982). Isolate F66 was used for pelleting Chinese aster and tomato seeds. It was selected out of 20 isolates obtained from heat-treated greenhouse soils (70 °C, 30 min) and was shown to inhibit growth of fungi and bacteria *in vitro* (Bollen and Van der Pol-Luiten, 1975).

For production of ascospores, an autoclaved soil-oatmeal medium (5% oatmeal, w/w) was inoculated with three 20-mm-discs from the edge of cultures on potato dextrose agar (PDA) plates. After two months, when cleistothecia were abundantly present, the soil-oatmeal culture was thoroughly dried and ground in a mill to particles smaller than

45 µm. In January 1976, the round culture medium containing 10.4 (±1.6) x 10^7 ascospores g^{-1} was incorporated in the pellet of seeds of Chinese aster (*Aster chinensis*, Super Choice Mixted) and tomato (*Solanum lycopersicum* cv. Primset). The pellet material consisted of the *T. flavus* culture (40%), quartz flour and a polymer binder. Pelleting was performed by a commercial company. The procedure was by the split pill process as being used for commercial pelleting of seeds with clay products. In the control treatment the seeds were pelleted following the same procedure but using autoclaved soil-oatmeal cultures in stead of living ones. Originally, the seeds were used in a pilot experiment on the efficacy of *T. flavus* in controlling damping-off pathogens. Density of the ascospore population in the pellet material after the coating procedure was not assessed. It was only afterwards that it was decided to assess the stored seeds for survival of the ascospores. In June 1993, the pellet material of seeds that had been stored in the dark at room temperature since 1976 was assayed for *T. flavus* spores by plating it onto PDA plates. A heat treatment as being used for selective isolation of *T. flavus* from soil (Boosalis, 1956; Bollen and Van der Pol-Luiten, 1975) was included. From the pellets, the seeds were removed and the coating material was pulverised with pestle and mortar and subsequently suspended in sterile, distilled water and given a heat treatment for 30 min at 60 °C in a thermostatic stirring water bath. The temperature was recorded by a thermocouple placed in the test tubes. Dilution series up to 10^3 were made of heat-treated and untreated samples. Aliquots of 0.4 ml of the suspensions were spread on PDA plates with 50 ppm oxytetracycline to prevent bacterial growth (five plates for each dilution). The plates were incubated in the dark at 28 °C for 5-7 days and the number of *T. flavus* colonies recorded.

Part of the population of *T. flavus* had survived 17 years storage in the pellet material of Chinese aster and tomato seeds (Table 1). The coating of aster and tomato seed contained approximately 1000 and 1200 spores per seed, respectively; this is about 0.5% of the amount initially applied to the coating material before the pelleting process. Since the assessment allow comparison of spore densities in the coating material before pelleting with those after the 17-years storage period, survival during the pelleting procedure and that during the subsequent storage period could not be distinguished separately. A long shelf life of the fungus is in line with the observation of Beuchat (1992) who found that in fruit powders, ascospores of *T. flavus* survived storage up to 30 months without loss of viability. In dual cultures the re-isolate inhibited growth of a number of soil fungi and bacteria in the same way as the original isolate did, demonstrating that production of antibiotics was unaffected. The fungi were *Fusarium oxysorum* f.sp. *melongenae*, *Phomopsis sclerotioides*, *Pythium sylvaticum*, *Rhizoctonia solani* and *Verticillium dahliae*; the bacteria were *Bacillus subtilis* and *Streptomyces* sp.

Part of the population had retained its heat resistance after the storage period (Table 1). The propagules in the substrate that was used for coating 17 years before were ascospores and had shown to endure the 60 °C treatment for 100%.

After removal of the coating material, seeds were incubated on wet filter paper for assessment of germinability. Germination was assessed in eight replicates with 50 seeds each, arranged as eight randomised blocks and placed in a climate room at 20 °C. None of the aster seeds and 90% of the tomato seeds germinated. Presence of *T. flavus* in the pelleting material during storage did not affect germination.

Table 1. Viable counts of *T. flavus* in coating material of pelleted seeds stored for 17 years and germination of the seeds.

Treatment	Tomato		Chinese aster	
	Coated with viable spores	Coated with autoclaved spores	Coated with viable spores	Coated with autoclaved spores
	Number x 10^3 g^{-1} on PDA			
None	34.7 ± 2.3[1]a[2]	0	137.5 ± 14.9 a	0
60 °C, 30 min	8.5 ± 0.9 b	0	25.4 ± 0.9 b	0
	Germination of seeds (%)			
None	92.7 ± 1.9	87.8 ± 1.1	0	0

[1] Mean and standard error of data of five replicate lots.
[2] Data followed by a different letter are different at P = 0.05 according to the Wilcoxon-Mann-Whitney test.

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Chapter 4

Colonisation of roots of potato and eggplant by *Talaromyces flavus* from coated seed

Abstract

Talaromyces flavus (Klöcker) Stolk & Samson, a fungus that has potential for biocontrol of *Verticillium dahliae*, colonised potato and eggplant roots. In pot experiments with field soils, the fungus moved from potato seed tubers and eggplant seeds coated with ascospores to developing roots, including the root tips, albeit at a low density. The population of *T. flavus* decreased log-linearly with distance from the seed and was higher with potatoes than with eggplants. Results suggest that passive carrying along the growing root is one of the main factors involved in colonisation by the fungus. A monomolecular model for root colonisation and soil inoculum density (R^2=0.94) and a negative exponential model for the relationship between *T. flavus* soil inoculum density and *V. dahliae* density on the roots (R^2=0.55) were fitted.

Introduction

Talaromyces flavus has been used in controlling Verticillium wilt of eggplant (e.g. Marois *et al.*, 1982) and has shown the ability to suppress early dying of potato under commercial production conditions (Fravel *et al.*, 1986; Nagtzaam, 1995). The fungus is a common inhabitant of cultivated soils (Fravel and Adams, 1986; Gams, 1992). The addition of spores of *T. flavus* to natural soils was followed by an increase of soil respiration and an increase of population of the fungus in soil (Marois and Fravel, 1983). A preference for the rhizosphere as a habitat, notably near the root tip (Tjamos and Fravel, 1995), has been reported for various solanaceous hosts (Marois *et al.*, 1984). Fahima and Henis (1990) noticed the spread to new roots and formation of conidiophores of *T. flavus* on microsclerotia of *Verticillium dahliae* Kleb. on the root surface. *T. flavus* proliferated on diseased roots as its populations on *V. dahliae*-infected roots were larger compared to healthy uninfected roots (Fahima and Henis, 1995).

Microsclerotia of *V. dahliae* infect the roots primarily near the root tip throughout the whole growing season without previous extensive colonisation of the root surface (Fitzell *et al.*, 1980; Gerik and Huisman, 1988). On the roots, outside the tip region, nematodes provide infection courts (Conroy *et al.*, 1972; Perry and Evert, 1983; Storey and Evans, 1987) or may increase susceptibility of the roots to *V. dahliae* (Faulkner *et al*, 1970). Infection of the roots early in the growing season leads to more severe yield depression than infection later in the season. Therefore, biological control should aim at establishing high densities of the antagonist on the root at the start of the growing season, in particular near the tips.

Application of antagonists is by delivering them either to seed or to soil. The aims of our study were to assess: (a) the ability of *T. flavus* to move from the seed along the root under different conditions of temperature and soil type and in presence or absence of pathogen and host plant; and (b) the ability to colonise the roots from soil inoculated with the fungus.

Materials and methods

T. flavus isolates

Isolates TN2, TN11, TN12, TN41 and F508 were obtained from field soil and TN43 from potato roots in The Netherlands. Isolate Tf-1 originated from a sclerotium of *Sclerotinia minor* Jagger in the U.S.A. (Dunn and Lumsden, 1981).

Preparation of talcum inoculum and coating of seeds

Ascospore suspensions were obtained from 4-week-old cultures grown on potato dextrose agar (PDA) by scraping cleistothecia from the agar surface and suspending them in sterile water. The suspension was crushed for 2 min at 8,000 rev min^{-1} to liberate ascospores from the cleistothecia. It was subsequently filtered through cheesecloth to remove hyphal debris and mixed with talcum powder. The mixture containing 10^7 ascospores g^{-1} talcum powder was dried at 20°C by forced air, sieved (≤0.2 mm), and stored at 4°C until used. The final population of inoculum, assessed by dilution-plating assay, was 10^6 colony forming units (cfu) g^{-1} talcum powder. Seeds and tubers were disinfected in 1% NaOCl for 30 and 10 min, respectively, washed in sterile water, air dried and coated by mixing them with talcum inoculum. Coated seeds of eggplant (*Solanum melongena* L.) cv. Black Beauty and minitubers (Lommen and Struik, 1992) of potato (*Solanum tuberosum* L.) cv. Element contained 2 or 200 mg talcum powder equalling 2×10^3 or 2×10^5 ascospores per seed or tuber. Control seeds were coated with autoclaved talcum powder without ascospores.

Preparation of alginate wheat-bran inoculum and infestation of soil

The inoculum was produced according to Fravel *et al.* (1985). Ten grams of sodium alginate (Janssen Chimica) and 110 g of powdered wheat bran were mixed with a 500 ml aqueous suspension containing 10^9 ascospores in total. The mixture was dripped into a solution of 100 mM $CaCl_2$ where pellets were formed by polymerisation. After air-drying, the pellets were ground in a Retsch grinding mill (1.0 mm pore size) and stored at 4 °C for up to 6 weeks before use. The inoculum was incorporated into air-dried soil. Control treatments received autoclaved alginate wheat-bran inoculum without ascospores.

Bioassay system and assessment of T. flavus and V. dahliae in the rhizosphere

The test plants were eggplant and potato. A sandy soil (pH-KCl 7.0, organic matter content 0.3%) and a loamy sand (pH-KCl 6.2, organic matter content 2.7%) were used. The moisture content of both soil types was adjusted to 10% (w/w) corresponding to water potentials of -6.2 and -9.1 kPa, respectively. The plants were grown in pots (4 x 4 x 12 cm) with a bottom of 22.4 μm mesh polyamide screen incubated in growth chambers at a light intensity of 29 W m^{-2} at different temperatures. Soil moisture was maintained by compensating for water losses by adding half-strength Hoagland nutrient solution from the bottom.

At harvest, roots were shaken gently to remove non-rhizosphere soil and population sizes of *V. dahliae* and *T. flavus* on and in the roots were assessed. Densities of *V. dahliae* were assessed after washing the roots for 20 min under running tap water followed by two rinses in sterile water, blotting dry on sterile filter paper and plating on ethanol agar medium (Nadakavukaren and Horner, 1959), amended with the bacteriostatic antibiotic oxytetracycline (50 mg l^{-1}). Populations of *T. flavus* were estimated using four methods:

1. Direct plating of the roots on Marois medium (Marois *et al.*, 1984) modified by adding quintozene (14 mg a.i. l^{-1}) and replacing chlorotetracycline, streptomycin sulphate and chloramphenicol by oxytetracycline (50 mg l^{-1});
2. Washing the roots under running tap water for 20 min followed by rinsing twice in sterile water, blotting dry on sterile filter paper and plating on modified Marois medium;
3. As for (2) but including disinfestation of the roots in 1% NaOCl for 1 min, prior to washing;
4. Washing the roots under running tap water and suspending them in sterile water and heating them at 60 °C for 30 min, followed by plating on PDA.

The plates with roots on Marois or PDA medium were incubated for 10 days at 30 °C in the dark. In seven experiments the colonisation of eggplant and potato roots was studied after inoculation of seed (Exps 1-5) or both seed and soil (Exps 6 and 7). Details of the experiments and factors involved are summarised in Table 1.

T. flavus was quantified in three or four sections of the root system. These sections were made according to depth from the soil surface. The sections were plated in separate dishes. Root length was determined by automatic image analysis using a Quantimet 570 (Zoon and Van Tienderen, 1990).

The colonisation pattern of *T. flavus* on the root was determined in more detail by using a qualitative detection method. In Exp. 2, four 2-cm root segments were cut, starting from the tip from the primary root which was carefully freed of excess soil and

secondary roots. Each segment was cut into 20 sub-segments and transferred to modified Marois medium. A segment or sub-segment was rated as colonised if at least one colony of *T. flavus* was recorded.

Data analysis

Treatments were arranged in a randomised block design with 10 replicates and a factorial treatment structure. Analysis of variance was used to test for significant differences for treatments. To homogenise variances, the number of colony forming units was transformed to $\log_{10}$(cfu+1) prior to ANOVA. The relationship between the population density on root segments and that at some distance from stem base was evaluated for linear and quadratic responses using polynomial contrast analysis. Differences between colonisation percentages of root sections were analysed by the G-test for goodness of fit (Sokal and Rohlf, 1981). Non-linear regression procedures were used for fitting monomolecular and exponential models. In Exp. 7, means of treatments were separated by Duncan's multiple range test. Significance was evaluated at $P<0.05$ for all tests.

Table 1. Synopsis of factors tested in the experiments.

Experiment. no.	*T. flavus* isolates	*V.dahliae*[a]	Test plant[b]	Growing substrate	Temperature (°C)	Plating procedure[c]	Experimental period (weeks)
			Colonisation of roots after inoculation of seeds[d]				
1	TN11	0	E	Sand	12.5, 15, 20	2,3,4	1
2	TN41	0	E	Sand	20	Segments, subsegments, 1	3
3	TN41	0	P, E	Sand, loamy sand	15, 20	2	3
4	TN41	0, 100	P, E	Sand, loamy sand	20	1,2,4	3
5	TN41	300	P	Sand	20	2	8
			Colonisation of roots after inoculation of seeds[d] and soil[e]				
6	TN2, TN11, TN41, TN43, F508, Tf-1 and a mixture[f]	10	E	Sand	20	4	8
			Colonisation of roots after inoculation of soil[e] only				
7	TN41	0, 250	E	Sand	15	2	3

[a] Number of microsclerotia of *V. dahliae* g^{-1} soil.

[b] E = eggplant; P = potato.

[c] 1 =direct plating of roots; 2 = plating of washed roots; 3 = the same as 2, but including disinfection of roots; 4 = plating of heat-treated roots (60 °C for 30 min). Details are described in Materials and Methods.

[d] Seeds were coated with *T. flavus* formulated in talcum

[e] Soil was amended with *T. flavus* in talcum (Exp. 6) or in alginate wheat bran (Exp. 7), both at 0.5% (w/w)

[f] Mixture of isolates TN11, TN12, TN41 and TN43.

Results

Colonisation of roots from coated seeds (Exp 1-5)

Tubers of potato (*Solanum tuberosum* L. cv. Element) and seeds of eggplant (*Solanum melongena* L. cv. Black Beauty) were treated with ascospores of *T. flavus* incorporated in talcum powder and sown in pots of soil infested or non-infested with *V. dahliae*. After 1-8 weeks, populations of *T. flavus* were assessed (Table 1).

Table 2. Effects of temperature and detection procedure on recovery of *T. flavus* strain TN11 from 1 week old seedlings of eggplant grown from coated seed (Exp. 1).

Temp (°C)	Detection procedure[a]	Cfu m^{-1} root[b]			Polynomial[c] contrasts	
		Distance along root from seed (cm)			Significance level[b]	
		0-3	3-6	>6	1st order	2nd order
12.5	4	0.3	0.1	0	0.0699	0.9234
15.0	4	1.7	0.8	0	0.0091	0.8312
20.0	2	2.1	0.1	0	0.0105	0.1486
	3	0.7	0.1	0.1	0.0255	0.1921
	4	7.7	1.0	1.0	0.0000	0.0004

[a]2 = plating of washed roots on selective medium; 3 = the same as 2, but including disinfestation of roots; 4 = plating of heat-treated roots (60 °C for 30 min). Details are described in Materials and Methods.
[b]Back-transformed values.
[c]Based on logarithmic values.

The modified Marois medium allowed to distinguish individual colonies of *T. flavus* to grow from plated roots. The numbers of *T. flavus* as mentioned in Tables 2 and 4 differed significantly (ANOVA, $P<0.05$) for the detection procedures applied. Highest numbers were obtained after heat treatment of the roots (Method 4). The other procedures yielded the same results as the standard procedure compromising direct plating of roots on modified Marois medium (Method 2).

In all experiments, and in particular with potato as a test plant, the rootlet region near the seed became readily colonised by *T. flavus*. Within 1 week after sowing, the fungus had established itself on the root of eggplant, albeit at a low density (Table 2). After 3 and 4 weeks colonisation had extended, but densities remained low. Numbers on the root were lower with increasing distance to the inoculum source (Tables 3 and 4, Fig. 1). After 8 weeks numbers were 48, 17, 9.9 and 8.1 cfu m^{-1} root at 0-2, 2-4, 4-6 and >6 cm distance from the potato tuber, respectively (Exp. 5, Table 5). In most experiments, the numbers of *T. flavus* on the root sections decreased log-linearly and in some, log-

exponentially with increasing distance from the inoculum source as indicated by polynomial contrasts. Higher numbers were recorded on potato than on eggplant but differences between densities on roots in loamy sand and in pure sand were not significant at P<0.05 (Table 4 and Fig. 1). The results do not allow a definite conclusion on the influence of temperature to be drawn. In Exp. 2 (Table 2), colonisation was more extended at 20 °C than at lower temperatures while it was not significantly different at 15 and 20 °C in Exp. 3 (Fig. 1). Populations of *T. flavus* presented in Table 4 were significantly (P<0.05) lower in *V. dahliae*-infested soil than in non-infested soil.

Table 3. Distribution of *T. flavus* TN41 on eggplant roots after incubation at 20 °C for 3 weeks (Exp. 2).

Root section	Percentage segments (2 cm) or subsegments (1 mm) colonised				G-test[b]
	Distance along root from the root tip (cm)				
	0-2	2-4	4-6	6-8	
Segments	29	29	21	0	+
Sub-segments	2.7	2.7	0.7	0	-

[a]Direct plating of roots (method 1). Details are described in Materials and Methods;
[b]G-test for goodness of fit; fractions differ significantly (+) or not (-) between root sections at P<0.05.

Colonisation of roots after inoculation of both seed and soil (Exp. 6)

In Exp. 6, seeds of eggplant coated with talcum inoculum of different *T. flavus* isolates were sown in soil amended with the talcum inoculum at a rate of 0.5% (dw/dw). Colonisation of roots was assessed after 8 weeks. With strain TN41, an additional treatment was included by coating only the seed and not applying inoculum to soil.

The amendment of the soil with talcum inoculum in addition to coating the seeds resulted in an increased root colonisation (Fig. 2). The isolates differed in colonisation intensity on roots. Application of a mixture of isolates did not result in a more intensive colonisation than with single isolates.

Colonisation of roots from inoculated soil (Exp. 7)

In Exp. 7, the sandy soil was infested with 250 viable microsclerotia of *V. dahliae* g^{-1} soil and amended with *T. flavus* alginate wheat-bran inoculum at rates of 0.001, 0.01, 0.1,

0.5 or 1.0% (dw/dw). Five additional treatments were included: (1) no additions at all; (2) addition of only *V. dahliae*; (3) addition of only *T. flavus* wheat-bran inoculum at 0.1%; (4) addition of only autoclaved alginate wheat-bran formulation product (1%); and (5) addition of 0.1% *T. flavus* wheat-bran inoculum combined with 0.9% autoclaved formulation product to assess the effect of alginate wheat-bran on *T. flavus*. The density of *T. flavus* in alginate wheat-bran inoculum was 10^6 cfu g^{-1}. One gram of inoculum contained 117000 particles of alginate wheat-bran product and 75% of the particles that were plated onto PDA yielded colonies of *T. flavus*.

A monomolecular model $y=K(1-Be^{-rx})$ according to Madden and Campbell (1990) was fitted for *T. flavus* root colonisation by non-linear regression in which y is the density of *T. flavus* on the root ($\log_{10}$ cfu+1) and x its soil inoculum density. Estimated values for the parameters were: K=0.45, B=0.993 and r=6.4 with R^2=0.94.

For the relationship between soil inoculum density of *T. flavus* and *V. dahliae* density on the roots, a negative exponential model $y=ae^{rx}$ was fitted, where y and x are the densities of *V. dahliae* on the root ($\log_{10}$ cfu+1) and of *T. flavus* in soil, respectively.

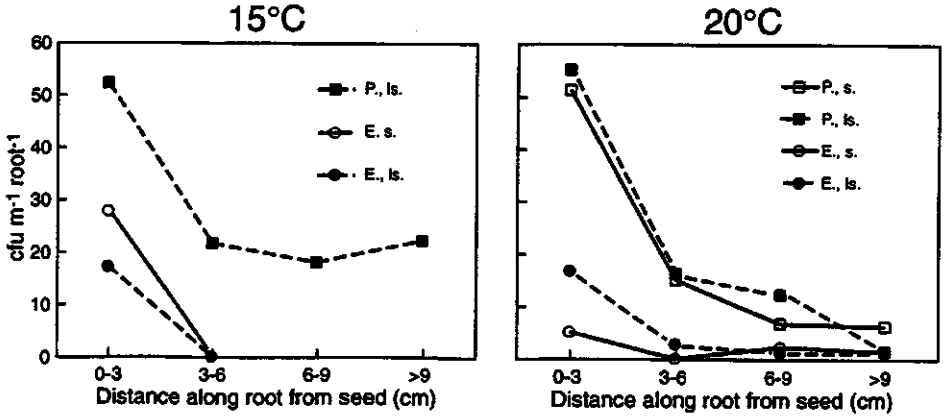

Fig. 1. Effect of temperature on colonisation of roots of eggplant (E) and potato (P) in sand (s) and loamy sand (ls) after inoculation of seeds by *T. flavus* TN41 (Exp. 3). Polynomial contrasts 1st and 2nd-order were significant ($P<0.05$) for all treatments; data for colonisation of eggplant roots in sandy soil and in loamy sand at 15 °C were not suitable for polynomials contrasts.

The parameter values estimated were: a= 0.20, r=-5.53 and R^2=0.55, respectively. Numbers of *T. flavus* and *V. dahliae* on the roots were negatively correlated (R^2=0.36 with a significance level of P=0.003).

Colonisation of roots by *V. dahliae* was also impeded by applying the formulation product alone at a concentration of 1% w/w autoclaved alginate wheat-bran granules (Table 6). It suppressed *V. dahliae* to the same extent as did 0.5 % formulated *T. flavus* inoculum. Autoclaved formulation product not only inhibited colonisation by *V. dahliae* but also that by *T. flavus* (Table 6; 0.1% *T. flavus* plus 0.9% alginate wheat-bran product compared to 0.1% *T. flavus* inoculum).

The fresh weights of eggplants were reduced by applying 0.1% *T. flavus* plus 0.9% alginate wheat-bran product or the autoclaved formulation product alone (Table 6). However, the presence of *T. flavus* in the formulation product mitigated this adverse effect on the growth because a greater fresh weight was obtained by applying *V. dahliae* and 1% *T. flavus* than by applying *V. dahliae*, 0.1% *T. flavus* and 0.9% formulation product. The presence of *V. dahliae* did not affect the growth of eggplant during the short experiment (3 weeks).

Discussion

Coating seeds with biocontrol agents requires substantially less inoculum than application to soil. Seed inoculants have the opportunity to be the first colonisers of roots and rhizosphere (Windels, 1981; Chao *et al.*, 1986). Our study clearly shows that *T. flavus* colonised roots originating from coated seeds in a non-sterile soil to at least 9 cm from the seed and that this population was maintained on the root for at least 7 weeks. Even with a small amount of inoculum on the eggplant seeds (2×10^3 spores $seed^{-1}$), *T. flavus* was recovered at >6 cm from the inoculum source (Table 4 and Fig. 1).

Colonisation of the root system from coated seed was fast, but the size of the antagonist population was low (<100 cfu m^{-1} root). To the best of our knowledge, no data are available on colonisation by antagonists from coated seeds where density is expressed per unit root length. Therefore, comparisons can only be made with data on numbers of cfu in the rhizosphere. Around roots of seedlings grown from coated seeds, Ahmad and Baker (1987) found 10^2-10^8 cfu g^{-1} rhizosphere soil for *Trichoderma harzianum* and Garibaldi *et al.* (1990) found 10^2-10^6 cfu g^{-1} for *Fusarium oxysporum*. However, the results of dilution plating of macerated root tissue or rhizosphere soil are probably strongly dependent on the procedures used. For *T. flavus*, a major factor is whether or not the cleistothecia or asci are crushed and ascospores are set free to yield individual colonies. Densities of 10^2-10^4 cfu g^{-1} root tissue or rhizosphere soil were found

Table 4. Effect of soil type, test plant, detection procedure and presence of *V. dahliae* on spread and recovery of seed-inoculated *T. flavus* strain TN41 from different root sections 3 weeks after sowing (Exp. 4).

Soil type	Test plant	*V. dahliae*[a]	Detection procedure[b]	Cfu m^{-1} root[c]				Polynomial[d] contrasts	
				Distance along root from seed (cm)				Significance level	
				0-2	2-4	4-6	>6	1st-order	2nd-order
Sand	Potato	-	1	22.3	7.1	4.3	6.6	0.000	0.000
		-	2	24.1	9.4	11.8	12.1	0.044	0.117
		+	2	19.3	7.4	4.4	5.7	0.034	0.210
		-	4	75.4	32.7	27.9	36.1	0.000	0.000
	Eggplant	-	2	1.1	2.0	1.9	0.9	-[e]	-
		+	2	1.0	0	0	0	-	-
Loamy sand	Potato	-	2	46.3	20.8	17.9	13.0	0.000	0.002
		+	2	29.8	13.0	9.5	12.4	0.000	0.000

[a] Addition of *V. dahliae* at a density of 100 microsclerotia g^{-1} soil (+) or not (-).
[b] 1 = Direct plating of roots; 2 = plating of washed roots; 4 = plating of heat-treated roots (60 °C for 30 min). Details are described in Materials and methods;
[c] Back-transformed values.
[d] Analyzed on logarithmic values.
[e] Data not suitable for polynomial contrasts.

with this technique for *T. flavus* (Marois *et al.*, 1984; Tjamos and Fravel, 1995; Fahima and Henis, 1995). In a rhizospere competence assay with pathogenic *Fusarium* spp., Ocamb and Kommedahl (1994) demonstrated the distinctions between both detection techniques as about 30 cfu m^{-1} root corresponded with up to 10^4 cfu g^{-1} rhizosphere soil. Nonetheless, the question remains as to whether such low numbers of *T. flavus* on the root(tip) are sufficient to impede colonisation and subsequent infection by *V. dahliae*. In Exps 3 and 4, densities on the potato at which root colonisation by the pathogen was significantly suppressed in the eggplant, were similar (Table 6). In the field, only 3.7 cfu m^{-1} of *T. flavus* were observed in the treatment which resulted in a significant suppression of *V. dahliae* by *T. flavus* (Nagtzaam, 1995). These findings suggest that colonisation of the roots from coated seed can contribute to suppression of *V. dahliae*. This is especially true if the additional effect of water percolation on migration of *T. flavus* along the root, which was excluded in our studies by carefully watering the plants from the bottom, is also considered. The low density of *T. flavus* on the roots suggests that direct interaction was not the principal factor in suppression of *V. dahliae*. It is possible that induced resistance in the host plants is involved.

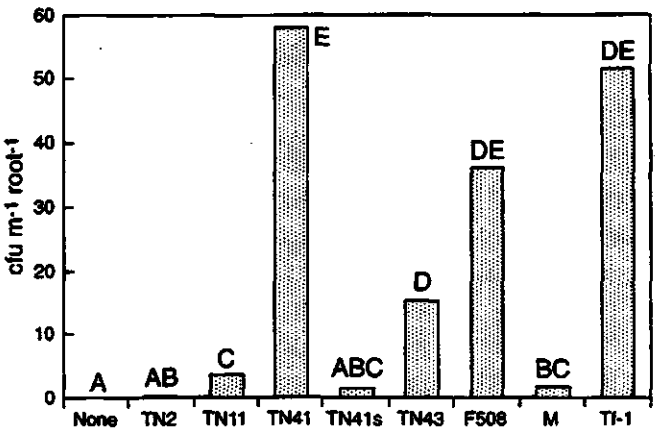

Fig. 2. Colonisation of roots of eggplant by seven isolates of *T. flavus* after 8 weeks in a sandy soil at 20 °C. M = a mixture of the isolates TN11, TN12, TN41 and TN43 (Exp. 6). Seed and soil were inoculated with talcum inoculum; TN41s = seed inoculation only; Values for each treatment labelled with the same letter are not significantly different according to Duncan's Multiple Range Test ($P<0.05$).

Fungi move along roots either by growth of mycelium or by passive movement of spores mediated by external factors, e.g. by soil, water or growing plant roots. Although our results strongly suggest that passive movement of *T. flavus* is involved, they do not provide definite evidence. The log-linear decrease in population density of *T. flavus* points to passive movement along the root since a rapidly-growing root and a slowly-growing micro-organism will lead to a decrease in population densities with increasing distance from the coated seed (Bowen, 1991). For bacterial movement along roots, this hypothesis was worked out in a mathematical model by Scott *et al.* (1995). They showed that bacterial densities decreased log-linearly along the growing root by carriage only. Passive transport, as a mechanism of colonisation by antagonists, has also been suggested for *Verticillium biguttatum*, a mycoparasite of *Rhizoctonia solani* (Van den Boogert, 1989). The densities of this mycoparasite indeed decreased with distance from the inoculum source. However, Ahmad and Baker (1987) working with *Trichoderma harzianum* and Garibaldi *et al.* (1990) working with *Fusarium* spp. obtained curves of a type (indicated as C-shaped by Ahmad and Baker, 1987) that is consistent with active spread. The presence of individual *T. flavus* colonies on remote parts of the root provides another indication of passive movement because growth implies that the root system would be continuously colonised between the seed and the youngest mycelium. If the root cap becomes colonised, fungal growth on the root is unlikely to be the only factor as

Table 5. Distribution of *T. flavus* TN41 on potato roots after incubation at 20 °C for 8 weeks (Exp. 5).

Treatment	Cfu m^{-1} root				Polynomial[a] contrasts	
	Distance along root from seed (cm)				Significance level	
	0-2	2-4	4-6	> 6	1st-order	2nd-order
T. flavus	47.9	17.4	9.9	8.1	0.000	0.043
Control	0	0	0	0	-[b]	-

[a] Analyzed on logarithmic values.
[b] Data not suitable for polynomial contrasts.

roots grow more rapidly (1 to 90 mm d^{-1} Lungley, 1973; Huisman, 1982) than *T. flavus* (on agar up to 3 mm d^{-1} at 20 °C; unpublished data). Fungal growth may be involved if the root cap is colonised by hyphae where, subsequently, conidia or asci are produced that might be passively transported by adherence of the spores to the root tips. Fahima and Henis (1990) observed conidiophores on microsclerotia of *V. dahliae* in superficial tissue of roots. We observed cleistothecia on roots of plants grown in test tubes under gnotobiotic conditions. Detection of *T. flavus* after a selective heat treatment which kills conidia and mycelium but not ascospores (Van der Spuy *et al.*, 1975), implies formation of cleistothecia with ascospores on roots grown in non-sterile soil. This is not unlikely as the exudation of sugars in the rhizosphere seems to provide the appropriate conditions for cleistothecia formation. Engelkes *et al.* (1993) recorded that a high C-to-N ratio stimulated ascospore production by *T. flavus*. Heat treatment activates germination of many soil-inhabiting ascomycetes (Warcup and Baker, 1963) and this applies also to *T. flavus* (Boosalis, 1956; Katan, 1985). Heat activation of ascospores explains why higher densities were recorded in detection procedures with heat treatment than in those without it (Tables 2 and 4).

Colonisation was not affected by the soil type or the temperature used in our study. We expected an effect by temperature since the growth rate of isolate TN41 on agar was 30% higher at 20°C than at 15°C (unpublished data). Davies and Whitbread (1989) emphasised that soil temperature has a complex effect on root colonisation as temperature influences not only the growth of a fungus, but also that of its competitors

Table 6. Colonisation of eggplant roots by *T. flavus* TN41 and *V. dahliae* after three weeks in soil infested with *V. dahliae* and inoculated with *T. flavus* at various densities (Exp. 7).

Treatment	Population density (Backtransformed cfu m^{-1} root)		Fresh weight of test plants (g)
	V. dahliae	*T. flavus*	
None	0 a[c]	0 a	0.19 bc
V. dahliae[a] only	66 c	0 a	0.15 b
V. dahliae and *T. flavus*[b] (% w/w)			
0.001	66 c	0 a	0.15 b
0.01	41 b	10 ab	0.18 bc
0.1	32 b	70 c	0.19 bc
0.5	5 a	40 d	0.20 bc
1	0 a	140 e	0.15 b
V. dahliae and formulation product only (1% w/w)	7 a	0 a	0.04 a
V. dahliae and *T. flavus* (0.1% w/w) and formulation product (0.9% w/w)	0 a	10 b	0.04 a
T. flavus (0.1% w/w) only	0 a	78 c	0.21 c

[a] 250 viable microsclerotia g^{-1} soil.
[b] Ascospores in alginate wheat-bran formulation product.
[c] Values followed by the same letter are different from each other according to Duncan's Multiple Range Test ($P<0.05$).

and root growth and exudation by the host plant. This may explain the difference in effects of temperature in the experiments. Passive colonisation may also interfere since it either is not, or is only slightly affected by temperature. An effect of temperature is only to be expected if both the population size of the antagonist on the root is influenced and an opportunity exists for contact between inoculum and the growing part of the root. The latter implies that the formation of antagonist propagules behind the root tip will not contribute to dispersion of the antagonist if transport by the downward flow of water is excluded by watering the plants from the bottom as was done in our experiments.

The monomolecular model fitted to the inoculum density of *T. flavus* in soil and its population density on the roots implies that the increase of number of colonies m^{-1} root declines with increasing amounts of soil inoculum. Our results indicate that augmenting the inoculum above 0.5% (w/w) does less to increase the density of *T. flavus* on the root than do lower application rates (Table 6). The value for parameter *K*, the

estimated maximum colonisation level, was 0.45. This implies that at most 180 colonies m^{-1} root can occur. The colonisation rate *sensu* Huisman (1988b), calculating the density on the root per applied fungal propagule to soil, is 14.5 for *T. flavus* and 15.9 for *V. dahliae* (10^{-3} colonies cm^{-1} root $propagules^{-1}$ g^{-1} soil). Under field conditions Huisman's colonisation rate is 0.9 for *T. flavus* and 5.5 for *V. dahliae* (calculated from data presented by Nagtzaam, 1995). In field experiments with cotton, Huisman (1988b) obtained colonisation rates of 0.6, 0.7 and 2.0 for *Trichoderma* spp., *Gliocladium* spp. and *V. dahliae*, respectively. In growth-chamber experiments, the colonisation rate of *T. flavus* was similar to that of *V. dahliae*. However, in the field, this potential of *T. flavus* apparently was not fully exploited since, under these conditions, *V. dahliae* was a more effective coloniser than *T. flavus*. *V. dahliae* is able to colonise the interior of the cortex (Gerik and Huisman, 1988). This opportunity to escape competition from other organisms on the root surface (Beckman and Talboys, 1981) may explain the higher colonisation rate of *V. dahliae* than *T. flavus* under field conditions.

We used talcum powder and alginate wheat-bran prill as carriers for *T. flavus*. Tjamos and Fravel (1995) have shown that drenching of soil with an ascospore suspension resulted in a more intensive colonisation of roots than inoculation with alginate prill. Fravel *et al.* (1995) found a significant interaction between formulation and soil type when studying survival and proliferation of *T. flavus* in soil. These results prompted Fravel *et al.* (1995) to state that formulations may need to be customised for soil type. The differences in the rate of colonisation of eggplant and potato indicate also the influence of the host plant. An obvious example, the surface structure of eggplant seeds and tubers of potato, differ considerably.

In greenhouse trials 6 weeks after inoculation of soil, Fahima and Henis (1990) found higher populations of *T. flavus* in *V. dahliae*-infested soil than in non-infested soil. In field experiments with potato, *V. dahliae* infestation did not affect populations of *T. flavus* on the root or in the soil (Spink and Rowe, 1989). In our study, colonisation by *T. flavus* was not affected (Table 6) or suppressed by *V. dahliae* (Exp. 4; Table 4). In Exp. 4, *T. flavus* was applied to the seed, in other experiments to soil. It is possible that the negative effect of *V. dahliae* on *T. flavus* is related to an extension of root length caused by the pathogen. For eggplant, mean root length significantly increased ($P<0.05$) by 2.4 and 0.9 m in *V. dahliae*-infested soil and in non-infested soil, respectively. A high elongation rate of roots may limit the opportunity for contact between antagonist inoculum on the root and the growing root tip, thus, may limit passive carriage of the newly formed propagules of the antagonist by the elongating root.

The addition of wheat bran as a formulation product to the sandy soil used in these experiment resulted in suppression of both the pathogen and its antagonist.

Perhaps this product provides an adequate nutrient source for a microflora antagonising both *T. flavus* and *V. dahliae*. A similar effect of alginate wheat-bran was occasionally found with *Rhizoctonia solani* and *Sclerotium rolfsii* (Lewis and Papavizas, 1987; Ristaino *et al.*, 1994).

In summary, *T. flavus* moved from coated seeds along the growing root in natural soil, probably mainly by passive movement on the elongating root. These results add to reported data on the ability of *T. flavus* to disperse along roots.

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Chapter 5

Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiments

Abstract

Talaromyces flavus reduced viability of microsclerotia of *Verticillium dahliae* on senescent potato stems collected from the field when applied as ascospores in carboxymethylcellulose or in talcum powder. Incorporating an alginate wheat-bran preparation of *T. flavus* in soil at a rate of 0.5% (w/w) was followed by a decrease of >90% of the populations of *V. dahliae* in soil at both 15 and 25 °C. Population densities of *V. dahliae* and *T. flavus* were negatively correlated (r=-0.50; P=0.001) with those of *T. flavus*. However, the population of *V. dahliae* was also reduced in soil with alginate wheat-bran alone. When incorporated in soil in alginate wheat-bran and simultaneously coated on seeds in talcum powder, *T. flavus* reduced colonisation of roots and infection of eggplants by *V. dahliae*. Although to a lesser extent than with the antagonist, alginate wheat-bran without *T. flavus* also reduced infection by the pathogen. Treatment with combinations of *T. flavus* with other biocontrol agents, viz. *Bacillus subtilis*, *Fusarium oxysporum* or *Gliocadium roseum*, containing half of the inoculum of the single application of each antagonist, gave similar control of root colonisation and stem infection by *V. dahliae* as application of the single antagonists. Population densities on the root of each antagonist were not or only slightly affected by the presence of the co-inoculated antagonist suggesting that the combinations were compatible.

Introduction

Verticillium dahliae survives from one season to the next as microsclerotia. Pathogen development is monocyclic (Schnatthorst, 1981). Fungus inoculum produced during one growing season does not incite additional infections during the same season. Therefore, severity of wilt caused by the pathogen is related to the initial size of the inoculum population. Disease control can be achieved in two ways. First, control measures should be aimed at either keeping the amount of inoculum arriving into soil at a minimum or by inactivation of the resident microsclerotia population. Secondly, the aim can be reducing the efficacy of disease induction by reducing root and stem colonisation through application of antagonists. The latter is particularly challenging for *V. dahliae* because the host plants are susceptible to infection during all stages of development.

In The Netherlands, the feasibility of biological control is being explored because of the lack of cost-effective and sustainable methods. We focused on inoculation of soil and seed with microbial antagonists. Most emphasis was placed on *T. flavus* because of the promising results in control of *V. dahliae* obtained by Marois *et*

al. (1982) and Fravel (1989). However, literature data on performance of this antagonist are controversial. Spink and Rowe (1989) did not obtain any control of the pathogen, even at high application rates of *T. flavus*. In previous trials, *T. flavus* was shown to be rhizosphere competent and to suppress colonisation of the roots by the pathogen (Nagtzaam and Bollen, 1997).

Application of combinations of antagonists may provide more dependable control than that of single species (Bollen, 1974; Cook and Baker, 1983; Deacon, 1994). Recently, Park *et al.* (1988), Lemanceau and Alabouvette (1991) and Pierson and Weller (1994) demonstrated the feasibility of this approach for soilborne pathogens by enhanced suppression of fusarium diseases and take-all by mixtures of antagonistic species or strains. Following the same approach, we combined *T. flavus* with other antagonists of *V. dahliae*. Efficacy against this pathogen has been established for *Bacillus subtilis* (Hall *et al.*, 1986; Azad *et al.*, 1987; Berg and Ballin, 1994), nonpathogenic strains of *Fusarium oxysporum* (Jorge *et al.*, 1992; Yamaguchi *et al.*, 1992) and *Gliocladium roseum* (Keinath *et al.*, 1991). A prerequisite for effectiveness is that co-inoculated antagonists should not inhibit development of one another. Therefore, population densities on the roots of either of the combined antagonists were measured.

Materials and methods

Soils

In experiments 1a and b, 2a and 3a-d, a sandy soil (pH-KCl 7.0, organic matter content 0.3%) was used. Moisture content was adjusted to 10% (w/w) equivalent to a matric potential of -6.2 kPa. In Exp. 2b a clay soil was used (pH-KCl 7.0, organic matter 3.5%) at a matric potential of -5.0 kPa.

Preparation of inoculum of Verticillium dahliae

Isolate G2 of *V. dahliae* was obtained from a naturally colonised potato plant in The Netherlands. Microsclerotia were produced on autoclaved rye seeds as described by Kotcon *et al.* (1984). The colonised seeds were ground with pestle and mortar, and filtered through nested 150, 75 and 20 µm mesh sieves. Germination percentage of microsclerotia retained on the 20 µm sieve was assessed by plating a 0.2 ml suspension with a known number of microsclerotia on ethanol medium (EA)(Nadakavukaren and Horner, 1959), or by transferring single microsclerotia to modified soil extract medium (MSEA) (Harris *et al.*, 1993) as described by Hawke and Lazarovits (1994). As a bacteriostatic antibiotic oxytetracycline (50 mg l^{-1}) was added to these media.

Preparation of antagonist inoculum

Isolates TN11 and TN41 of *T. flavus* were obtained from farming land in The Netherlands. Alginate wheat-bran inoculum was produced according the method described by Fravel *et al.* (1985) for encapsulating biocontrol agents. Ascospore suspensions of *T. flavus* were obtained from 4-weeks-old cultures grown on PDA by scraping cleistothecia from the agar surface and suspending them in sterile water. The suspension was crushed for 2 min at 8,000 r.p.m. in an Ultra-Turrax homogenizer to liberate ascospores from the cleistothecia and filtered through cheese cloth to remove hyphal debris. Ten grams of sodium alginate (Janssen Chimica) and 110 g of powdered wheat bran were mixed with a 500 ml aqueous suspension containing in total 10^9 ascospores. This mixture was allowed to drip into a solution of $CaCl_2$ where pellets were formed by polymerisation.

After air-drying, the pellets were ground in a Retsch grinding mill (1.0 mm pore size). The percentage of pellet fragments yielding *T. flavus* colonies was assessed by transferring single fragments to modified Marois medium plates, 40-50 fragments on one plate, and counting the number of colonies after incubation for at least 4 days at 30 °C in the dark. The number of fragments g^{-1} inoculum was estimated under a binocular microscope. The inoculum contained 8×10^6 ascospores g^{-1}, 3×10^6 fragments g^{-1} and c. 75% of the plated pellets on PDA yielded colonies of *T. flavus*. Talcum inoculum was produced by mixing an ascospore suspension with talcum powder resulting in 1×10^6 cfu g^{-1} air-dried talcum powder.

B. subtilis inoculum (FZB24) was a formulated product obtained from FZB Biotechnik GmbH and kindly provided by Dr. G. Schmiedeknecht, Humboldt-Universität Berlin, Germany. The product is spore-based and yields 7×10^{10} cfu g^{-1}.

F. oxysporum isolate Fo47b10, originating from suppressive soil in the Châteaurenard region of France (Eparvier *et al.*, 1991) was kindly provided by Dr. C. Alabouvette, I.N.R.A., Dijon, France. It was grown in shake culture on malt extract broth (20 g l^{-1}). After incubation for 10 days at 25 °C with agitation (150 r.p.m.), the growth medium was removed by centrifugation at 2000 r.p.m. for 30 min, the remaining fungal biomass washed with sterile water, resuspended in sterile water and mixed with talcum powder. The mixture was dried at room temperature by forced air, sieved through a mesh of 0.2 µm and stored at 4 °C. The population density in the talcum inoculum was 1×10^5 cfu g^{-1}.

G. roseum isolate GHK, obtained from sclerotia of *Sclerotinia sclerotiorum*, was kindly provided by Dr. C.J. Kok of the DLO Research Institute for Plant Protection, Wageningen, The Netherlands. Biomass was prepared by growing the fungus in Czapek Dox broth (Oxoid). One hundred ml of the growth medium was added to a 300 ml

Erlenmeyer flask and autoclaved. Five disks of a 21 days old culture grown on malt extract agar (50 g l^{-1}) were added to each flask. Flasks were kept at 25 °C with agitation (150 r.p.m.) for 7 days. Fungal biomass was separated from the aqueous broth by filtration through cotton muslin and air dried. Alginate wheat-bran pellets were prepared by the method of Lewis *et al.* (1993). Sodium alginate was dissolved in one portion of distilled water (2.8 g 120 ml^{-1}) and powdered wheat-bran was suspended in another (10 g 60 ml^{-1}). The suspensions were autoclaved, cooled and blended together with air-dried, ground *G. roseum* (0.33 g). Then pellets were produced as described above for *T. flavus*. The population density in the pellets was 1 x 10^3 *cfu* g^{-1}. Talcum inoculum was prepared from a conidia suspension obtained from 9-day-old cultures grown on malt extract agar (Oxoid; 50 g l^{-1}). This suspension was mixed with talcum powder resulting in 1 x 10^7 cfu g^{-1} air-dried talcum.

Assessment of populations of V. dahliae and antagonists

Population densities of *V. dahliae* and antagonists on and in the roots with adhering soil were assessed after gently shaking of roots to remove non-rhizosphere soil. Densities of *V. dahliae* were assessed as described in Nagtzaam *et al.* (1997): roots were washed for 20 min under running tap water, rinsed two times in sterile water, blotted dry on sterile filter paper and plated onto EA. Colonisation density was assessed by plating sap extracted from stems on solidified EA (Nagtzaam *et al.*, 1997).

Population densities of antagonists on or in the roots were determined by maceration of roots in a buffer (0.1 M $MgSO_4$) using a pestle and mortar. The macerate was diluted and plated on selective media. For *B. subtilis*, the macerate was heated at 80 °C for 10 min. (Sneath *et al.*, 1986) and then plated on nutrient agar (Lab Lemco Agar, Oxoid, 23 g l^{-1}). In addition to normal procedure of cleaning materials by 96% ethanol, 10% NaOCl was used.

The benomyl resistant isolate Fo47b10 of *F. oxysporum* was recovered by plating the macerate on Komada-agar (Komada, 1975) supplied with benomyl (5 mg l^{-1}).

G. roseum was assessed using the selective medium of Park *et al.* (1992). A minimal salts medium was prepared consisting of distilled water (1 l), glucose (3 g), $MgSO_4.7H_2O$ (0.2 g), K_2HPO_4 (1.0 g), KCl (0.5 g), $NaNO_3$ (1.0 g), $FeSO_4.7H_2O$ (0.01 g) and Bacto agar (20 g). Before autoclaving the pH of the minimal medium was adjusted to 6.0 by phosphoric acid (25%). After cooling down, rose bengal (10 mg l^{-1}, Sigma), oxytetracycline (50 mg l^{-1}, Sigma), benomyl (0.25 mg l^{-1}), sodiumproprionate (500 mg l^{-1}, B.D.H.), acriflavine (60 mg l^{-1}, Sigma) and quintozene (14 mg a.i. l^{-1}) were added.

T. flavus was assessed on Marois medium slightly modified as described above.

The plates for detection of *B. subtilis*, *F. oxysporum*, *G. roseum* and *T. flavus* were incubated at 37, 20, 20 and 30 °C, respectively. In all treatments dry weight of roots ml^{-1} macerate in each replicate was determined by pouring a measured volume of the macerate on a preweighed paper filter. After filtration of the macerate of the roots and adhering soil, the filter paper with root and rhizosphere soil was placed in a glass petri dish, dried at 60°C for at least 1 day, and reweighed.

Experiments

Experiments were designed to assess the potential of *T. flavus* to reduce the amount of inoculum of *V. dahliae* that arrives into the soil (Exps 1a and 1b) or the survival of the inoculum already present in soil (Exps 2a and 2b). Effects of antagonists on colonisation of roots and stems by *V. dahliae* were studied by adding *T. flavus* alone or in combination with other antagonists to soil and seed (Exps 3a-d).

Experiment 1a. Effect of treatment of microsclerotia of V. dahliae with aqueous ascospore suspensions of T. flavus on microsclerotia germination.

The potential of *T. flavus* to inactivate microsclerotia was studied using the nylon fabric technique described by Lumsden (1981). Viable microsclerotia of *V. dahliae* were vacuum-infiltrated into nylon filter discs (poresize 45 µm, diameter 48 mm, Millipore) resulting in about 960 microsclerotia per filter. Then the filter was divided in 16 equal segments (c. 1 cm^2) each containing about 60 microsclerotia. These segments were exposed to an ascospore suspension of *T. flavus* TN41 for 30 min, retrieved, washed in sterile water, blotted dry and plated on MSEA in a petri dish. After incubation for at least 10 days at 20°C, the filter segments were examined microscopically for growth of *V. dahliae* from viable microsclerotia. Viability was assessed as formation of microsclerotia in the agar. The numbers of colonies of *V. dahliae* per segment was recorded.

Experiment 1b. Effect of T. flavus on germination of microsclerotia on potato stems collected in the field

Potato stems colonised by *V. dahliae* were collected in the field, dried at room temperature and cut into 2-4 cm pieces. The pieces were sprayed with an ascospore suspension of *T. flavus* TN41 (2×10^4 spores ml^{-1}) in water amended with carboxymethylcellulose sodium salt (CMC) (1.0 g 100 ml^{-1}) or dusted with talcum inoculum of *T. flavus* TN41 (10^6 cfu g^{-1} talcum inoculum). Control treatments were included by exposure of stem pieces to formulation products without *T. flavus*.

Each stem piece was placed on sterilised sandy soil in a petri dish, one piece in a dish. The dishes were placed in boxes (25 x 25 x 40 cm) and incubated at 100% R.H. and 20 °C for 3 or 5 weeks.

At harvest the stem pieces were macerated with a pestle and mortar in sterile water and the suspension was poured through nested 106 and 20 µm mesh sieves. Material retained on the 20 µm-sieve was resuspended in tap water and dried on filter paper at room temperature. Single microsclerotia were transferred according to the procedure described by Hawke and Lazarovits (1994). Of each stem piece 40-50 microsclerotia were transferred to both MSEA and adapted Marois medium to assess *V. dahliae* and *T. flavus*, respectively. For percentages data arcsine transformations were performed before analysis of variance. A randomised factorial design with five blocks was used. Each block was made up by one incubation box.

Experiments 2a and 2b. Effect of T. flavus on germination of microsclerotia in soil

In Exp. 2a filter segments (1 cm^2) with microsclerotia prepared as in Exp. 1a were buried in sand amended with alginate wheat-bran inoculum of *T. flavus* TN41 at rates of 0.001, 0.01, 0.1, 0.5 or 1.0% (w/w). This procedure was preferred to adding the microsclerotia free to soil because it avoided the laborious retrieval of microsclerotia and platings. Moreover, the latter procedure has given highly variable results (Termorshuizen, 1995; Wheeler and Rowe, 1995). A randomised complete block design with 10 replicates was used. Each replicate consisted of four filter segments buried in black plastic cylinders (diam. 3 cm, height 5 cm) with c. 30 g soil. The cylinders of one block were placed in a box covered by Parafilm to prevent drying out of the soil. Water was added at 3-4 days intervals to compensate for moisture losses. The cylinders were kept at 15 or 25 °C for 7 days or 10 weeks. Filter segments were retrieved, washed in sterile water, blotted dry, and two of them were plated on MSEA and two on adapted Marois medium to detect *V. dahliae* and *T. flavus*, respectively. In control treatments, microsclerotia were incubated in soil without *T. flavus* (control) or with a 1% autoclaved formulation product only. For each replicate the mean number of cfu segment^{-1} was calculated and logarithmically transformed before analysis of variance. This transformed number of cfu of *T. flavus* and *V. dahliae* per segment were regressed on logarithmically transformed inoculum density of *T. flavus* in soil. The *t*-test was applied to test the hypothesis that the slopes of the lines were the same at 15 and 25 °C.

In Exp. 2b microsclerotia obtained from naturally colonised potato stems were incorporated at a rate of 215 viable microsclerotia g^{-1} dried clay field soil. Alginate wheat-bran inoculum of *T. flavus* TN11 was incorporated in soil at a density of 0.5% w/w. In control treatments autoclaved TN11 inoculum (0.5% w/w) was added to soil infested or

Table 1. Treatments in Exps 3a-d to assess the potential of microbial antagonists, applied single or in combinations, in preventing infection and colonisation of eggplant or potato by *V. dahliae*.

Treatment	*V. dahliae* [a]	Antagonists				
		T. flavus		Other antagonist		
		Soil [b]	Seed [c]	Soil	Seed	Exp.[f]
Antagonists						
T. flavus (Tf)	300[a]	0.5	+	-	-	abcd
G. roseum (Gr)	300	-	-	0.5	+	acd
F. oxysporum (Fo)	300	-	-	0.5[d]	+	bd
B. subtilis (Bs)	300	-	-	Spores[e]	+	ac
Tf + Gr	300	0.25	+	0.25	+	acd
Tf + Fo	300	0.25	+	0.25	+	d
Tf + Bs	300	0.25	+	Spores[e]	+	c
Control treatments						
No formulation, antagonist or pathogen	-	-	-	-	-	abcd
V. dahliae-check	300	-	-	-	-	abcd
Wheat-bran (0.5% w/w)	300	-	-	-	-	cd
Talcum (0.5% w/w)	300	-	-	-	-	cd
Wheat-bran (0.25% w/w) + talcum (0.25% w/w)	300	-	-	-	-	d

[a] Number of viable microsclerotia g^{-1} soil; [b] Percentage (w/w) of alginate wheat-bran inoculum incorporated in soil; [c] Coating of seeds with talcum inoculum (+) or incubating the seeds in water suspensions of the biocontrol products (+), or untreated (-); [d] Talcum inoculum with *F. oxysporum* in stead of alginate wheat-bran pellets was used; [e] suspension containing 0.3 g preparation l^{-1}; in combination with *T. flavus* 0.15 g preparationl^{-1}; [f] Experiment.

non-infested with *V. dahliae*. Moreover, viable TN11 inoculum was added to soil without microsclerotia. The soil was incubated at 15 °C for 4 weeks. Quantification of the density of microsclerotia in soil was largely based on the method of Harris *et al.* (1993). Soil samples were air dried at ambient conditions for 14 days and ground. A subsample of 12.5 g air-dry soil was wet-sieved over 150 and 20 µm mesh screens. The part of the soil samples retained on the 20 µm sieve was suspended in 50 ml of a 0.08% water agar solution. This solution was used to avoid precipitation of microsclerotia. On each of 10 plates per sample, 0.8 ml soil suspension was pipetted. Nutrient medium was prepared by sterilising 0.1 g $MgSO_4.7H_20$, 1.7 g KH_2PO_4, 4.0 g K_2HPO_4, 0.002 g $FeSO_4.7H_20$, 0.4 g $NaNO_3$, 0.1 g KCl, 24 ml soil extract, 12 g agar (Oxoid No. 3), 2.0 g pectate (Bulmer, Hereford, U.K.), 1.0 ml tergitol (NP-10) in 1000 ml distilled water. The soil extract was prepared by boiling 1 kg sandy garden soil in 1 l water for 30 min followed by paper filtration. To the autoclaved medium, 6 mg biotin and 50 mg oxytetracycline were added in filter-sterilised suspensions.

The soil suspension was spread over the agar plate and allowed to dry in a flow cabinet for c. 15 min. Plates were incubated in plastic bags for the first 3 days to enhance germination of microsclerotia. Assessment of the plates took place after incubation for 4 weeks at 23°C. Prior to assessment of colony numbers, soil residues on the agar surface were removed by washing with tap water.

Population densities of *T. flavus* in soil were assessed according to Boosalis (1956). A soil suspension of 6.25 g in 50 ml water was heated at 60 °C for 30 min and 0.8 ml of the suspension was plated onto PDA with oxytetracycline (50 mg l^{-1}).

Exp. 3a-d. Effect of application of single species or combinations of antagonists on root colonisation *and incidence of stem infection by V. dahliae*

Four experiments (Exp. 3a-d) were performed to evaluate single and combined application of potential antagonists (Table 1). In treatments combining two antagonists half of the inoculum of the single application of each antagonist was used (Table 1). Inoculum of isolates of *F. oxysporum*, *G. roseum* and *T. flavus* was incorporated in soil at 0.5% (w/w). Of *B. subtilis*, an aqueous suspension of the preparation was mixed with soil. The amount was based on the recommended application of FZB Biotechnik GmbH, which is 2 l suspension of *B. subtilis* m^{-2} soil (0.3 g preparation l^{-1}). In combinations with *G. roseum* and *F. oxysporum*, seeds were first coated with talcum inoculum of *T. flavus* and then with talcum inoculum of the other fungi. In combination with *B. subtilis*, the suspension was applied prior to the talcum inoculum of *T. flavus*.

Soil was infested with *V. dahliae* at a density of 300 viable microsclerotia g^{-1} soil. The microsclerotia were obtained from naturally colonised potato stems (Exp. 3a-b)

or they were artificially produced on rye seed (Exp. 3c-d). The bioassay was described in detail by Nagtzaam *et al.* (1997). Three coated eggplant seeds were placed in one pot and after emergence the number was reduced to one. In Exp. 3c, besides eggplant (seeds), potato (minitubers as described in Lommen and Struik, 1992) was used as a test plant. The pots were incubated for 8 weeks at 20 °C in a controlled environment chamber. Populations of *V. dahliae* and antagonists on the roots were then assessed. Stem infection and colonisation of *V. dahliae* were determined as described above. A randomised complete block design was used with 10-12 replicates.

Results

Experiments 1a and 1b. Effect of T. flavus on germination of microsclerotia of V. dahliae.
Germination percentage was low; in the control treatment without antagonist preparation, less than 20% of the microsclerotia in the filter segments formed colonies in the agar. Exposure of microsclerotia to a suspension of ascospores of TN41 and subsequent *in vitro* incubation reduced germination of microsclerotia at 10^3 ascospores ml^{-1} and completely suppressed it at 10^7 ascospores ml^{-1} (Fig. 1). Autoclaved ascospore suspensions (control treatment) did not affect germination.

T. flavus reduced significantly (P=0.05) the viability of microsclerotia on potato stems after incubation for five weeks (Table 2); although there was no reduction after incubation for three weeks.

Experiments 2a and 2b. Effect of T. flavus on germination of microsclerotia in soil
T. flavus inoculum consisting of ascospores incorporated in alginate wheat-bran significantly reduced the number of cfu originating from microsclerotia of *V. dahliae* embedded in filters in soil at 15 and 25 °C (Exp. 2a; Fig. 2). However, alginate wheat-bran (1% w/w) without *T. flavus* also significantly reduced the number of cfu originating from microsclerotia. Depending on the amount of the formulated preparation of *T. flavus* in soil, viability of microsclerotia was affected. Incubation temperature significantly affected development of *T. flavus* in soil and viability of *V. dahliae* as shown by a significant interaction (P<0.05) between inoculum density and incubation temperature for colonisation of the segments by *T. flavus* and number of cfu originating from microsclerotia embedded in filters.

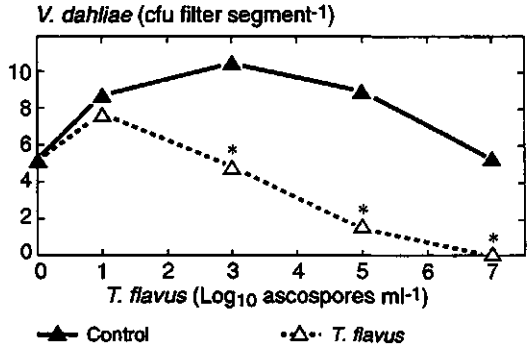

Fig. 1. Germination capacity of microsclerotia embedded in filter segments after exposure for 30 min to aqueous ascospore suspensions of *T. flavus* TN41 (Exp. 1a). Each segment contained 60 microsclerotia. * Significantly different from control treatment (autoclaved spore suspension) at $P<0.05$.

Table 2. Viability of microsclerotia of *V. dahliae* from potato stems after incubation of naturally infested potato stems treated with *T. flavus* or with formulation product only, applying two formulation products (Exp. 1b).

Type of *T. flavus*-inoculum		Percentage of germinable microsclerotia	
		After 3 weeks	After 5 weeks
CMC[a]	+[b]	18	20[d]
	-	35	28
Talcum[c]	+	30	23[d]
powder	-	30	38

[a] Carboxymethylcellulose sodium salt (2×10^4 spores ml^{-1}); [b] Formulation with (+) or without (-) *T. flavus*; [d] 10^6 cfu g^{-1} talcum inoculum;[d] Different from the corresponding mean without *T. flavus* at $P \leq 0.06$ and $P \leq 0.05$, respectively, according to Wilcoxon-Mann-Whitney test.

The slopes of the regression lines between log_{10}-transformed inoculum density of *T. flavus* or *V. dahliae* developing on the filter segments and log_{10}-transformed inoculum density of *T. flavus* in soil differed significantly (P=0.05) at the two temperatures. Negative correlations (r=-0.50 and r=-0.51; P=0.001) were found between the log cfu *T. flavus* filter^{-1} and the log cfu *V. dahliae* filter^{-1} at 15 and 25 °C, respectively.

T. flavus reduced the population density of *V. dahliae* also in the clay field soil (Exp. 2b; Table 3). Unlike in the sandy soil (Exp. 2a), the autoclaved alginate wheat-bran formulation decreased the population of *V. dahliae* much less than the product containing the antagonist.

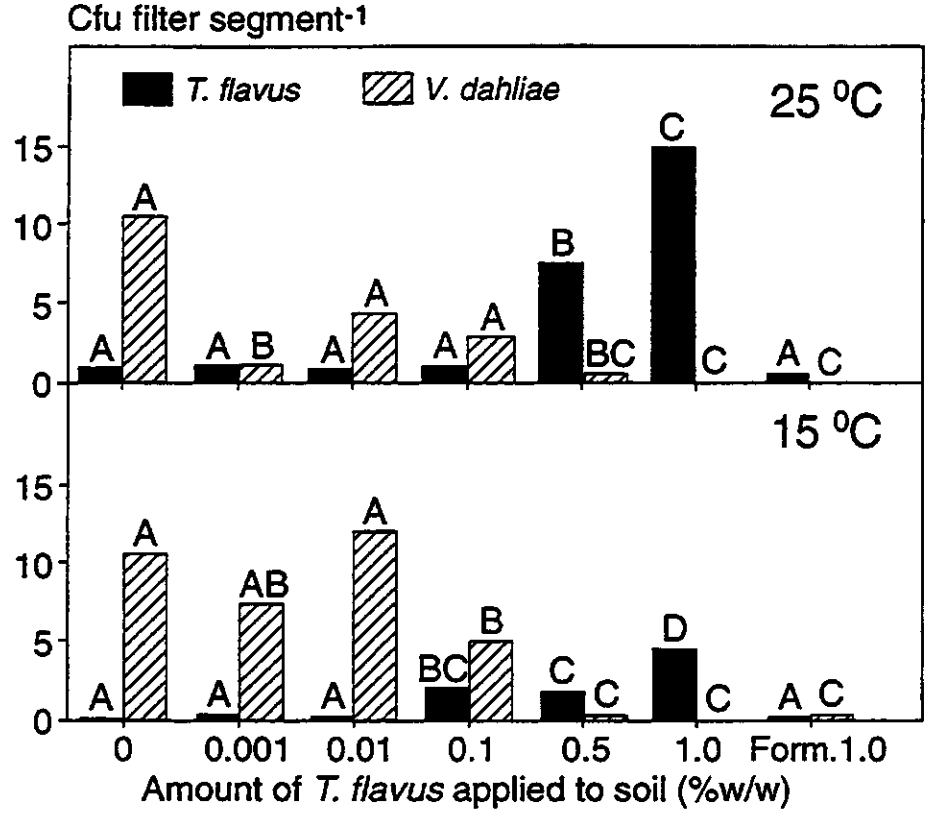

Fig. 2. Germination capacity of microsclerotia of *V. dahliae* embedded in filters and population densities of *T. flavus* on filters buried in sandy soil supplied with or without *T. flavus* (Exp. 2a). Different letters denote a significant difference between treatments with different inoculum densities of *T. flavus* incorporated in soil at P<0.05 according to Duncan's Multiple Range Test. Form. 1.0 = formulation product of alginate wheat-bran (1% w/w) only.

Exp. 3a-d. Effect of application of single species or combinations of antagonists on root colonisation *and incidence of stem infection by V. dahliae*

Inoculum of all four antagonists reduced root or stem colonisation by *V. dahliae* in at least one experiment (Table 4 and Fig. 3). The formulation check of alginate wheat-bran or talcum powder also affected root colonisation by *V. dahliae*.

Inoculum of *T. flavus* reduced root colonisation by *V. dahliae* in three out of the four series, *G. roseum* in all three series and *F. oxysporum* and *B. subtilis* in one of the two series in which they were applied. Reduction of root colonisation by *V. dahliae* was consistently associated with reduction of stem colonisation (Table 4 and Fig. 3). *T. flavus* combined with one of the other antagonists was usually as suppressive against *V. dahliae* as *T. flavus* alone or another antagonist alone. In contrast to the effects on *V. dahliae* in eggplant, antagonists failed to reduce colonisation of potato stems (Exp. 3c; data not shown). In all experiments the control plants were not contaminated by *V. dahliae*.

Table 3. Survival of microsclerotia of *V. dahliae* after incubation for 4 weeks at 15 °C in a natural clay soil amended with *T. flavus* (Exp. 2b).

Treatment		*V. dahliae*	*T. flavus*
V. dahliae	*T. flavus*	(Cfu g^{-1} soil)[a]	(Cfu g^{-1} soil)
Viable	Viable	14.6c[b]	73b
Viable	Autoclaved	64.2d	1a
None	Viable	2.2ab	233b
None	Autoclaved	0.2a	1a
Autoclaved	Viable	3.9b	167b

[a] Backtransformed from logarithmic values; [b] Different letters denote significant difference according to Duncan's Multiple Range Test ($P<0.05$).

Apparently antagonists did not inhibit each other as the combinations performed well (Table 4, Fig. 3). In most cases numbers of each antagonist on the root were not influenced by the presence of a co-inoculated antagonist (Table 5). In two out of the five test series, densities of *T. flavus* were even significantly higher after co-inoculation with

another antagonist than after single inoculation. Population density of *B. subtilis* on the roots was higher when the antagonist was combined with *T. flavus* than when it was applied alone. Taking into consideration that in combinations half of the inoculum was used as that in single applications of each antagonist, densities were higher than expected.

Discussion

Application of *T. flavus* in CMC or in talcum powder to potato stems covered with microsclerotia of *V. dahliae* and collected from the field reduced the viability of the microsclerotia by 28 and 44%, respectively (Table 2). Therefore, we would recommend treatment of infested potato stems left on the field after harvest with *T. flavus* as a measure to diminish the number of viable microsclerotia arriving into soil. From the results of the *in vitro* experiment, where microsclerotia were exposed to *T. flavus* ascospores at various densities (Fig. 1), it can be inferred that the efficacy depends on the relative densities of pathogen and antagonist.

A considerable amount of biocontrol propagules is introduced into soil when *T. flavus*-treated crop residues are ploughed under at the end of the growing season. With 10^6 microsclerotia g^{-1} aerial stem and a haulm dry weight of 6.8 g m^{-2} as has been recorded for 'Element' (Mol *et al.*, 1996), a colonisation level of 10% of the microsclerotia by *T. flavus* will result in about three *T. flavus*-colonised microsclerotia cm^{-3} soil. The colonised microsclerotia might perform as initial centres of growth for *T. flavus* in the next growing season and inactivate other microsclerotia present in the soil. Adams and Fravel (1990) showed the potential of this approach when they established control of *Sclerotinia minor* after spraying *Sporidesmium sclerotivorum* onto plants.

Under controlled conditions, the initial population density of *V. dahliae* in soil can be substantially reduced by incorporating *T. flavus* alginate wheat-bran inoculum in soil. This was demonstrated in a sandy soil (Fig. 2) and a natural, clay soil as well (Table 3), even at 15 °C being a rather low temperature for *T. flavus* a fungus which has an optimum temperature for *in vitro* growth of 34 °C (Nagtzaam, unpublished). In Wageningen, mean soil temperatures are 15.1 °C during the whole growing season and 18.7 °C during the warmest month (measured at a depth of 10 cm in a field with a standing crop and averaged over 12 years). At 15 °C, the population of *V. dahliae* was reduced in soil supplied with *T. flavus* inoculum at 0.1% and not at 0.01%. An application rate of 0.1% corresponds with 2000 kg ha^{-1}. The large amount of antagonist preparation needed for biocontrol of *V.dahliae* will restrict its use to high-value crops but may restrain a large scale implementation of the method in extensively grown field crops.

Table 4. Vascular colonisation of eggplant by *V. dahliae* after application of antagonists single or in combination with *T. flavus* (Exp. 3c-d).

	Colonisation (cfu ml^{-1} sap) [a, b]	
Treatment	Exp. 3c	Exp. 3d
V. dahliae (Vd)	932a	21a
Vd + *T. flavus* (Tf)	244de	4bc
Vd + *G. roseum* (Gr)	144ef	7bc
Vd + *F. oxysporum* (Fo)	-	12ab
Vd + *B. subtilis* (Bs)	467bc	-
Vd + Tf + Gr	123f	0c
Vd + Tf + Fo	-	0c
Vd + Tf + Bs	338cd	-
Vd + alginate wheat-bran	524abc	1bc
Vd + talcum	830ab	6bc
Vd + alginate wheat-bran + talcum	660ab	-

[a] Backtransformed values for colony forming units ml^{-1} stem sap; [b] A different letter denotes a significant difference (P=0.05) within a column according to Duncan's Multiple Range Test; - = Treatment not incorporated in this experiment.

In the control treatment with 1% (w/w) alginate wheat-bran without *T. flavus*, the population of *V. dahliae* was reduced in the sandy soil (Fig. 2), but not in the clay soil (Table 3). The effect of the formulation product complicates interpretation of the results. Although a highly significant negative correlation was found for the development of *T. flavus* and the number of germinable microsclerotia in soil (Fig. 2), we are unable to evaluate the capacity of the antagonist alone. Suppression of pathogen development by a formulation product as in the present study with alginate wheat-bran, stands not its

own. Fahima *et al.* (1992) found a reduced germination capacity of microsclerotia in soil after addition of a sterilised wheat-bran preparation, although the effect was less pronounced than in the present study.

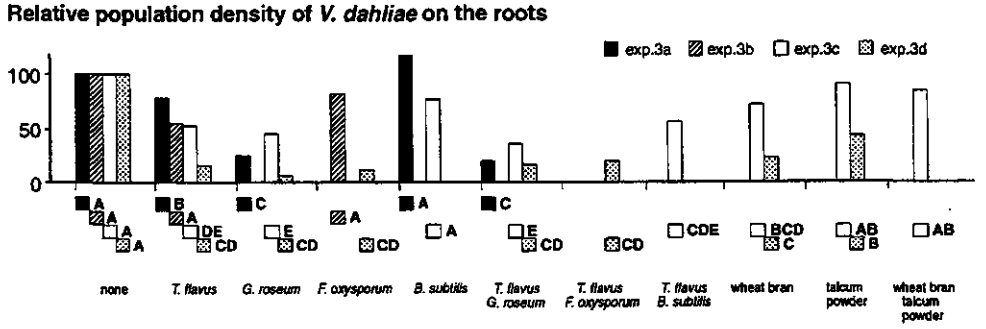

Fig. 3. Population densities of *V. dahliae* on eggplant roots after applying four antagonists, single or combined with *T. flavus*. Root colonisation is expressed in percentages relative to the *V. dahliae*-check in the corresponding experiment. Densities after single inoculation of *V. dahliae* were 0.95, 1.3, 2.2 and 0.3 cfu cm^{-1} root for Exp. 3a, 3b, 3c and 3d, respectively (backtransformed values). Different letters denote a significant difference between treatments in the corresponding experiment at $P<0.05$ according to Duncan's Multiple Range Test. Absence of bar means that a treatment had not been included.

It has been known for many years that addition of organic amendments to soil infested with *V. dahliae* can reduce disease severity (Jordan *et al.*, 1972; Harrison, 1976). The effect is attributed to supply of energy to indigenous, microbial antagonists present in soil. The association with a specific soil microflora may explain the different performance of wheat-bran and other organic formulation products in different soils as has been observed by Fravel *et al.* (1995). Supplementary effects of formulation products with antagonists compared to those of formulation products only, were noticed, e.g. in the effects on the *V. dahliae* populations in stems (Exp. 3c, Table 4).

A range of micro-organisms were shown to be compatible in combined application (Table 5). Apparently, these organisms occupy different habitats because of heterogenous distribution on the root resulting in spatial separation and a trivial probability of interaction between their populations (Bowen, 1980) or they occupy

different ecological niches because of different nutritional or environmental requirements. The first hypothesis cannot explain the increased antagonist densities on the root in combined application as compared to single inoculation. The co-inoculated antagonist may have increased the accessibility of nutrients by cross-feeding. Moreover, densities may be indirectly enhanced by increased root exudates induced by a co-inoculated antagonist (Bowen and Theodorou, 1979; Park *et al.*; 1988). Another possibility may be protection of the combined antagonist by the co-inoculated organism by detoxifying antibiotics or physical protection of its structures (Bowen and Theodorou, 1979).

Table 5. Relative population densities of four antagonists of *V. dahliae* on eggplant roots applied alone or in combination with *T. flavus* (Exp. 3a-d).

	Population density (%) on the root relative to density when applied alone[a]							
	T. flavus				Other antagonist			
Treatment	3a	3b	3c	3d	3a	3b	3c	3d
T. flavus + *G. roseum*	97 a[b]	-[c]	152 b	87 b	101 a	-	103 a	87 a
T. flavus + *F. oxysporum*	-	-	-	80 b	-	-	-	79 a
T. flavus + *B. subtilis*	-	-	149 b	-	-	-	136 b	-

[a] Densities after single inoculation were 4 x 10^5, 13, 3 x10^3 and 1 x 10^4 for *T. flavus* (Tf), 1 x 10^5, 5 x 10^4 and 2 x 10^4 for *G. roseum* (Gr), 3 x 10^3 and 8 x 10^4 for *F. oxysporum* (Fo) and 4 x 10^6 and 6 x 10^6 for *B. subtilis* (Bs) cfu g^{-1} dry weight of roots, for the Exp. 3a-d, respectively. Details on treatments are presented in Table 1; [b] Different letters denote a significant difference between treatments in the corresponding series of experiments at $P<0.05$ according to Duncan's Multiple Range Test; [c] Treatment not included.

It seems that the antagonists act independently on *V. dahliae* as *T. flavus* combined with an another antagonist was usually as suppressive against *V. dahliae* as *T. flavus* or another antagonist alone (Table 4 and Fig. 3). Combining compatible antagonists with different biocontrol properties, adapted to diverse environmental and biological conditions, and simultaneously application of different biocontrol methods might lead to a more consistent biocontrol. The search for adequate combinations is in particular relevant for *T. flavus* since field results on control of *V. dahliae* by application

of this antagonist alone have been inconsistent, ranging from no control (Fravel, 1989; Spink and Rowe, 1989) to moderate (Davis *et al.*, 1986) or good control (Marois *et al.*, 1982; Fravel, 1989). First, it should be studied if performance in the field confirms the results with combinations obtained under controlled conditions. The next step is the search for better combinations of species or strains of biocontrol agents in a mixture for improvement of control of *V. dahliae* in the field. However, application of several antagonists requires costly procedures in pesticide approval regulations for each of the organisms. Therefore it seems opportune to combine *T. flavus* with organisms which have already received approval.

Chapter 6

The effect of spatial distribution of fungal mycoparasites on their performance: a modelling approach

Abstract

The distribution of propagules in soil is an important factor in determining the performance of mycoparasites to control soilborne plant pathogens. To evaluate the importance of spatial distribution patterns of propagules of a mycoparasite and its fungal host, the assumption of an uniform distribution was compared with the assumption of a random distribution. It is shown that the two types of distributions have comparable shapes. Quantitatively, average distances among propagules appear to be 40-50% smaller for the case of random distribution. The consequences of the two assumptions for epidemic curves of the mycoparasite were evaluated by using a simple model of omnidirectional and constant growth of the mycoparasite. Random distribution of propagules indicated a considerably slower rate of progress of infected hosts than uniform distribution. Parameters for epidemic curves were estimated from published data on the inactivation of sclerotia of *Sclerotinia minor* by different densities of macroconidia of *Sporidesmium sclerotivorum* for both types of distribution. The differences between the two types of distribution were shown to be quite small. The average distance among propagules of mycoparasite and host fungus was better correlated with the area under the disease progress curve than the density of the mycoparasite in soil. It is argued that in studying the dynamics of antagonists it may be epidemiologically more significant to base different treatments on differences in average distances among propagules than on differences in densities per unit of volume.

Introduction

The selection of fungal mycoparasites able to control soilborne plant pathogens is one approach to develop environmentally benign farming systems in intensive agriculture (Whipps and Budge, 1990). However, obvious successes in this approach have been reported only rarely. Understanding the dynamics of mycoparasites of plant pathogens may be essential to design consistent biocontrol strategies which are commercially attainable. Modelling approaches have been undertaken to improve the understanding of mycoparasite performance (Stack *et al.*, 1988; Gubbins and Gilligan, 1997), but patterns of spatial distribution have rarely been considered in this context. Among other things, the optimal performance of a mycoparasite will be related to its own spatial distribution and that of the host, the pathogen (Hassel, 1976; Allen *et al.*, 1985).

The types of spatial distribution of mycoparasite and of its host determine the distance distribution between them. McCoy and Powelson (1974) have calculated this distance distribution assuming an uniform distribution of both types of propagules in soil. In this paper we will study this distance distribution with the more realistic assumption of

random distribution of both types of propagules. For both distance distributions, the efficacy of the mycoparasite will be assessed by a simple model. Basically, growth of the mycoparasite is represented as increasing spheres of influence surrounding the mycoparasite which will be referred to as the "mycoparasitic zones". Within these spheres, it is assumed that propagules of the host are inactivated. Predictions of the model will be compared with experiments reported in the literature on the mycoparasite-host system *Sporidesmium sclerotivorum* and *Sclerotinia minor* (Adams *et al.*, 1984). The fungus *S. sclerotivorum* is a wide-spread ecologically obligate parasite of sclerotia of many fungal species (Ayers and Adams, 1979a). Macroconidia germinate in the presence of sclerotia of hosts and are able to infect and kill them (Ayers and Adams, 1979b). *S. sclerotivorum* is claimed to be a good candidate for commercial biocontrol of lettuce drop, caused by *S. minor* (Adams, 1990; Adams and Ayers, 1982). Adams *et al.*(1984) determined the survival of sclerotia of *S. minor* as a function of density of macroconidia of *S. sclerotivorum* and incubation time.

The model

First we will derive the distance distribution among randomly distributed propagules and compare that with that of uniformly distributed propagules as derived by McCoy and Powelson (1974). Subsequently, for both types of distribution, the epidemic curves of the mycoparasite will be calculated. Symbols are explained in Table 1. The meaning of the symbols used is summarised in Table 2.

Table 1. Assumptions underlying both models.

- Mycoparasite and pathogen propagules are distributed uniformly or randomly in soil.
- No growth occurs of pathogen propagules.
- Outgrowth of propagules of the mycoparasite takes place immediately and continuously at a constant growth rate.
- No passive spread takes place of mycoparasite and pathogen propagules (*e.g.* dispersal by water movement or by animals).
- All pathogen propagules occurring in the mycoparasitic zone are inactivated.
- The performance and growth of the mycoparasite is independent of the density of the pathogen.

The average distance between uniformly distributed propagules

McCoy and Powelson (1974) showed that the average distance between uniformly distributed propagules, $\overline{R}_{reg}$, is given by:

$$\overline{R}_{reg} = 1.1225\ ID_M^{-\frac{1}{3}}, \quad (1)$$

where ID_M is propagule density.

The distance between randomly distributed propagules

The volume of a sphere with radius R (L) surrounding one propagule equals:

$$V = \frac{4}{3}\pi R^3. \quad (2)$$

Since propagules are distributed randomly, the expected number of propagules, N, in volume V (L^3) of the sphere is:

$$N = ID_M \frac{4}{3}\pi R^3. \quad (3)$$

The probability that in this sphere no propagule is present, P_0, equals according to the Poisson distribution:

$$P_0 = e^{-ID_M \frac{4}{3}\pi R^3}. \quad (4)$$

The distribution of the probability that at least one propagule is present in the sphere with radius R, $F(R)$, is then given by:

$$F(R) = 1 - e^{-ID_M \frac{4}{3}\pi R^3}. \quad (5)$$

Taking the derivative with respect to R we find the probability density of the distance between propagules, $f(R)$, to be:

$$f(R) = 4\, ID_M \pi R^2 e^{-ID_M \frac{4}{3}\pi R^3}. \quad (6)$$

Figure 1 shows some examples of the probability density for ID_M values of 200, 2000 and 20000.

The density function, *f(R)*, is used to calculate the average and variance of the distance between propagules. For the average distance we find:

$$\overline{R} = \int_0^\infty Rf(R)dR = \Gamma\left(\frac{4}{3}\right)\left(\frac{4}{3}\pi\right)^{-\frac{1}{3}} ID_M^{-\frac{1}{3}}, \quad (7)$$

where $\Gamma(x)$ represents the gamma function tabulated in, *e.g.*, Larson and Hostetler (1986). Using this table the average distance between propagules is approximated by:

$$\overline{R} \approx 0.5540\ ID_M^{-\frac{1}{3}}. \quad (8)$$

In Figure 2, the average distance between propagules is shown as function of propagule density for both types of propagule distribution.

For the variance of the distance between propagules, s^2, we obtain:

$$\sigma^2 = \int_0^\infty R^2 f(R)dR - \overline{R}^2 = \left\{\Gamma\left(\frac{5}{3}\right) - \Gamma^2\left(\frac{4}{3}\right)\right\}\left(\frac{4}{3}\pi\right) ID_M^{-\frac{1}{3}}, \quad (9)$$

and using the tabulated gamma function the standard deviation of the distance between propagules is approximately:

$$\sigma \approx 0.20\ ID_M^{-\frac{1}{3}}. \quad (10)$$

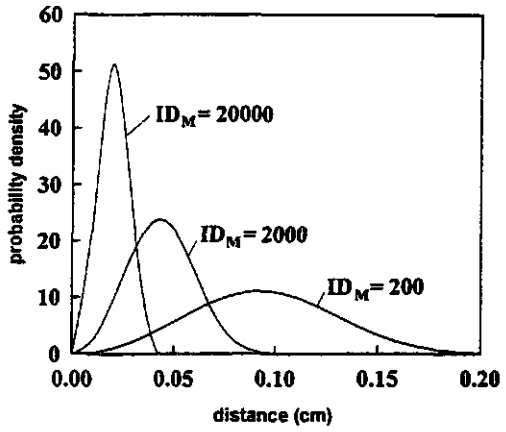

Fig. 1. Probability density functions of the distance between propagules in randomly distributed propagules for propagule densities, ID_M, of 200, 2000 and 20000 propagules per cm^{-3}.

Epidemic curves of mycoparasitic dynamics

Here, we aim to calculate the proportion of host propagules inactivated by the mycoparasite as a function of time for both the uniform and random types of distribution of propagules. It is assumed that the mycoparasite is able to germinate and grow to reach and infect propagules of its host. We assume that the mycoparasite grows equally in all directions, resulting in a sphere of influence indicated as the mycoparasitic zone. Once a propagule of the host is present in the mycoparasitic zone, it is assumed to be inactivated. Assuming a constant growth rate of the mycoparasite, the change of the radius of the mycoparasitic zone in time, $R(T)$, is:

$$R(T) = \lambda T, \qquad (11)$$

where λ is the growth rate of mycoparasite in soil.

Epidemic curve for the case of uniformly distributed propagules

The propagules of the mycoparasite are arranged in cubic lattices, which implies that the centres of the mycoparasitic zones are positioned on the vertices of the cubic lattices. The distance, d, between the centres of two neighbouring mycoparasites is related to inoculum density, ID_M, since $d = ID_M^{-1/3}$. Four separate growth stages need to be considered.

A. Mycoparasitic zones do not overlap as long as their radii do not exceed $\frac{1}{2}d$. The proportion of the total volume occupied by mycoparasitic zones then equals: $ID_M 4/3\pi R^3$.

B. If the radii of the mycoparasitic zones R exceed $\frac{1}{2}d$, neighbouring propagules overlap. To calculate the total mycoparasitic zone the overlapping zones should be counted only once. This holds as long as $R<\frac{1}{2}\sqrt{2}d$.

C. If the radii of the mycoparasitic zones R exceed $\frac{1}{2}\sqrt{2}d$., all mycoparasitic zones at vertices on the sides of a cube overlap and, again, these overlapping zones should be counted only once.

D. Finally, if the radii of the mycoparasitic zones R exceed $\frac{1}{2}\sqrt{3}$, the space is completely filled with the mycoparasite.

Stages B and C can be calculated using standard integration techniques (Larson and Hostetler, 1986). For the calculation of case C, the resulting integral was evaluated using the computer program DERIVE. Putting all pieces together we obtain:

$$A = 1-\frac{4}{3}\pi r^3$$

$$B = \frac{1}{12}\left(\pi(32r^3-36r^2+3)+12\right)$$

$$C = \frac{1}{6}(16r^3+12r^2-1)\operatorname{atan}\left(\frac{\sqrt{2}(4r^2+2r-1)}{2\sqrt{2r^2-1}}\right) - \frac{1}{6}(16r^3-12r^2+1)\operatorname{atan}\left(\frac{\sqrt{2}(4r^2-2r-1)}{2\sqrt{2r^2-1}}\right)$$

$$- \frac{1}{6}(16r^3+12r^2-1)\operatorname{atan}\left(2\sqrt{2}r\sqrt{2r^2-1}+4r^2-1\right)$$

$$- \frac{1}{6}(16r^3-12r^2+1)\operatorname{atan}\left(2\sqrt{2}r\sqrt{2r^2-1}-4r^2+1\right) + \frac{1}{3}(12r^2-1)\operatorname{atan}\left(\sqrt{2}\sqrt{2r^2-1}\right)$$

$$+ \frac{1}{3}(1-12r^2)\operatorname{atan}\left(\frac{\sqrt{2}}{2\sqrt{2r^2-1}}\right) - \left(\sqrt{2}\sqrt{2r^2-1}-1\right)$$

$$D = 1$$

with $r = R\left(ID_m\right)^{-\frac{1}{3}}$ (12)

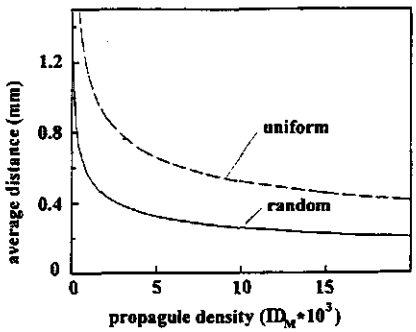

Fig. 2. Relation between propagule density (L^{-3}) and average distance (L) between propagules in soil, for a uniform and random distribution.

Epidemic curve for the case of randomly distributed propagules

For randomly distributed propagules, the proportion of pathogens infected equals the probability density, *F(R)* (Eq. 5). Substituting the assumption of constant rate of radial growth (Eq. 11) we find:

$$F(T) = 1 - e^{-ID_M \frac{4}{3}\pi(\lambda T)^3} . \qquad (13)$$

Figure 3 shows the proportion of inactivated hosts as a function of a scaled-time unit (*i.e.* $\lambda T\ (ID_M)^{1/3}$) for both the uniform and the random types of distribution. By doing so, all possible shapes of the epidemic curve can be shown in one graph. Given a value for the mycoparasite density, ID_M, and for the growth rate of the mycoparasite, λ, the epidemic curves can be constructed by rescaling the x-axes.

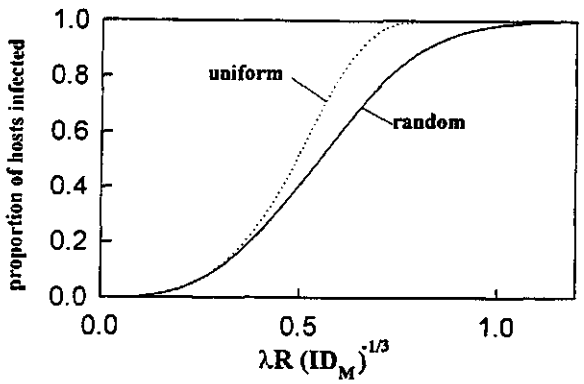

Fig. 3. Proportion of hosts infected for the uniform (Eq. 12) and for the random (Eq. 13) distribution of propagules in soil.

Empirical verification of model predictions

Data on the number of sclerotia surviving were taken from Figure 2 of Adams *et al.* (1984). Both model equation (12) for the regular distributed and equation (13) for the randomly distributed mycoparasites were fitted to the data using a non-linear least squares procedure. For the regular distribution it was estimated that λ=0.020±0.002 and for the random distribution λ=0.022±0.002. Figure 4 shows the proportion of parasitized sclerotia of *S. minor* at different densities of *S. sclerotivorum*. The best fit was obtained at relative short incubation periods (< 5 weeks) and at a density of 65 mycoparasite propagules cm^{-3}.

Discussion

The results show that differences between assumptions of uniform and random distributions in soils have qualitatively little influence on average distances between propagules and epidemic curves: all figures show lines having similar shapes. However, quantitatively there are some considerable differences.

For both types of distribution decrease in average distances levels off from densities higher than approximately 5000 propagules cm^{-3}. Average distances of randomly distributed propagules are approx. 40-50% of those of uniformly distributed propagules (Fig. 2). In density-dependent biocontrol experiments only those densities are likely to be relevant which result in significantly different average distances, *i.e.* at propagule densities <5000 cm^{-3}. The density-dependent experiment by Adams *et al.* (1984) was performed at the most appropriate densities varying between 0 and 5000 macroconidia of *S. sclerotivorum* g^{-1} (equalling approx. 0-6500 macroconidia cm^{-3}) of soil. Average distances between propagules of mycoparasite and those of its host may be more epidemiologically significant than propagule densities. Thus, the area under the disease progress curve of Figure 4 calculated from Adams *et al.* (1984) shows a better correlation with the average distance between mycoparasite and host (r^2=-0.98) than with the inoculum density of the mycoparasite (r^2=0.83). This is explained by the non-linear relation between inoculum density and average distance between propagules (Eq. 8). Above the density of 5000 propagules cm^{-3} of soil the costs of applying more propagules should be weighed against the additional effect of biocontrol.

From a theoretical point of view, it may be questioned which type of distribution, uniform or random, results in best biocontrol. When considering Figure 2, one would conclude that the situation which leads to the smallest average distance would result in best biocontrol. However, from Figure 3 the opposite can be concluded. The epidemic

curves for the uniform and random type of distribution of propagules are in the initial stages practically equal (Fig. 4), being caused by the absence of overlap of mycoparasitic zones originating from different propagules. However, quite soon the space- filling of the randomly distributed propagules progresses slower than that of the uniformly distributed propagules, because overlapping mycoparasitic zones occur earlier

Table 2. Glossary of symbols used to represent variables and parameters in the text. L=units of length, T=units of time.

Variable or parameter		Definition
Mycoparasite	ID_M	Density of mycoparasite propagules in soil (propagules L^{-3} soil).
	T	Growth period (T) of mycoparasite.
	λ	Growth rate of mycoparasite in soil ($L\ T^{-1}$).
	d	Distance between two neighbouring propagules (L).
Pathogen/ mycoparasite	R	Radius of mycoparasitic zone and distance between propagules in soil (L).
	V	Volume of the mycoparasitic zone with a radius R (L^3).
	P_0	Probability that no propagule occurs in the mycoparasitic zone.
	F(R)	Probability that one or more propagules occur in the mycoparasitic zone at a mycoparasite density of ID_M.
	f(R)	Probability distribution of distance between propagules in soil.

for the randomly distributed propagules, since a portion of propagules will occur close together. For the same reason the epidemic curve for the randomly distributed propagules levels off earlier than the uniformly distributed propagules because in the random type of distribution always some propagules will occur relatively isolated. The differences of the epidemic curves may also be explained from Figure 2, which shows that the average distance between uniformly distributed propagules is larger than for the randomly distributed propagules. Consequently, propagules which are situated in a uniform pattern have a larger nonoverlapping, 'unique', sphere of influence than

propagules which are randomly arranged. In addition to the random type of distribution, an aggregated distribution of propagules of the pathogen may occur. Also biocontrol agents may be applied in an aggregated manner, *e.g.* by applying them to planting holes. We aim to return to this mathematically complex subject in the future.

The epidemic curves based on the uniform and random distribution of propagules show almost equal results when applied to the data of Adams *et al.* (1984) (Fig. 4). At the low inoculum densities of *S. sclerotivorum*, 0.65 and 6.5 macroconidia cm^{-3}, overlapping mycoparasitic zones are rare for both types of distribution. At the higher inoculum densities, 650 and 6500 macroconidia cm^{-3}, propagules apparently occur so close together that overlapping occurs quickly for both types of distribution. Only at 65 macroconidia cm^{-3} a picture equal to that of Figure 1 appears. At lower inoculum densities of *S. sclerotivorum*, the performance of the model is rather good, although the surviving number of sclerotia of *S. minor* is underestimated by the model at 6.5 macroconidia cm^{-3}. This might be caused by increased growth rate of the mycoparasite due to consumption of energy acquired from the colonised sclerotia. At 650 and 6500 macroconidia cm^{-3}, numbers of infected sclerotia are overestimated. Perhaps at high densities, the mycoparasite propagules start to compete with each other. It seems therefore that density-dependent phenomena need more experimental work in the study of ecology of mycoparasites.

The model predicts the numbers of surviving sclerotia rather well in the initial stages of the epidemic but at later stages larger departures from data are found, most notably at the higher inoculum densities of *S. sclerotivorum*. This is likely to be due to our assumption of equal and constant growth of the mycoparasite in all directions. Although equal omnidirectional growth has been reported for *Trichoderma harzianum* in sterile soil (Knudsen and Bin, 1990) and for *Rhizoctonia solani* in nonsterile soil (Neate and Berger, 1995), it seems to be the exception rather than the rule in the heterogenous environment of the soil. Detailed information about growth patterns of fungi in soil and about mechanisms explaining fungal growth in soil is not available. The assumption of a constant growth rate of the mycoparasite is unlikely to hold, since growth rate depends of the amount and quality of internal and external energy sources. If no external energy (*e.g.* host nutrients or nutrients added to the carrier substrate) becomes available to the mycoparasite, growth is likely to cease due to the presence of fungistasis in natural soils (Lockwood, 1977). Thus, Stack *et al.* (1987) reported that growth of fungal antagonists in nonsterile soil was influenced significantly by carbon and nitrogen source and C-N ratio of the carrier substrate. Similar results were obtained by studying the effect of propagule size on the infection efficiency of root pathogens (Henis and Ben-Yephet, 1970; Wilkinson *et al.*, 1985), which was attributed to the differences in energy available to the

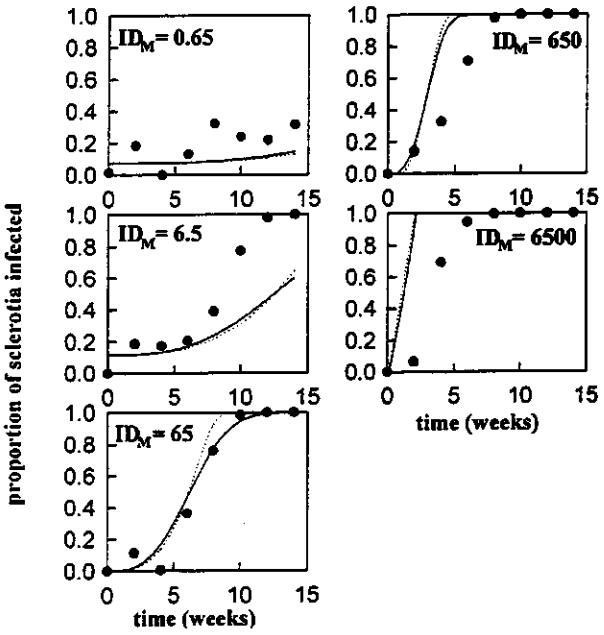

Fig. 4. Proportion of sclerotia of *Sclerotinia minor* infected in soil at a range of initial densities of *Sclerotinia minor* as observed in experiments (Adams *et al.*, 1984) and predicted by dynamic models based on a random ( ) or uniform (----) distribution of propagules of the mycoparasite.

inoculum propagules. Accordingly we observed that development of *T. flavus* in soil was related to the size (=energy) of the propagules incorporated (Nagtzaam, unpublished). We will elaborate further on the concept of energy in modelling the dynamics of mycoparasites in a subsequent paper.

Finally, the assumption that all propagules of the host which are present in the mycoparasitic zone are inactivated is not realistic. This assumption is related to the omnidirectional equal presence of the mycoparasite in its mycoparasitic zone. However, it may be realistic when asymmetrical growth in soil is modelled, but, as stated above, better alternatives are not available yet. If more realistic growth models were available, the assumption that all propagules of the host which are present in the mycoparasitic zone are inactivated may be true if the mycoparasitic zone is simply defined as the locations where the mycoparasite is present.

In spite of the evident departures of reality discussed above, the results of the present analysis show that simple mycoparasite-host models lead to meaningful and interpretable results. Epidemic curves do not differ strongly at the values of growth rate and inoculum density of the mycoparasite studied here, but implications about distance distributions are considerable.

Chapter 7

Field application of *Talaromyces flavus* to control *Verticillium dahliae* in potato

Abstract

The potential of *Talaromyces flavus* in controlling *Verticillium dahliae* was evaluated in two independent field experiments with potato, each lasting two growing seasons. Incidence of stem infection by *V. dahliae* and yield of tubers showed a clear dose-response relation to the amount of pathogen inoculum applied to soil (R^2=0.69 and 0.40, respectively). Incidence of stem infection and density of stem colonisation by the pathogen gradually increased during the season. After application of a *T. flavus* preparation, stems were less densely colonised by *V. dahliae* in the treated plots than in the control plots in the first growing season of Exp. 1 and in the second growing season of Exp. 2. The reduction in pathogen colonisation was not followed by higher tuber yields. Although population density of *T. flavus* in soil did not increase during the growing season, it remained at a higher level in *T. flavus*-treated plots than in untreated plots (P=0.05), also in the second year after introduction. In one experiment with a control treatment of alginate wheat-bran without *T. flavus*, a reduced pathogen colonisation of stems was found. The inconsistency of performance in the present study, and the contradictory data reported in the literature, demonstrate the need to identify conditions required for biocontrol of *V. dahliae* by *T. flavus*.

Introduction

Verticillium dahliae is the primary pathogen involved in early dying (Verticillium wilt) of potato in The Netherlands and can cause yield losses of up to 25% (Haverkort *et al.*, 1989). In the so-called Multi Year Crop Protection Plan, the Dutch government aims at a substantial reduction in the use of pesticides, especially of soil fumigants (Anonymous, 1990). This may lead to an increased incidence of diseases that were of minor importance in previous frequently fumigated fields (Bollen, 1993). Fumigation applied for eradication of the potato cyst nematode not only controls these nematodes but also reduces the population of free-living nematodes that predispose the roots to infection with *V. dahliae* (Bollen, 1993) Therefore, any reduction in fumigation of potato fields implies the need for developing alternative control methods for this pathogen. A wider crop rotation is economically not feasible because of the time required for sufficient reduction of inoculum of *V. dahliae* in soil (Mol *et al.*, 1995). The cultivation of tolerant potato cultivars only is not a realistic, economic alternative. Control by soil solarization as advocated by Katan (1981) and Melero-Vara *et al.* (1995) in warmer climes is not an option under the climatic conditions in The Netherlands.

The potential for biological control of *V. dahliae* under field conditions was demonstrated for *Aspergillus alutaceus* (Marois *et al.*, 1982), *Gliocladium roseum* (Johnston *et al.*, 1994), *Talaromyces flavus* (Marois *et al.*, 1982; Davis *et al.*, 1986; Fravel *et al.*, 1986), *Trichoderma harzianum* (Ordentlich *et al.*, 1990), *T. koningii* (Georgieva, 1992) and *T. viride* (Marois *et al.*, 1982). *T. flavus* has several properties that makes it an interesting candidate for biocontrol of *V. dahliae*. Firstly, it is resistant to dry conditions and has a long shelf life when incorporated in a clay formulation or an alginate-clay matrix (Fravel *et al.*, 1985; Papavizas *et al.*, 1987; Nagtzaam and Bollen, 1994). Secondly, following a single application *T. flavus* can establish itself in an agricultural soil and suppress pathogens in the second year after application (Fravel *et al.*, 1986; McLaren *et al.*, 1994). Thirdly, *T. flavus* has been combined successfully with other microbial antagonists (Johnston *et al.*, 1994; McLaren *et al.*, 1994; Nagtzaam *et al.*, 1998), which provides possibilities for a more consistent and effective control as *T. flavus* sometimes fails to control *V. dahliae* when applied as single biocontrol agent (Spink and Rowe, 1989; Keinath*et al.*, 1990).

The objectives of this study were to determine the potential of field application of *T. flavus* to control *V. dahliae*. Effects were monitored in two consecutive potato crops in two separate experiments after a single field application of the antagonist. To adequately interpret the effect of *T. flavus* on colonisation of the plant by *V. dahliae* and crop yield, the effect of the pathogen at different soil infestation levels on potato plants was also studied in the first experiment.

Material and methods

Preparation of pathogen and antagonist inocula

Air-dried potato stems, which were naturally colonised by *V. dahliae*, were used as source of pathogen inoculum. The stems were ground into a fine powder. The number of viable microsclerotia g^{-1} was assessed according to methods described by Nagtzaam *et al.* (1997).

Antagonist inoculum consisted of ascospores incorporated in alginate wheat-bran. Wheat bran was shown to be a good carrier for *T. flavus* (Papavizas *et al.*, 1987). In Exp. 1, it was prepared by mixing ascospores of *T. flavus* isolates TN12, TN41 and TN43, water, alginate and wheat bran; in Exp. 2, isolate TN11 was added to this combination of isolates. Isolates TN11, TN12 and TN41 were obtained from field soil and TN43 from potato roots in The Netherlands. Inoculum was prepared according to the procedure developed by Fravel *et al.*(1985), but using wheat bran instead of pyrophyllite as a carrier for *T. flavus*. After drying, the alginate wheat-bran granules were ground in a

Retsch grinding mill (1.0 mm pore size) to powder to increase the number of particles g^{-1} inoculum. With this procedure, the particle density in soil was predicted to be sufficiently high to establish contact between antagonist and pathogen during the growing season (Chapter 6). The ground product contained 115,000 particles g^{-1}, compromising 1.0×10^7 and 0.5×10^7 ascospores g^{-1}, producing 1.5×10^6 and 0.8×10^6 colony forming units (cfu) g^{-1} on Marois medium (Marois *et al.*, 1984) in 1993 (Exp. 1) and 1994 (Exp. 2), respectively. Talcum inoculum of *T. flavus* was prepared by mixing talcum powder with 0.5×10^7 ascospores g^{-1} powder. After air-drying, the talcum inocula of the different isolates were combined. Densities were 2.4×10^6 and 3.0×10^6 cfu g^{-1} after plating on Marois medium (Marois *et al.*, 1984) in 1993 and 1994, respectively.

Soil and plant assays

V. dahliae was quantified in soil by collecting 15 samples between rows from each plot to a depth of 20 cm with a 2.5-cm-diameter auger. For each plot, the samples were bulked, mixed thoroughly, air-dried at room temperature (20-25 °C) for 3-4 weeks and then ground in a Retsch grinding mill (1.0 mm pore size). In a subsample of 12.5 g (dry weight), the density of *V. dahliae* was estimated using the procedure described by Nagtzaam *et al.* (1998), largely based on the method of Harris *et al.* (1993). After wet-sieving, a suspension of soil was plated on a selective medium. Except for Exp. 1 (autumn 1992 and spring 1993) where ethanol agar (EA; Nadakavukaren and Horner, 1959) was used, the suspensions were plated on modified soil extract agar (MSEA; Harris *et al.*, 1993). A flocculation agent (0.1% sodium pyrophosphate) was added to the soil suspension. The population densities of *V. dahliae* on and in the roots were estimated in a sample comprising the entire root mass of 10 plants per plot (Nagtzaam *et al.*, 1997). Subsamples of the soil and root samples were assayed for *T. flavus* by including a heat treatment (60 °C for 30 min.) in the procedure and by plating them on Marois medium (Nagtzaam and Bollen, 1997). At harvest, presence of *T. flavus* in tubers was estimated by plating segments of surface-sterilised tubers on Marois medium.

Stem infection and stem colonisation by *V. dahliae* was determined as described by Nagtzaam *et al.* (1997). Twenty stems were collected, one per plant, from each plot. The two methods, viz. plating stem segments or sap from stems, were compared for their effectiveness in detecting *V. dahliae* in stems.

The initial inoculum densities of *V. dahliae* and *T. flavus* in the upper 20 cm of soil from the experimental fields were below the detection limits of 0.2 microsclerotia g^{-1} and 1 cfu g^{-1} of air-dried soil, respectively.

Because *Pratylenchus* spp. may predispose potato plants to infection by *V. dahliae* (Rowe *et al.*, 1985), roots were analysed for presence of these nematodes using

the funnel spray method (Oostenbrink, 1960). *Pratylenchus penetrans* and *P. crenatus* were not detected in five random samples from the field (average fresh root weight sample^{-1} 0.6 g).

Experimental design and treatments

During the years 1992-1995 the efficacy of *T. flavus* was studied in two separate field experiments, each lasting two growing seasons (Exp. 1 in 1993 and 1994, and Exp. 2 in 1994 and 1995). The fields were located on a clayey soil (pH-KCl 7.0, organic matter 3.5%) in Wageningen, The Netherlands. Seed potatoes of cv. Element were grown according to commercial farmers' practices. Plant density was 40,000 plants ha^{-1} with a distance between rows of 0.75 m. Each of five replicate plots measured 12 x 12 m or 6 x 6 m in Exp. 1 and 2, respectively. Plants in the outside rows were not assessed. Irrigation was not applied. Soil temperature at 10-cm during the growing season were monitored at the Wageningen meteorological station 'De Haarweg', 3 km from the experimental site.

In Exp. 1, the soil was artificially infested with *V. dahliae* at a rate of 50 viable microsclerotia g^{-1} soil (calculated for a 20-cm layer) in November 1992 or April 1993. The autumn application was made in the same period when *V. dahliae*-infected potato parts usually are ploughed into soil in commercial practice. The spring application was made prior to planting. In Exp. 1 additional treatments were included to study the relationship between artificial infestation of field plots with *V. dahliae* and colonisation of the plant by the pathogen and crop yield. Understanding of this relation is required to establish indirectly the biocontrol performance of *T. flavus* against *V. dahliae* by assessing pathogen colonisation of the plant and this pathogens' effect on yield. In spring 1993, five replicate plots were infested with 0, 5, 10 and 100 microsclerotia g^{-1} soil. In Exp. 2, 10 or 30 viable microsclerotia g^{-1} soil were applied in spring 1994 only.

In both experiments, the soil was amended with 43 kg ha^{-1} of alginate wheat-bran granules containing ascospores of *T. flavus* 1-3 days before planting. In addition, seed potatoes were treated with ascospores mixed in talcum powder (10 g powder kg^{-1}) just before planting. Four control treatments were included: (1) no addition of *T. flavus* or *V. dahliae*, (2) addition of *V. dahliae* only, (3) addition of *T. flavus* only, and (4) addition of the formulation products without *T. flavus* to plots with or without *V. dahliae*. In both experiments, inocula of pathogen and antagonist were incorporated in soil by disc-harrowing 20 cm deep.

The long-term effect of *T. flavus* on Verticillium wilt was studied by replanting the plots with seed potatoes cv. Element in the next growing season (Exp. 1, 1994 and Exp. 2, 1995). The plots were not reinfested with *V. dahliae* or *T. flavus* in the second growing season.

In both experiments during the years 1992-1995, population densities of *V. dahliae* or *T. flavus* were estimated after standardised periods of days after planting (DAP) the potatoes; in the soil (1 DAP); on the roots (35-40 DAP); and in the stems (100-110 DAP). In Exp. 2, root colonisation by both *V. dahliae* and *T. flavus* and stem colonisation by *V. dahliae* only was measured six times during the growing season in samples from three out of the five plots with 10 microsclerotia g^{-1} soil and treated with or without *T. flavus*. At the end of the first season of Exp. 2, tubers from plots treated with *T. flavus* or from untreated plots were assessed for presence of the antagonist.

Data analysis

The experiments were conducted arranged as a randomised block design in five replicates. Data of root colonisation and densities in soil of *V. dahliae* and *T. flavus* were analysed after $\log_{10}(x+1)$ transformation. Colonisation of the stems by *V. dahliae* was evaluated after calculating the median of cfu of *V. dahliae* of 20 stems in each plot. Analysis of variance and general linear contrasts were used for examining the data. Two methods were compared to detect *V. dahliae* in stems: plating stem segments and plating extracted stem sap on selective medium. The fraction of infected stems in a plot was assessed using both methods. These data were compared by the sign test (Sokal and Rohlf, 1981). Backtransformed data are presented in the tables and figures.

Results

The relationship between artificial infestation of field plots with V. dahliae and colonisation of the plant by the pathogen and crop yield

Colonisation of roots and incidence of stem infection by *V. dahliae* increased linearly with the amount of inoculum added to soil ($P<0.01$; Fig. 1a, b). Since most stems were infected at the end of the growing season (153 DAP), the relation between the proportion of infected stems and the inoculum density was most apparent early in the season, R^2 =0.69, 0.70 and 0.40 at 62, 83 and 153 DAP, respectively.

At 104 DAP, fresh weight of leaves but not fresh weight of tubers showed a significant linear relation with the amount of *V. dahliae* added to soil ($P<0.01$ and 0.07, and R^2 =0.47 and 0.14 , respectively; Fig. 1c-d).

The effect on tuber yield became apparent at the end of the growing season, R^2 =0.04, 0.04, 0.02, 0.14, 0.01 and 0.40 at 41, 62, 83, 104, 125 and 153 DAP, respectively. At final harvest only, fresh weight of tubers showed a significant linear relationship with the amount of inoculum added before planting ($P<0.01$). At 100 ms g^{-1}

(55.8 tons of tubers ha^{-1}), yield loss was 24% compared to the control treatment (73.6 tons ha^{-1}) (Fig. 1d).

Effects of field application of V. dahliae, T. flavus and its formulation on V. dahliae in soil and on roots

The increased population levels of *V. dahliae* in pathogen infested plots persisted in the second year after application, irrespective of harvesting, ploughing and other tillage practices (Table 1). In both experiments, *V. dahliae* was detected also in some of the control plots. In Exp. 1, data of soil inoculum density of *V. dahliae* are presented for the second year only. In the first year, when the suspensions of soil were plated on EA and not on MSEA, almost no colonies of *V. dahliae* had developed on the plates.

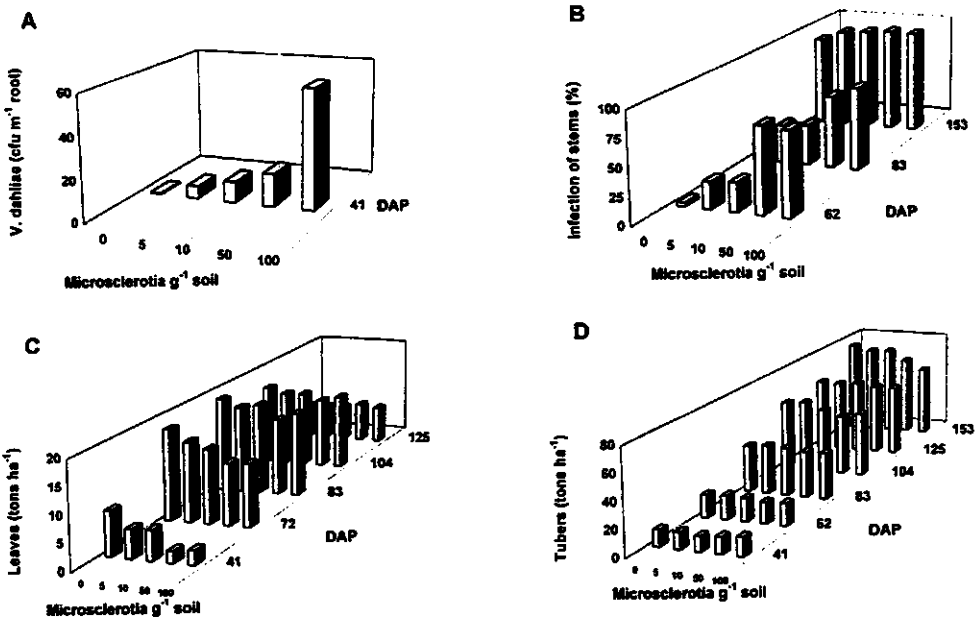

Fig. 1. Root colonisation (A), incidence of stem infection of *V. dahliae* (B) and fresh weight of leaves (C) and tubers (D) at five microsclerotial levels in soil in Exp. 1 during the 1993 growing season. DAP= Days after planting.

Table 1. Populations of *V. dahliae* in soil and on potato roots in plots that were artificially infested in November 1992 or April 1993 (Exp. 1) and April 1994 (Exp. 2).

Treatment	In soil, 1 DAP[a]		On roots, 30-40 DAP	
	Year 1	Year 2	Year 1	Year 2
Experiment 1				
Wheat bran + alginate and talc powder in spring 1993[b]	-[c]	1.5	0.9	-
T. flavus in wheat bran + alginate and in talc powder in spring 1993	-	0.8	0.2	-
V. dahliae in autumn 1992, wheat bran + alginate and talc powder in spring 1993	-	6.4	3.5	3.5
V. dahliae in autumn 1992, *T. flavus* in wheat bran + alginate and in talc powder in spring	-	3.7	1.5	1.7
V. dahliae in spring 1993, wheat bran + alginate and talc powder in spring 1993	-	4.3	33.6	-
V. dahliae in spring 1993, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	-	4.6	43.5	-
General contrasts[d]				
Formulation		-	-	
Antagonist		NS	NS	NS
Interaction[e]		NS	**	
Experiment 2				
Control, no *V. dahliae*, *T. flavus* or formulation product	1.0	3.7	0.3	4.3
Wheat bran + alginate and talc powder in spring 1994	1.6	7.2	0.4	2.8
T. flavus in wheat bran + alginate and in talc powder in spring 1994	1.3	5.6	1.1	3.8
V. dahliae at a low level in spring 1994	10.8	31.4	16.9	19.0
V. dahliae at a low level in spring 1994, wheat bran + alginate and talc powder in spring 1994	5.9	25.3	9.4	14.1
V. dahliae at a low level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder	10.3	19.4	12.6	25.9
V. dahliae at a high level in spring 1994	19.9	45.8	28.6	31.4
V. dahliae at a high level in spring 1994, wheat bran + alginate and talc powder in spring	-	-	-	-
V. dahliae at a high level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder	12.4	31.4	21.8	18.5
General contrasts[d]				
Formulation	*	NS	NS	NS
Antagonist	NS	*	NS	NS
Interaction[e]	**	**	**	**

[a] DAP = days after planting; [b] Wheat bran + alginate with or without *T. flavus* was applied to soil, talc powder with or without *T. flavus* was applied to tuber; [c] Treatment not assessed or not included; [d] General contrasts by Students t-test. NS = not significant, * and ** significant difference at P=0.05 and P=0.01, respectively; 'Formulation' = effect of formulation product on *V. dahliae*; 'Antagonist' = effect of formulated *T. flavus* product on *V. dahliae*; [e] Interaction between the factors *V. dahliae* (df=2) and *T. flavus* (df=1) in analysis of variance.

Colonisation of roots by *V. dahliae* was 3.5.-33.6 cfu m^{-1} root (Table 1). During the growing season, root colonisation density increased only slightly (Fig. 2a). Assessment of the populations of *V. dahliae* in soil and on roots was done early in the season, at 1 and 30-40 DAP, respectively. At these sampling dates, periods of exposure of the pathogen to the antagonist were 1-4 d, 30-34 d, and >1 year with the assessment in the second growing season. The results of Table 1 show that the populations of *V. dahliae* in soil or on the roots were not consistently affected by the antagonist, neither at the low nor at the high level of pathogen inoculum. On two occasions only was a reduction observed. In Exp. 2, at 110 DAP, roots were significantly less densely colonised by the pathogen in the plots with *T. flavus* preparation than in those without the antagonist (Fig. 2a). In Exp. 2, in the second year, the pathogen population was significantly lower in soils of plots treated with the antagonist than in untreated plots (Table 1).

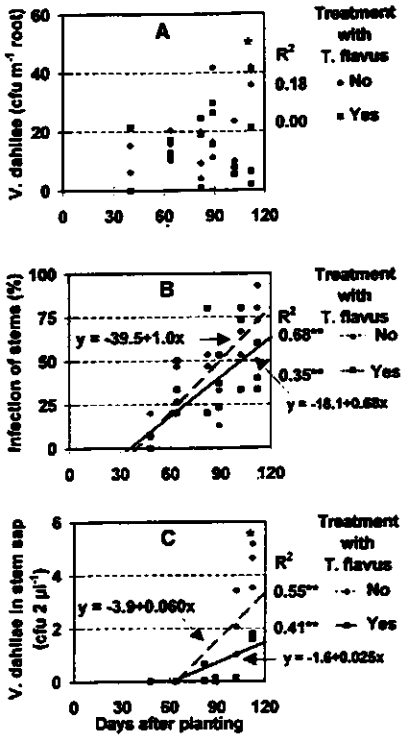

Fig. 2. Root colonisation (A), incidence of stem infection (B) and stem colonisation (C) by *V. dahliae* in plots with 10 microsclerotia g^{-1} soil in Exp. 2 during the 1994 growing season (year 1). An asterisk (*) indicates significant difference by analysis of variance ($P<0.05$) between values in treatments with *V. dahliae* only and in treatments with *T. flavus* and *V. dahliae*.

In Exp. 2 the inclusion of two control treatments (one in which the formulation products were applied and one in which nothing was added to the plots) allowed the assessment of the effects of the formulation products. Development of *V. dahliae* in soil and on the roots was reduced by applying alginate wheat-bran for application of *T. flavus* to soil and talcum powder for application of this antagonist to seed tubers, but differences were not statistically significant except for soil inoculum density in the first growing season in Exp. 2 (Table 1).

Effects of field application of V. dahliae, T. flavus and its formulation on colonisation of the plant by V. dahliae

The incidence of stem infection and rate of colonisation by *V. dahliae* were higher in pathogen infested plots than in the control plots in the first year after inoculation (Table 2). Incidence of stem infection gradually increased from the first date of assessment (Fig. 2b). Population density in the sap of the stem showed a marked increase from 100 days after planting on (Fig. 2c). Significant interactions were observed ($P<0.05$) between *T. flavus* preparation and the two levels of initial *V. dahliae* inoculum applied on the incidence of stem infection as well as the pathogen populations in the stem (Table 2). However, the direction of interaction was not consistent. Except for Exp. 1, year 1, platings of extracted stem sap gave significantly higher numbers of infected stems than segment platings ($P<0.001$; data not shown).

Incidence of stem infection was not affected by applying *T. flavus* preparation (Table 2), but colonisation of the stem was lower ($P<0.05$) in the first season of Exp. 1 and in the second season of Exp. 2. This significant difference in level of stem colonisation by the pathogen persisted between plots with antagonist preparation and those without the antagonist in Exp. 2 if non-infected stems were excluded from the analysis.The effect of formulation products was assessed in Exp. 2, where colonisation was reduced by the products ($P<0.01$), but stem infection was not.

Effects of field application of V. dahliae, T. flavus and formulation products on tuber yield

The lower stem infection or colonisation of *V. dahliae* in plots treated with *T. flavus* or formulation products was not followed by an increase in tuber yield (Table 3).

Table 2. Incidence of stem infection and colonisation by *V. dahliae*.

Treatment	Incidence of infection (% infected stem segments 100-110 DAP[a])		Colonisation (cfu 200 µl^{-1} sap 100-110 DAP)	
	Year 1	Year 2	Year 1	Year 2
Experiment 1				
Control, no *V. dahliae*, *T. flavus* or formulation product	19	-[c]	-	1.3
Wheat bran + alginate and talc powder in spring 1993[b]	19	45	-	6.8
T. flavus in wheat bran + alginate and in talc powder in spring 1993	12	51	-	-
V. dahliae in autumn 1992, wheat bran + alginate and talc powder in spring 1993	66	36	110	16.6
V. dahliae in autumn 1992, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	66	39	58	15.6
V. dahliae in spring 1993	67	-	-	-
V. dahliae in spring 1993, wheat bran + alginate and talc powder in spring 1993	65	31	-	5.8
V. dahliae in spring 1993, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	68	29	-	18.1
General contrasts[d]				
Formulation	NS	-	-	-
Antagonist	NS	NS	*	NS
Interaction[e]	**	NS	-	NS
Experiment 2				
Control, no *V. dahliae*, *T. flavus* or formulation product	10	75	0.3	52.7
Wheat bran + alginate and talc powder in spring 1994	8	74	0	85.5
T. flavus in wheat bran + alginate and in talc powder in spring 1994	27	76	0.3	44.7
V. dahliae at a low level in spring 1994	73	80	128.6	692
V. dahliae at a low level in spring 1994, wheat bran + alginate and talc powder in spring 1994	68	92	25.1	152.4
V. dahliae at a low level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	43	83	24.5	80.3
V. dahliae at a high level in spring 1994	55	79	57.8	206.2
V. dahliae at a high level in spring 1994, wheat bran + alginate and talc powder in spring 1994	-	-	-	-
V. dahliae at a high level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	73	80	98.6	33.8
General contrasts[d]				
Formulation	NS	NS	**	NS
Antagonist	NS	NS	NS	*
Interaction[e]	**	NS	**	**

[a] [b] [c] [d] [e] See footnotes Table 1.

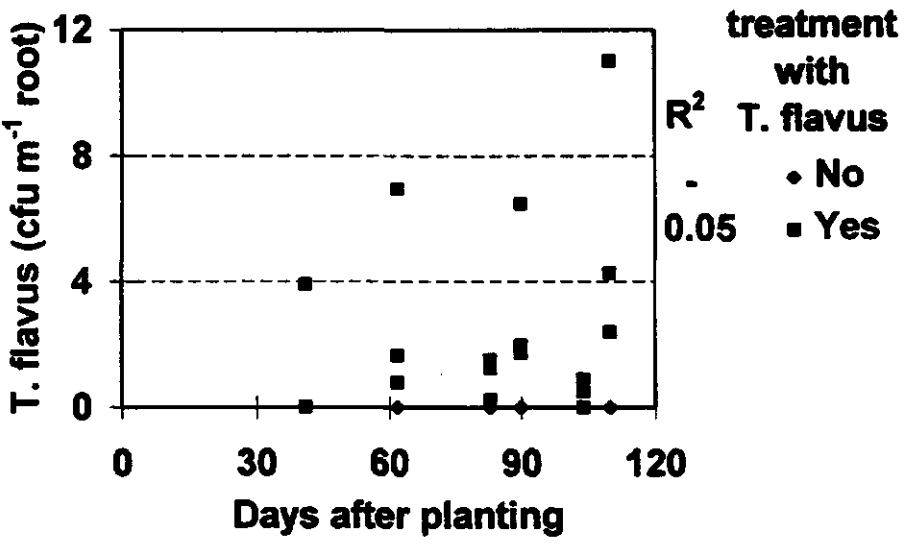

Fig. 3. Root colonisation by *T. flavus* of plots infested with 10 microsclerotia g^{-1} soil in Exp. 2, year 1, during the 1994 growing season.

Effect of field application of T. flavus on its establishment in soil and its colonisation of roots and tubers

T. flavus was readily recovered from soil but hardly from roots (Table 4). After 110 DAP, only about 6 cfu m^{-1} root were recovered (Fig. 3). The treatment with *T. flavus* increased its populations in the soil and on the roots in both Exp. 1 and Exp. 2 (Table 4). Increased antagonist populations in the soil of treated plots were recovered again in the second year after application. Significant interactions were observed ($P<0.05$) between *T. flavus* preparation and the two levels of initial *V. dahliae* inoculum applied on the populatons of *T. flavus* in soil and on potato roots. However, the pattern of interaction was not consistent.Tubers remained almost free of *T. flavus*; the antagonist did not appear on 210 plated segments of tubers from treated plots and on only 1 out of 105 tubers of untreated plots.

Table 3. Tuber yield of plots in Experiments 1 and 2.

Treatment	Yield (ton ha^{-1})	
	Year 1	Year 2
Experiment 1		
Control, no *V. dahliae*, *T. flavus* or formulation product	73.6	-[c]
Wheat bran + alginate and talc powder in spring 1993[b]	67.0	34.1
T. flavus in wheat bran + alginate and in talc powder in spring 1993	62.8	38.0
V. dahliae in autumn 1992, wheat bran + alginate and talc powder in spring 1993	60.0	33.1
V. dahliae in autumn 1992, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	62.6	34.9
V. dahliae in spring 1993	62.8	-
V. dahliae in spring 1993, wheat bran + alginate and talc powder in spring 1993	63.2	36.5
V. dahliae in spring 1993, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	60.8	32.7
General contrasts[d]		
Formulation	NS	-
Antagonist	NS	NS
Interaction[e]	NS	*
Experiment 2		
Control, no *V. dahliae*, *T. flavus* or formulation product	46.0	43.1
Wheat bran + alginate and talc powder in spring 1994	48.5	43.5
T. flavus in wheat bran + alginate and in talc powder in spring 1994	47.1	45.5
V. dahliae at a low level in spring 1994	40.8	42.0
V. dahliae at a low level in spring 1994, wheat bran + alginate and talc powder in spring 1994	40.5	41.3
V. dahliae at a low level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	37.9	42.7
V. dahliae at a high level in spring 1994	38.1	40.3
V. dahliae at a high level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	36.2	39.0
General contrasts[d]		
Formulation	NS	NS
Antagonist	NS	NS
Interaction[e]	**	NS

[b c d e] See footnotes, Table 1.

Discussion

Colonisation of the plant by *V. dahliae* and fresh weight of leaves and tubers showed a clear dose-response relation to the amount of pathogen inoculum incorporated in soil (Fig. 1). Increase of root colonisation and incidence of stem infection started early in the season, also in plots that were infected with the lowest amount of inoculum (5 ms g^{-1} soil). Effects on tuber yield became apparent at the end of the season. A similar pattern of host colonisation during the growing season and a retarded effect on tuber yield were noticed in earlier studies (Nicot and Rouse, 1987; Bollen *et al.*, 1989). From the data presented in Fig. 1, it can be inferred that under the circumstances that prevailed in the experiments, colonisation of roots and stems rather than tuber yield will be affected by an agent or an other factor restricting pathogen development.

After application of a *T. flavus* preparation, stems were less densely colonised by *V. dahliae* in the treated plots than in the control plots in the first growing season of Exp. 1 and in the second growing season of Exp. 2. Significant interactions ($P<0.05$) were observed between *T. flavus* preparation and the two levels of initial *V. dahliae* inoculum. As the direction of the interaction was not consistent for the experiments a biological interpretation of the interaction cannot be provided. A long-term suppression of the pathogen by *T. flavus* is consistent with results of McLaren *et al.* (1994) and Fravel *et al.* (1986), who observed a lower yield reduction more than one year after inoculation of *T. flavus*. Multi-year effects of antagonists have been found for other mycoparasites as well, e.g. *Coniothyrium minitans* on *Sclerotium cepivorum* (Budge and Whipps, 1991; Gerlagh *et al.*, 1995) and *Sporidesmium sclerotivorum* on *Sclerotinia minor* (Adams and Ayers, 1982). *T. flavus* has been claimed to suppress *V. dahliae* by production of glucose oxidase (Fravel and Roberts, 1991) or by mycoparasitism (Fahima and Henis, 1990). Recently, Madi *et al.* (1997) suggested that production of antifungal compounds and glucose-oxidase activity cause retardation of germination, of hyphal growth and of melanization of microsclerotia. Which of these mechanisms predominates when the antagonist is operating under field conditions still needs to be elucidated. The nutritional status of the soil may determine which of the potentential biocontrol mechanisms is most active. The formulation with wheat bran as an organic carrier of *T. flavus* may adversely have affected it's performance (Fravel *et al.*, 1995). It remains questionable whether the observed suppressive effect on *V. dahliae* was due to *T. flavus* since the suppression was not associated with a marked increase of antagonist population in soil or on roots (Table 4). However, both timing and placement of the antagonist and a high level of antibiotic production may be more critical for biocontrol performance than the population size

Table 4. Populations of *Talaromyces flavus* in soil and on potato roots.

Treatment	In soil, 1 DAP[a] (cfu g^{-1} of soil)				On roots, 30-40 DAP (cfu m^{-1} root)	
	Year 1		Year 2		Year 1	Year 2
Experiment 1						
Wheat bran + alginate and talc powder in spring 1993	0	[b]A	3.0	B	0.1	0
T. flavus, wheat bran + alginate and talc powder in spring 1993 [b]	59.4	A	66.0	A	5.6	0.6
V. dahliae in autumn 1992	-		-		-	0
V. dahliae in autumn 1992, wheat bran + alginate and talc powder in spring 1993	7.8	A	4.7	A	0.1	0
V. dahliae in autumn 1992, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	49.0	A	51.7	A	3.7	1.7
V. dahliae in spring 1993	-		-		-	0.2
V. dahliae in spring 1993, wheat bran + alginate and talc powder in spring 1993	20.7	A	28.5	A	0	-
V. dahliae in spring 1993, *T. flavus* in wheat bran + alginate and in talc powder in spring 1993	103.2	A	49.2	A	12.6	0.7
General contrasts[d]						
Formulation	-		-		-	NS
Antagonist	*		**		**	NS
Interaction[e]	**		**		**	NS
Experiment 2						
Control, no *V. dahliae*, *T. flavus* or formulation product	2.3	A	1.5	A	0	0
Wheat bran + alginate and talc powder in spring 1994	7.7	A	1.2	B	0	0
T. flavus in wheat bran + alginate and in talc powder in spring 1994	9.3	A	3.0	B	0.6	0
V. dahliae at a low level in spring 1994	0.7	A	1.2	A	0	0
V. dahliae at a low level in spring 1994, wheat bran + alginate and talc powder in spring 1994	3.0	A	1.1	B	0	0
V. dahliae at a low level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	7.7	A	2.2	B	1.7	0.3
V. dahliae at a high level in spring 1994	1.0	A	1.4	A	0.2	0
V. dahliae at a high level in spring 1994, wheat bran + alginate and talc powder in spring 1994	-	A	-		-	-
V. dahliae at a high level in spring 1994, *T. flavus* in wheat bran + alginate and in talc powder in spring 1994	3.7	A	1.9	A	0.7	0.5
General contrasts[d]						
Formulation	*		*		NS	NS
Antagonist	**		**		**	**
Interaction[e]	**		**		NS	NS

[a][b][c][d][e] See footnotes, Table 1; [b] Different capital letters denote significant difference at different times across rows via Duncan's Multiple Range Test, P=0.05.

(Papavizas, 1985). Furthermore, growth of a mycoparasite requires time and therefore the probability of parasitism lags behind changes in host density. This may confound attempts to correlate current levels of mycoparasitism with current levels of pathogen density (Turchin, 1990). In addition, a mycoparasite may not increase or even persist unless host density exceeds a threshold level (Jaffee, 1993).

The density of *V. dahliae* on roots (in general less than 50 cfu m^{-1} root) remained at a constant level throughout the season (Fig. 2a). Huisman (1988a) found a similar pattern for colonisation of cotton roots. It is unlikely that the lower density of the pathogen on roots at the end of the season in plots treated with *T. flavus* preparation in Exp. 2 (Fig. 2a) is caused by direct interaction in or on the roots. A direct contact between pathogen and antagonist is unlikely because of the low density of both the antagonist (<15 cfu m^{-1} root) and pathogen on the roots (Fig. 2 and 3). Involvement of induced resistance by the antagonist seems to be plausible. Circumstantial evidence is provided by the observation that the sap of infected stems in plots treated with *T. flavus* contained less cfu of *V. dahliae* than in untreated plots. Apparently, development of *V. dahliae* in the plant is affected.

Alginate wheat-bran as a formulation product had some biocontrol effect of its own. In Exp. 2 it reduced pathogen colonisation of stems. In growth chamber experiments the same phenomenon was more pronounced when the product was added in higher amounts (1% w/w) than in the present field experiment (Nagtzaam and Bollen, 1997). Reduction of inoculum in soil and control of Verticillium wilt after application of organic products has been reported several times (Green and Papavizas, 1969; Jordan *et al.*, 1972; Harrison, 1976; Dutta and Isaac, 1979). Perhaps unidentified antagonists were activated by the nutrients in the wheat bran. Rapid exhausting of the easily accessible nutrients implies that an effect of wheat bran will be restricted to a narrow zone around the particles and the effect will be short-lived, which might explain that it appeared in the first but not in the second year (Exp. 2, Table 2).

Population densities of *T. flavus* in soil correspond with those reported by Keinath (1990) but were low compared to those of Fravel *et al.* (1986). Populations of *T. flavus* remained at a higher level in antagonist-treated plots than in untreated plots in the second year after introduction. Apparently, the fungus survived soil freezing during winter. Its ascospores are known for persistence under adverse conditions (Beuchat, 1992; Nagtzaam and Bollen, 1994). However, antagonist density did not significantly increase. Recently, Daigle and Cotty (1997) demonstrated that a minimum density of *Aspergillus flavus* spores must be present in alginate pellets to ensure that the agent excludes competitors and successfully converts the energy in the pellets into biocontrol biomass. Perhaps the granules were ground in too small particles or the density of

T. flavus in the particles was too low to successfully compete with the indigenous soil microflora.

T. flavus occurred in non-inoculated control plots in the second year of Exp. 2 (Table 4). Fravel *et al.* (1986), Spink and Rowe (1989) and Keinath *et al.* (1990) experienced similar infestation problems in their control plots. At the start of the experiments*T. flavus* was not detected in soil of the plots. Contamination by seed tubers colonised by *T. flavus* is not likely as the antagonist did not appear on tubers from *T. flavus* - treated plots.

Reduction in tuber yield caused by *V. dahliae* is more severe at relative high temperatures and when the crop suffers from drought stress than when conditions are cool and wet late in the growing season (Johnson, 1988; Haverkort *et al.*, 1989). Soil temperature and precipitation close to the experimental site are given in Fig. 4. Weather conditions were conducive for the disease in 1994 and 1995 but not in 1993. Nevertheless, in 1994 and 1995 the reduced stem colonisation by *V. dahliae* in plots with introduced *T. flavus* was not followed by an increased tuber yield.

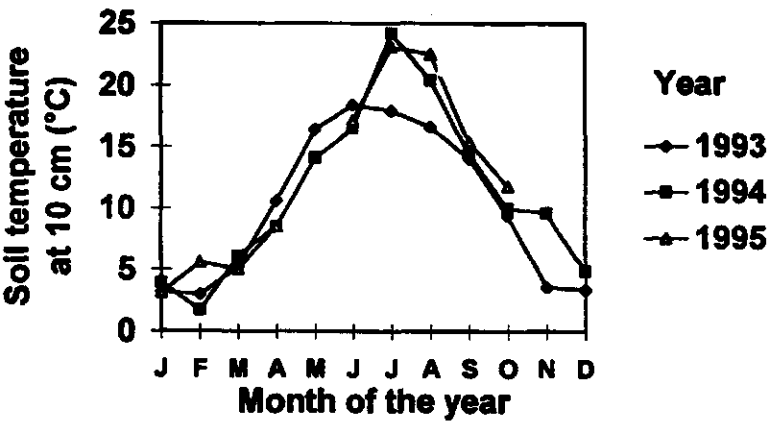

Fig. 4. Monthly average temperature (°C) at 10-cm soil depth from 1993 to 1995 at a meteorological station a 3 km from the field plots.

Apart from a few inconsistent suppressive effects on *V. dahliae*, our results are in line with those of Spink and Rowe (1989), who found that disease development was not suppressed and tuber yield was not increased by applying *T. flavus* preparation, even so in plots where the population of the antagonist had increased to high levels. However, several authors (Marois *et al.*, 1982; Davis *et al.*, 1986; Fravel *et al.*, 1986; Zeise, 1997) demonstrated the potential of *T. flavus* for control of Verticillium wilt in the field. These variable results are probably related to insufficient understanding of

environmental conditions on the growth and survival of both pathogen and potential Verticillium antagonists in field soil. Further studies should concentrate on identification of the conditions required for efficacy of biocontrol in order to evaluate the feasibility of *T. flavus* for biocontrol of *V. dahliae* in the field.

Chapter 8

General discussion

The issue raised in this thesis is whether *T. flavus* has sufficient potential for biocontrol of *V. dahliae*. This topic was approached by considering three ways in which biocontrol can be achieved. The first aim was to decrease the amount of pathogen inoculum that enters the soil. A second aim was inactivation of the microsclerotia population resident in soil. The third aim was prevention of infection of the host by restraining colonisation of roots and stems by the pathogen. The results of the experiments on potential of *T. flavus* as a control agent will be evaluated for each of the three aims. Subsequently, the opportunities of biocontrol of *V. dahliae* as such will be discussed.

Restricting enter and build-up of pathogen inoculum into soil

In single-cycle vascular wilt diseases, fungus inoculum produced during one growing season seldom incites additional infections during the same growing season (Schnathorst, 1981). The results shown in Fig. 1 of Chapter 2 demonstrate that severity of wilt caused by *V. dahliae* is related to the size of the inoculum population at the start of the growing season. These results are in line with those of Ashworth *et al.* (1979), Bollen *et al.* (1989) and Davis *et al.* (1994). Although pathogen inoculum density in soil is a major one, it is not the only determinant of disease severity. Due to differences in cultural practices, soil type, environmental conditions, host cultivars and virulence of *V. dahliae* isolates, a clear relationship between initial inoculum size and disease severity is not always apparent (Davis and Everson, 1986; Paplomatas *et al.*, 1992; Bejarano-Alcázar *et al.*, 1995).

Treatments of infected crop debris with antagonists

Impeding the build-up of inoculum in soil is achieved by growing resistant instead of susceptible cultivars and removal of debris of diseased potato crops from the field (Davis *et al.*, 1994). However, the degree of the reduction achieved in this way is not sufficient for adequate disease control in a susceptible crop (Mol *et al.*, 1995). Another approach is by applying antagonists to crop debris which rapidly colonise the plant tissue and reduce saprophytic growth of the pathogen, thus preventing formation of microsclerotia. This approach makes effective use of the fact that *V. dahliae* is a weak competitor for its food source. The antagonists might also inactivate the microsclerotia that are already present on and in the plant remains.

From the results of the experiments where microsclerotia were exposed to *T. flavus* ascospores at various densities, it can be inferred that the efficacy of killing

microsclerotia depends on the density of the antagonist population (Chapter 5). At high densities, the entire population of microsclerotia was inactivated. Therefore, inactivation of microsclerotia by applying antagonists to plant debris seems a promising approach.

Spraying senescent potato stems, after harvest of a diseased crop, with a spore suspension of *T. flavus* at high densities resulted in a reduction, but not complete suppression of the microsclerotia population (Chapter 5, Table 2). Tjamos and Fravel (1995) observed a reduced germinability of microsclerotia on stems of *Verticillium*-infected cotton plants treated with *T. flavus*. The less pronounced effect, compared with microsclerotia incubated *in vitro* or in soil, is probably due to the harsher conditions for antagonist development above ground due to rapid fluctuations in water potential, extreme temperatures and UV-radiation. Although ascospores of *T. flavus* are expected to survive these conditions, biocontrol will only occur when survival is followed by growth of the antagonist.

Prolonged effect of biocontrol application

Treatment with biocontrol agents instead of chemicals for inactivation of microsclerotia can have the advantage of a prolonged effect. When plant debris is ploughed under at the end of the growing season, microsclerotia that were colonised by the antagonist can serve as initial centres of growth for the antagonist and inactivate other microsclerotia in soil.

In one of the two field experiments described in Chapter 7, stem colonisation by *V. dahliae* was also reduced in the second year following application of *T. flavus* (Table 2). In the second year, the plots were replanted by seed potatoes and not reinfested with *V. dahliae* or *T. flavus*. Long-term suppression of the pathogen by *T. flavus* was consistent with the results of McLaren *et al.* (1994) and Fravel *et al.* (1986), who observed a lower yield reduction in the second year after inoculation of *T. flavus*. Working with *Coniothyrium minitans*, Gerlagh *et al.* (1995) demonstrated that one application of the mycoparasite provided control of white mould caused by *Sclerotinia sclerotiorum* for four years after the year of application in various crops.

Inactivating the resident pathogen inoculum in soil

In The Netherlands, potatoes are cultivated on 120,000 hectares corresponding with about 20% of the total area of arable crops in 1996. Probably, most of these soils are infested with *V. dahliae* because of spread of pathogen propagules from infected potato fields by wind, water, machinery and animals and persistence of the infestation because of the long term survival of the microsclerotia. Therefore, control of Verticillium wilt

should aim at inactivation of the resident pathogen inoculum in soil rather than at prevention of infestation.

Elimination of soil-borne inoculum was focussed on inactivation of the microsclerotia rather than on parasitism of the hyphae that emerge from the microsclerotia. Establishment of parasitism of hyphae can involve only a very limited volume of soil as the ephemeral germinating hyphae have an insignificant maximum length of 0.1 mm (Fitzell *et al.*, 1980). Moreover, the time available for parasitism of hyphae is short as microsclerotia germinate mainly in the vicinity of the root tip (Mol and Van Riessen, 1995).

Density related biocontrol effects

Under controlled conditions, in both a sandy and a natural clay soil, the initial microsclerotial population of *V. dahliae* was substantially reduced by incorporating *T. flavus* alginate wheat-bran (Chapter 5, Fig. 2, Table 3) into the soil. Viability of microsclerotia was reduced, even at 15 °C which is a low temperature for *T. flavus* as the optimum temperature for its *in vitro* growth is 34 °C. Fravel *et al.* (1986) succeeded in obtaining control of *V. dahliae* in the field when applying 36 kg antagonist inoculum ha^{-1} (about 0.002% w/w), but they provided no data about pathogen density in soil. At about the same population density, we failed to establish an effect on the pathogen population in field soil (Chapter 7, Table 1). Spink and Rowe (1989) were unable to show an effect on populations of *V. dahliae* in field soil even when they applied up to 1,523 kg ha^{-1} of pellets with ascospores of *T. flavus*. Other data on effects of antagonists on field soil inoculum densities of *V. dahliae* were not found in the literature. The labour intensive detection methods may be one of the reasons for this paucity of data. Moreover, recovery of *V. dahliae* from soils is characterised by inconsistent and highly variable results (Wheeler and Rowe, 1995). Difficulties were also encountered in estimating the pathogen population size in soil. In 1993, it was not possible to obtain a reliable quantification, although after modifying the medium used for plating soil suspensions reasonable estimates of the microsclerotial population were obtained one year later (Chapter 7).

Pathogen populations in soil are reduced only at high densities of *T. flavus* (Chapter 5, Table 3, Fig. 2). At high densities of an antagonist, distances between pathogen and antagonist propagules in soil are small (Chapter 6). A small distance is a prerequisite for suppression of *V. dahliae* by *T. flavus*, viz. by production of glucose oxidase (Fravel and Roberts, 1991) and other products (Madi *et al.*, 1997) or by mycoparasitism of microsclerotia (Fahima and Henis, 1990; Fahima *et al.*, 1992). Effectivity of metabolites including glucose oxidase involved in antibiosis is limited to the

vicinity of the antagonist as these chemicals are bound to soil particles or inactivated (Brian, 1957). Mycoparasitism demands unconditionally direct contact between antagonist and pathogen.

Growth of *T. flavus* increases the opportunity for contact between pathogen and antagonist. To estimate its growth rate, a testing system was developed taking into account an uneven development of the antagonist in soil. At 25-30 °C in incubators a growth rate of 0.1-1.1 mm day^{-1} was found in a sandy and a loamy sand (Nagtzaam and Termorshuizen, unpublished). Growth in soil does not accord with the view of Adams (1990), who classified *T. flavus* as a passive mycoparasite that does not grow out from an infected sclerotium in natural soil. In Chapter 6, a model is described to estimate distances between fungal propagules in soil at given inoculum densities. Using the model, the average distance was calculated to be 4 mm in the plow layer of the plots in the field experiments of Chapter 7, where 43 kg ha^{-1} of antagonist inoculum was applied (about 0.002% w/w). The average fungal growth rate mentioned above applies to growth at 25 °C, whereas the mean soil temperature during the growing season of potato in The Netherlands is 15 °C. In laboratory experiments, *in vitro* growth on agar was shown to be four times as much at 25 °C than at 15 °C. Therefore, it is unlikely that in the field *T. flavus* could bridge the distance of 4 mm between propagules in order to reach Verticillium microsclerotia in time, i.e. before they had germinated in the rhizosphere and infected the root. This implies that more antagonist inoculum would have been needed than was applied in the field experiment. The density related effect is shown in an experiment under controlled conditions where in soil supplied with *T. flavus* and incubated at 15 °C for 10 weeks, the population of *V. dahliae* was reduced at a density of 0.1% but not at 0.001 % of pathogen inoculum (Chapter 5, Fig. 2). If this information had been available before the start of the field experiment, a higher level of antagonist would have been applied, however because of the 2-year scheduling of the field experiments, the amount of antagonist inoculum was based only on literature data. In particular, the data of Fravel *et al.* (1986) were used as a source. These authors recorded successful control of *V. dahliae* in the field with *T. flavus* inoculum at a rate of 43 kg ha^{-1}.

Selection of more effective antagonists

In future research on control of *V. dahliae* by fungal antagonists the selection procedure merits further attention. Control may be improved by the use of other species or other isolates of *T. flavus*. The isolate used in the present study was selected out of 17 isolates that had been recognised as fungal antagonists on soil plates or dilution plates. Three isolates were obtained from roots and fourteen from soil originating from widely separated areas in various countries. The selection was based on the width of inhibition

zone on agar against *V. dahliae* and other pathogens, and on performance against *V. dahliae* in growth chamber experiments.

Biocontrol agents against *V. dahliae* have been isolated from roots and rhizosphere (Berg, 1997; McLaren *et al.*, 1986; Wadi and Easton, 1985), diseased plant material (Ordentlich *et al.*, 1990), a sclerotium of another pathogen (Dunn and Lumsden, 1981), or from soil as was done in the present thesis. According to Harman and Hadar (1983), most successful agents originate from an environment similar to that in which they are expected to act. Therefore, most efficacy in elimination of inoculum in soil can be expected from *T. flavus* or other antagonists that occur in soil, preferably on microsclerotia. An interesting candidate has recently been recovered from soil in Alaska. It is a cold tolerant strain of *Trichoderma atroviride* that parasites conidia, mycelia and microsclerotia of *V. dahliae* (McBeath, 1994).

Biocontrol in the second growing season

In the second growing season, the pathogen population in soil consists of the remaining resident microsclerotia from the previous seasons and the microsclerotia that are released from infected debris of the last crop that became incorporated into soil. The total number of microsclerotia from the crop residues depends on the level of colonisation of the plants by the pathogen and the amount of stems and leaves ploughed into the soil. As has been shown in Fig. 1 of Chapter 7, the amount of crop residue decreases with increasing pathogen density in soil. This phenomenon explains the finding of Mol *et al.* (1996) that soil inoculum density increases faster with low rather than high levels of infestation in potato-cropped soils. It also may affect biocontrol effects in multiyear experiments. At the end of the second growing season more microsclerotia in soil may be present in plots which had a reduced pathogen level the first year, as more crop residues, containing microsclerotia, are ploughed into soil than in plots with a higher pathogen level. In the first year of field experiment 2, a significant difference in pathogen microsclerotia level in soil was demonstrated for fields treated with a low and high level of *V. dahliae*. This difference had disappeared in the second year (Chapter 7, Table 1). Induced suppressiveness to Verticillium wilt by repeated cropping of the same soil (Keinath and Fravel, 1992) may also interact with effects of treatment with selected biocontrol agents.

Notwithstanding the complications mentioned above, it is relevant to monitor effects of biocontrol agents against *V. dahliae* for several years after application. Presumptive evidence for the potential of *T. flavus* to carry over as a biocontrol agent to the second growing season is provided by the results shown in Table 2 of Chapter 7, and those of Fravel *et al.* (1986). The phenomenon has been well documented for other

antagonist-pathogen systems by e.g. Adams and Ayers (1982), McLaren *et al.* (1994) and Gerlagh *et al.* (1995).

Reducing pathogen colonisation of the plant

The effects of biocontrol agents on *V. dahliae* in soil and rhizosphere are reflected in the densities of the pathogen on the root or in the plant. It is presumed that a quantitative variable like the degree of plant colonisation has more discriminating power than a qualitative one such as percentage of infected plants. In Chapter 7, the two methods were compared for their effectiveness in detecting *V. dahliae* in stems of field grown potatoes. Using a qualitative method an assessment of whether or not a stem segment was infected was done by plating of the whole segment on agar. Using a quantitative method the number of individual colonies after plating sap from stems on agar was counted. It was demonstrated that platings of extracted sap gave significantly higher numbers of infected stems than segment platings (Chapter 7). Chapter 2 provides the first experimental evidence for a relation between the level of colonisation of the stem and the inoculum density of *V. dahliae* in soil. A significant correlation between densities in root and sap was established also. Estimating population densities in stem sap in addition to those on the root or the incidence of stem infection provides extra information in studies on effects of environmental factors on the dynamics of *V. dahliae*. This is exemplified in Tables 1 and 2 of Chapter 7 where significant differences were observed between pathogen levels in vascular tissue of field grown plants, but not in incidence of infection or level of root colonisation.

Preventing root infection

Ideally an antagonist should completely prevent infection of the roots by *V. dahliae*. However, in potato any reduction in pathogen development is valuable as yield depression depends on the amount of pathogen inoculum in soil (Chapter 7; Ashworth *et al.*, 1979, Nnudo and Harrison, 1979). In this respect, potato differs from roses and hardy nursery stock where partial control is not relevant because they cannot be traded, irrespective of the level of infection.

To prevent infection, *T. flavus* should be co-located at the same site as *V. dahliae* when the pathogen is about to penetrate the root. This can be attempted by delivering the antagonist to seed or to soil. It was demonstrated that *T. flavus* moved from seeds coated with antagonist to developing roots, including the root tips where *V. dahliae* primarily enters (Chapter 4, Tables 2-5, Fig. 1). Densities of the antagonist on the roots were low and decreased log-linearly with distance from the seed. The log-linear decrease of population density of *T. flavus* points to passive movement along the root.

Passive movement of bacteria along roots was described in a mathematical model by Scott *et al.* (1995). They showed that bacterial densities decreased log-linearly along the growing root by carriage only. Passive transport as a mechanism of colonisation by antagonists has also been suggested for *Verticillium biguttatum,* a mycoparasite of *Rhizoctonia solani* (Van den Boogert, 1989). The presence of individual *T. flavus* colonies on remote parts of the root provides another indication of passive movement because growth implies that the root system would continuously be colonised between the seed and the young mycelium. Fungal growth may be involved if the root cap is colonised by mycelium where subsequently conidia or asci are produced that might passively be transported by adherence of the spores to the root tips. Fahima and Henis (1990) observed conidiophores of *T. flavus* on microsclerotia of *V. dahliae* in superficial tissue of roots. Cleistothecia have been observed on roots of plants grown in test tubes under gnotobiotic conditions (Nagtzaam, unpublished). Detection of *T. flavus* after a selective heat treatment of roots (Chapter 4, Table 2), which kills conidia and mycelium but not ascospores (Van der Spuy *et al.*, 1975), implies formation of cleistothecia with ascospores on roots grown in non-sterile soil. This is likely as the exudation of sugars in the rhizosphere seems to provide the appropriate conditions for cleistothecia formation, as Engelkes *et al.* (1997) recorded that a high C:N ratio stimulated ascospore production by *T. flavus.* It is recommended to study the effect of percolating water on movement of *T. flavus* on the root, because downward flow of water implies that the formation of antagonist propagules behind the root tip will contribute to the dispersion of the antagonist.

Tjamos and Fravel (1995, 1997) observed that recovery of *T. flavus* was significantly greater in root tips when populations were expressed as colony forming units g^{-1} fresh weight of root and subsequently concluded that *T. flavus* has a preference for the root tip. However, they also observed that the percentage of root segments other than tips colonised by *T. flavus* was significantly greater than that of root tips. As mentioned above, the occurrence of cleistothecia on roots can be deduced from data presented in Chapter 4. Engelkes *et al.* (1997) recorded that a high C:N ratio stimulated ascospore production by *T. flavus.* Rovira and Davey (1974) and Curl and Truelove (1986) demonstrated that the zone of elongation is the main site of release of glucose from the roots. Combining these findings it is assumed that the formation of the teleomorphic stage of *T. flavus* is induced and more cleistothecia are formed in the vicinity of the tip than in other areas of the root. When assessing population densities in plant tissue or soil, cleistothecia and asci are crushed and ascospores are set free to yield individual colonies. As a result higher densities in the root tip zone may be found although the population density (number of conidiophores, cleistothecia, asci or separate

hyphal colonies per area of root) on the whole root system may be the same. Indeed, assessing population densities, without crushing cleistothecia and asci, do not provide evidence for preferential colonisation of the root tip by *T. flavus* (Chapter 4, Tables 2-4 and Fig. 1).

From the results presented in Chapter 4 (Tables 2-6, Fig. 1) it is concluded that seed coating does not create antagonist densities on the root that are sufficiently high to completely prevent infection but that, at least, a reduction in infection can be achieved.

Colonisation of the root from soil results in higher densities of *T. flavus* on the root tips. In soil, the majority of *T. flavus* propagules are supposed to be in a dormant state. They will germinate at high levels of root exudates which are excreted from the zone of root elongation and might consequently colonise the root tip. As with *V. dahliae* (Huisman, 1982) on older root tissue, the combination of higher microbial metabolism and a lower exudation rate will result in a lower level of colonisation behind the root tip. Presumptive evidence for a similar pattern in colonisation of the root by *T. flavus* and *V. dahliae* is obtained by the observation that both fungi have about the same colonisation rate (Chapter 4).

Mechanisms of biocontrol on the root

Incorporating a formulated *T. flavus* product in soil diminished infection of roots at a density of 0,01% (w/w) (Chapter 4, Table 6). To reduce the population of *V. dahliae* in soil higher densities of the antagonist population were needed (Chapters 5 and 6). On the root other mechanisms of biocontrol may operate than in the soil. Besides parasitism and antibiosis operating in the soil and on roots, induced resistance and competition for infection sites or exudates may occur on and in the roots. Competition for infections sites is likely to take place on the root tip because both *V. dahliae* (Fitzell *et al.*, 1980; Gerik and Huisman, 1988) and probably also *T. flavus* (Tjamos and Fravel, 1997) have a preference for the root tip. The low density of *T. flavus* on the roots suggests that direct interaction by competition, antibiosis or mycoparasitism is not the principal factor in suppression of *V. dahliae*. Induced resistance in the host plants seems to be more likely. An ability of the fungus to colonise not only the surface but also the cortical tissue of the root would support this hypothesis. Evidence for internal colonisation is provided by presence of *T. flavus* in superficially sterilised root material. Washing the roots under running tap water for 20 min. and disinfestation of the roots in 1% NaOCl for 1 min. yielded the same densities of *T. flavus* on the root as direct plating of the roots on selective medium for *T. flavus* (Chapter 4, Tables 2 and 4).

In conclusion, protection of the plant by reducing root infection seems to be a more appropriate strategy to follow than aiming for elimination of the resident pathogen

population in soil, as in the latter approach much higher densities of the antagonist are needed.

Combining antagonists

As well as *T. flavus, G. roseum, F. oxysporum* and *B. subtilis* reduced root and stem colonisation by *V. dahliae* (Chapters 4 and 5) which is in line with the results of Marois *et al.* (1982) and Fahima and Henis (1990, 1995). It seems that the antagonists act independently on *V. dahliae*, as *T. flavus* combined with another antagonist was usually as suppressive against *V. dahliae* as *T. flavus* or another antagonist alone (Chapter 5, Table 4 and Fig. 3). With a combination of *T. flavus* and *G. roseum*, Johnston *et al.* (1994) obtained higher yields than with either antagonist alone, which suggests that the obtained effect is not solely an overlap of action. Combinations of *T. flavus* with *G. roseum, F. oxysporum* or *B. subtilis* were mostly compatible in their efficacy (Chapter 5, Table 5). In addition, applying the fungus-bacterium combination of *T. flavus* and *B. subtilis* resulted in higher densities of each of the antagonists on the root than after inoculation with the single species. Perhaps, these organisms occupy different habitats because of a heterogenous distribution on the root resulting in spatial separation and a trivial probability of interaction between their populations (Bowen, 1980). Another possibility is that they occupy different ecological niches because of different nutritional or environmental requirements. However, both hypotheses cannot explain the increased antagonist densities on the root in a combined application as compared to a single inoculation. The co-inoculated antagonist may have increased the accessibility of nutrients. Moreover, densities may be indirectly enhanced by increased root exudates induced by the co-inoculated antagonist (Bowen and Theodorou, 1979; Park *et al.*, 1988). Another possibility may be protection of one antagonist by the co-inoculated organism through detoxification of antibiotics or physical protection of its structures (Bowen and Theodorou, 1979). The increased densities of both *T. flavus* and *B. subtilis* as compared to single inoculations do not support the theory of Fravel and Roberts (1991) of glucose oxidase involvement in the biocontrol of *V. dahliae* on the root. Glucose oxidase catalyses the oxidation of glucose, in the presence of oxygen, to gluconate and hydrogen peroxide. The latter product is highly toxic to *V. dahliae* (Kim *et al.*, 1988). *T. flavus* was shown to have a high tolerance level to glucose oxidase in the presence of glucose, but *B. subtilis* was as much sensitive to this metabolite as *V. dahliae* (Kim *et al.*, 1990). If hydrogen peroxide occurs on the root due to presence of *T. flavus*, population densities of *B. subtilis* would tend to be lower in combination with this fungus than after single inoculation.

The results with combinations of antagonists with different modes of action or environmental optima for activity, raise the prospects for better control of *V. dahliae*.

Preventing invasion of the vascular system from infected cortical root tissue

Very few of the successful root infections of *V. dahliae* reach the vascular system (Huisman, 1982). Huisman and Gerik (1989) calculated around 5,000 cortical colonies to each systemic infection. Increasing this ratio may be an effective means of controlling wilt than reducing pathogen colonisation of the root. Attempting to prevent invasion of the vascular system from an infected cortex is a virtually unexplored research field. The internal tissues of the host have probably been ignored in biocontrol because it is almost impossible to achieve adequate spread of an external applied antagonist through the plant and subsequently obtain sufficient direct contact between antagonist and pathogen in the plant environment. In a growing plant, the probability of contact between antagonist and pathogen decreases as the vascular volume increases in time. Reproduction of the antagonist may partly compensate this. This implies that the probability of mycoparasitism and/or antibiosis to occur in the internal tissues of the plant is rather low which did perhaps lead to the focus on biocontrol of *V. dahliae* prior to its entrance and establishment in the host. However, the isolation of endophytic bacterial antagonists against *V. dahliae* (Tjamos *et al.*, 1997) and the development of molecular markers for pathogen and antagonist (Hu *et al.*, 1993; Tjamos *et al.*, 1997) open interesting prospects to study the opportunities for control of the pathogen within the plant.

The feasibility of influencing pathogen development once entry in the plant is gained, can be deduced from the results of Chapter 7. It was demonstrated that sap of infected stems from plots treated with *T. flavus* contained fewer propagules of *V. dahliae* than stems from untreated plots, even if the non-infected stems were excluded from the analysis. Moreover, the low population densities of *T. flavus* on the root and in the soil cannot explain the reduction in vascular colonisation by the pathogen in treated plots in the field (Chapters 4, 5 and 7). This provides circumstantial evidence for the involvement of induced resistance by *T. flavus*. The antagonist was never found in the vascular system of plants (Nagtzaam, unpublished), but this is not required for induction of resistance. Défago *et al.* (1990) and Maurhofer *et al.* (1994) hypothesised that subinhibitory quantities of antifungal metabolites induce the stress necessary to activate plant defence mechanisms. So, antibiotics, other metabolites (e.g. Fuska *et al.*, 1979; Kim *et al.*, 1988) and enzymes (Madi *et al.*, 1992) produced by *T. flavus* in root tissue may trigger induced resistance. Plant-mediated control of *V. dahliae* has been reported several times (Melouk

and Horner, 1975; Price and Sackston, 1989; Schnathorst and Mathre, 1966). These studies were never conclusive as direct interactions between the inducer and the pathogen could not be excluded.

To conclude, it seems that the internal plant environment merits more attention. One line in future research could be to find out whether vascular colonising, non-pathogenic, isolates of *V. dahliae* or other Verticillium species exist and, if so, to test them for their effectiveness against *V. dahliae*. The chance of success will be greater if antagonists are ecologically and behaviourally more similar to the pathogen (Deacon, 1991).

Opportunities for biocontrol of *V. dahliae*

The results of the experiments presented in this thesis and in the literature (*Fravel et al.*, 1986; Ordentlich *et al.*, 1990; Zeise ,1997) demonstrate that *T. flavus* has potential for biocontrol of *V. dahliae*. However, thus far the results with this antagonist tend to be inconsistent (Chapter 7). Control is often partial or even fails completely (Spink and Rowe, 1989; Keinath *et al.*, 1990,1991a,b). With the present inconsistency of results, application of *T. flavus* has little commercial value. The next step in further research should focus on the nature and sources of this inconsistency.

One of the strategies that merit further exploration is inoculation of infected crop debris with antagonists before the crop remains are ploughed under. Improvement of the results obtained with the present isolate of *T. flavus* (Chapter 5, Table 2) might be achieved in various ways. First combinations of *T. flavus* with other species can be used. As shown in Chapter 5 (Table 4) *T. flavus* is compatible with various other antagonists. A second option is to focus on selection for more effective isolates of *T. flavus* itself. As shown in Chapter 4 (Fig. 2) variance between isolates exists in ecological properties. Madi *et al.* (1997) demonstrated that antagonism to *V. dahliae* was increased by fungicide-induced mutation of a wild type *T. flavus*. In the search for new antagonists, the microflora occurring on microsclerotia of *V. dahliae* should be systemically screened. Systematic screening of the species in this habitat has never be done. Likewise it is relevant to initiate studies on the existence of suppressive soils to *V. dahliae* which may be a rich source of potential biocontrol agents.

The opportunities for control of the pathogen within the plant should be explored. In this thesis presumptive evidence is presented that pathogen development in the plant is influenced by *T. flavus*. Recently, bacteria colonising endorhizosphere and vascular tissue of potato have been claimed as effective agents against *V. dahliae* (Tjamos *et al.*, 1997). The development of molecular techniques makes it possible to study behaviour of pathogen and antagonist in vascular tissues.

Thus far, a single method for consistent and effective control of *V. dahliae* compatible with the aims of sustainable agriculture has not become available. For the time being, control has to be integrated from partially effective methods. Biocontrol should be considered as a method to be applied in combination with cultural methods (Mol *et al.*, 1995), biological soil disinfestation (Blok *et al.*, 1995), the use of tolerant cultivars (Mol *et al.*, 1996) or the selective application of chemical control.

References

Adams, P.B., 1990. The potential of mycoparasites for biological control of plant diseases. Annual Review of Phytopathology 28: 59-72.

Adams, P.B. and Ayers, W.A., 1982. Biological control of Sclerotinia lettuce drop in the field by *Sporidesmium sclerotivorum*. Phytopathology 72: 485-488.

Adams, P.B. and Fravel, D.R., 1990: Economical biological control of Sclerotinia lettuce drop by *Sporidesmium sclerotivorum*. Phytopathology 80: 1120-1124.

Adams, P.B., Marois, J. and Ayers, W.A., 1984. Population dynamics of the mycoparasite *Sporidesmium sclerotivorum*, and its host, *Sclerotinia minor*, in soil. Soil Biology & Biochemistry 16: 627-633.

Ahmad J. S. and Baker, R., 1987. Rhizosphere competence of *Trichoderma harzianum*. *Phytopathology* 77: 182-189.

Allen, M.F., Boosalis, M.G., Kerr, E.D., Muldoon, A.E. and Larsen, H.J., 1985. Population dynamics of sugar beets, *Rhizoctonia solani*, and *Laetisaria arvalis*: Responses of a host, plant pathogen, and hyperparasite to perturbation in the field. Applied Environmental Microbiology 50: 1123-1127.

Allen, S.J., 1994. The effects of various cultural practices on the epidemiology of Verticillium wilt of cotton in New South Wales. Proceedings of 6th International Verticillium Symposium, Dead Sea, Israel.

Anonymous, 1990. Multi Year Crop Protection Plan. Ministry of Agriculture, Nature conservation and Fisheries, The Hague, The Netherlands.

Ashworth, L.J., Huisman, O.C., Harper, D.M. and Stromberg, L.K., 1974. Free and bound microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 64: 563-564.

Ashworth, L.J., Jr., Huisman, O.C., Harper, D.M. and Stromberg, L.K., 1979. Verticillium wilt disease of tomato: influence of inoculum density and root extension upon disease severity. Phytopathology 69: 490-492.

Ashworth, L.J., McCutcheon, O.D. and George, A.G., 1972. *Verticillium albo-atrum*: the quantitative relationship between inoculum density and infection of cotton. Phytopathology 62: 901-903.

Ashworth, L.J., Waters, J.E. Jr., George, A.G. and McCutcheon, O.D., 1972. Assessment of microsclerotia of *Verticillium albo-atrum* in field soils. Phytopathology 62: 715-719.

Ausher R., Katan, J. and Ovadia, S., 1975. An improved selective medium for the isolation of *Verticillium dahliae*. Phytoparasitica 3: 133-137.

Ayers, W.A. and Adams, P.B,. 1979a. Mycoparasitism of sclerotia of *Sclerotinia* and *Sclerotium* species by *Sporidesmium sclerotivorum*. Canadian Journal of Microbiology 25: 17-23.

Ayers, W.A. and Adams, P.B., 1979b. Factors affecting germination, mycoparasitism, and survival of *Sporidesmium sclerotivorum*. Canadian Journal of Microbiology 25: 1021-1026.

Azad, H.R., Davis, J.R., Schnathorst, W.C. and Kado, C.I., 1987. Influence of Verticillium wilt resistant and susceptible potato genotypes on populations of antagonistic rhizosphere and rhizoplane bacteria and free nitrogen fixers. Applied Microbiology and Biotechnology 26: 99-104.

Baker, K.F. and Cook, R.J, 1974. Biological control of plant pathogens. San Francisco, Freeman.

Beckman, C.H. and Talboys, P.W, 1981. Anatomy of resistance. In: Fungal wilt diseases of plants, M.E. Mace, A.A. Bell and C.H. Beckman (eds.), pp. 487-521; Academic Press, New York.

Bejarano-Alcázar, J., Melero-Vara, J.M., Blanco-López, M.A. and Jiménez-Diaz, R.M., 1995. Influence of inoculum density of defoliating and nondefoliating pathotypes of *Verticillium dahliae* on epidemics of Verticillium wilt of cotton in southern spain. Phytopathology 85: 1474-1481.

Ben-Yephet, Y. and Szmulewich, Y., 1985. Inoculum levels of *Verticillium dahliae* in the soils of the hot semi-arid Negev region of Israel. Phytoparasitica 13: 193-200.

Berg, G., 1997. Bacterial antagonists to *Verticillium dahliae* var. Longisporum Stark in the rhizosphere of oil seed rape. 7th International Verticillium Symposium, Athens, Greece, p64.

Berg, G. and Ballin, G., 1994. Bacterial antagonists to *Verticillium dahliae* Kleb. J. Phytopathology 141: 99-110.

Beuchat, L.R., 1992. Survival of *Neosartorya fischeri* and *Talaromyces flavus* ascospores in fruit powders. Letters in Applied Microbiology 14: 238-240.

Blok, W.J., 1997. Early decline of asparagus in The Netherlands: etiology, epidemiology and management (Chapter 8). Ph.D. Thesis Wageningen Agricultural University, 177 pp.

Blok, W.J., Lamers, J.G. and Bollen, G.J., 1995. Control of *Fusarium oxysporum* f.sp. *asparagi* in field soil amended with crop residues and tarped with plastic sheeting. European Journal of Plant Pathology. Supplement, Proceedings of the 13th International Plant Protection Congress, Abstract No. 955.

Bollen, G.J., 1974. Fungal recolonisation of heat-treated glasshouse soils. Agro-Ecosystems 1: 139-155.

Bollen, G.J., 1993. Mechanisms involved in nontarget effects of pesticides on soil-borne pathogens. In: Pesticide interactions in crop production. Beneficial and deleterious effects, J.L. Altman (ed.), pp. 281-301; CRC Press, Boca Raton.

Bollen, G.J. and Van der Pol-Luiten, B., 1975. Mesophilic heat-resistant soil fungi. Acta Botanica Neerlandica 24: 254-255.

Bollen, G.J. and Volker, D., 1995. Phytohygienic aspects of composting of plant residues. In: The Science of composting, M. de Bertoldi, P. Sequi, B. Lemmes and T. Papi (eds.), pp 233-246; Blackie Academic & Professional, Glasgow.

Bollen, G.J., Hoekstra, O., Scholte, K., Hofman, T.W., Celetti, M.J. and Schirring, A., 1989. Incidence of soilborne pathogens in potato related to the frequency of potato growing on a clay loam. In: Effects of crop rotation on potato production in the temperate zones, J. Vos, C.D. van Loon and G.J. Bollen (eds.), pp. 202-222; Kluwer Academic Publishers, Dordrecht.

Boogert, P.H.J.F,. van den, 1989. Colonisation of roots and stolons of potato by the mycoparasitic fungus *Verticillium biguttatum*. Soil Biology & Biochemistry 21: 255-262.

Boosalis, M.G., 1956. Effect of soil temperature and green-manure amendment of unsterilized soil on parasitism of *Rhizoctonia solani* by *Penicillium vermiculatum* and Trichoderma sp. Phytopathology 46: 473-478.

Bowden, R.L. and Rouse, D.I., 1991. Effects of *Verticillium dahliae* on gas exchange of potato. Phytopathology 81: 293-301.

Bowen, G.D., 1980. Misconceptions, concepts and approaches in rhizosphere biology. In: Comtemporary microbial ecology, D.C. Ellwood, M.J. Latham, J.H. Slater, J.N. Hedger and J.M. Lynch (eds.), pp 283-304, Academic Press, London.

Bowen, G.D., 1991. Microbial dynamics in the rhizosphere: Possible strategies in managing rhizosphere populations. In: The rhizosphere and plant growth, D.L. Keister and P.B. Cregan (eds.), pp 25-32; Kluwer Academic Publishers, Dordrecht.

Bowen, G.D. and Theodorou, C., 1979. Interactions between bacteria and ectomycorrhizal fungi. Soil Biology & Biochemistry 11: 119-126.

Bowers, R.C., 1982. Commercialisation of microbial biological control agents. In: Biological control of weeds with plant pathogens, R. Charudattan and H.L. Walker (eds.), pp. 157-173; John Wiley & Sons, New York.

Brian, P.W., 1957. The ecological significance of antibiotic production. In: Microbial ecology, R.E.O. Williams and C.C. Spencer (eds.), pp 168-188; Cambridge University Press, Cambridge.

Brinkerhoff, L.A., 1973. Effects of environment on the pathogen and the disease. Proceedings of a work conference: Verticillium wilt of cotton, Texas, U.S:, 78-88.

Budge, S.P. and Whipps, J.M., 1991. Glasshouse trials of *Coniothyrium minitans* and *Trichoderma* species for the biological control of *Sclerotinia sclerotiorum* in celery and lettuce. Plant Pathology 40: 59-66.

Busch, L.V. and Schooley, H.D., 1970. Environmental influence on symptom expression in Verticillium wilt of chrysanthemum. Canadian Journal of Botany 48: 1939-1941.

Cappaert, M.R., Powelson, M.L., Christensen, N.W. and Crowe, F.J., 1992. Influence of irrigation on severity of potato early dying and tuber yield. Phytopathology 82: 1448-1453.

Catani, S.C. and Peterson, J.L., 1967. Antagonistic relationship between *Verticillium dahliae* and fungi isolated from the rhizosphere of *Acer platonoides*. Phytopathology 57: 363-366.

Chao, W.L., Nelson, E.B, Harman, G.E. and Hoch, H.C., 1986. Colonisation of the rhizosphere by biological control agents applied to seeds. Phytopathology 76: 60-65.

Conroy, J.J., Green, R.J. and Ferris, J.M., 1972. Interaction of *Verticillium albo-atrum* and the root lesion nematode, *Pratylenchus penetrans*, in tomato roots at controlled inoculum densities. Phytopathology 62: 362-366.

Cook, R.J., 1973. Influence of low plant and soil water potential on diseases caused by soilborne fungi. Phytopathology 63: 451-458.

Cook, R.J. and Baker, K.F., 1983: The Nature and Practice of Biological Control of Plant Pathogens. The American Phytopathological Society, St. Paul.

Curl, E.A. and Truelove, G., 1986. The Rhizosphere. Advances Series in Agricultural Sciences 15, Springer Verlag, Berlin.

Daigle, D.J. and Cotty, P.J., 1997. The effect of sterilisation, pH, filler and spore inoculum concentration on the preparation of alginate pellets. Biocontrol Science and Technology 7: 3-10.

Davies, K.G. and Whitbread, R., 1989. Factors affecting the colonisation of a root system by fluorescent Pseudomonads: the effects of water, temperature and soil microflora. Plant and Soil 116: 247-256.

Davis, J.R. and Everson, D.O., 1986. Relation of *Verticillium dahliae* in soil and potato tissue, irrigation method, and N-fertility to Verticillium wilt of potato. Phytopathology 76: 730-736.

Davis, J.R., Everson, D.O., Fravel, D., Sorensen, L.H. and Schneider, A.T., 1997a. Associations of *Verticillium tricorpus* with soil suppressiveness of Verticillium wilt of potato. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 90.

Davis, J.R., Fravel, D.R., Marois, J.J., and Sorensen, L.H., 1986. Effect of soil fumigation and seedpiece treatment with *Talaromyces flavus* on wilt incidence and yield 1983. Biological and Cultural Tests 1: 18.

Davis, J.R., Huisman, O.C., Sorensen, L.H. and Schneider, A.T., 1997b. Field studies comparing the susceptibility between crops for the colonisation of *Verticilium dahliae* in plant tissue. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 78.

Davis, J.R., Huisman, O.C., Westermann, D.T., Everson, D.O., Sorensen, L.H. and Schneider, A.T., 1997c. Alternative approaches for control of Verticillium wilt of potato with sudangrass and correlations with related factors. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 74.

Davis, J.R., Huisman, O.C., Westermann, D.T., Hafez, S.L., Everson, D.O., Sorensen, L.H. and Schneider A.T., 1996. Effects of green manures on Verticillium wilt of potato. Phytopathology 86: 444-453.

Davis, J.R., Pavek, J.J. and Corsini, D.L., 1983. A sensitive method for quantifying *Verticillium dahliae* colonisation in plant tissue and evaluating resistance among potato genotyped. Phytopathology 73: 1009-1014.

Davis, J.R., Pavek, J.J., Corsini, D.L., Sorensen, L.H., Schneider, A.T., Everson, D.O., Westermann, D.T. and Huisman, O.C., 1994. Influence of continuous cropping of several potato clones on the epidemiology of Verticillium wilt of potato. Phytopathology 84: 207-214.

Deacon, J.W., 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology 1: 5-20.

Deacon, J.W., 1994. Rhizosphere constraints affecting biocontrol organisms applied to seeds. BCPC Monograph 57: 315-326.

Défago, G., Berling, C.H., Burger, U., Haas, D., Kahr, G., Keel, C., Voisard, C., Wirthner, P. and Würhrich, B., 1990. Suppression of black root rot of tobacco and other root diseases by strains of *Pseudomonas fluorescens*: Potential applications and mechanisms. In: Biological control of soil-borne plant pathogens, D. Hornby, R.J. Cook, Y. Henis, W.H. Ko, A.D. Rovira, B. Schippers and P.R. Scott (eds.), pp 93-108; CAB International, Oxon.

DeVay, J.E., Forester, L.L., Garber, R.H. and Butterfield, E.J., 1974. Characteristics and concentration of propagules of *Verticillium dahliae* in air-dried field soils in relation to prevalence of Verticillium. Phytopathology 64: 22-29.

Dunn, M. T. and Lumsden, R. D., 1981. Fungi associated with sclerotia of *Sclerotia minor* buried in various soils. Phytopathology 71: 766.

Dutta, B.K. and Isaac, I., 1979. Effects of organic amendments to soil on the rhizosphere microflora of antirrhinum infected with *Verticillium dahliae* Kleb. Plant and Soil 53: 99-103.

Easton, G.D., Nagle, M.E. and Bailey, D.L., 1969. A method of estimating *Verticillium albo-atrum* propagules in field soil and irrigation waste water. Phytopathology 59: 1171-1172.

Easton, G.D., Nagle, M.E. and Seymour, M.D., 1992. Potato production and incidence of *Verticillium dahliae* following rotation to nonhost crops and soil fumigation in the state of Washington. American Potato Journal 69: 489-502.

Elad, Y., Katan, J. and Chet I., 1980. Physical, biological and chemical control integrated for soilborne diseases in potatoes. Phytopathology 70: 418-422.

Elmer, W.H. and Stoner, K.A., 1993. Effect of straw and compost on early dying and colorado potato beetle of potato, 1992. Biological and cultural tests 8: 50.

Engelkes, C. A., Nuclo, R. L. and Fravel, D.R., 1993. Effect of source and concentration of carbon and nitrogen on biomass and ascospore production of the biocontrol agent *Talaromyces flavus*. Phytopathology 83: 1365.

Engelkes, C.A., Nuclo, R.L. and Fravel, D.R., 1997. Effect of carbon, nitrogen, and C:N ratio on growth, sporulation, and biocontrol efficacy of *Talaromyces flavus*. Phytopathology 87: 500-505.

Eparvier, A., Lemanceau, P. and Alabouvette, C., 1991: Population dynamics of non-pathogenic Fusarium and fluorescent Pseudomonas strains in rockwool, a substratum for soilless culture. FEMS Microbiology Ecology 86: 177-184.

Evans, G. and Gleeson, A.C., 1973. Observations on the origin and nature of *Verticillium dahliae* colonising plant roots. Australian Journal of Biological Sciences 26: 151-61.

Evans G, McKeen, C.D. and Gleeson, A.C., 1974. A quantitative bioassay for determining low numbers of microsclerotia of *Verticillium dahliae* in field soils. Canadian Journal of Microbiology 20: 119-124.

Fahima, T. and Henis, Y., 1990. Interactions between pathogen host and biocontrol agent: multiplication of *Trichoderma hamatum* and *Talaromyces flavus* on roots of diseased and healthy hosts. In: Biological control of soilborne plant pathogens, D. Hornby D (ed.), pp. 165-180; CAB International, Wallingford

Fahima, T. and Henis, Y., 1995. Quantitative assessment of the interaction between the antagonistic fungus *Talaromyces flavus* and the wilt pathogen *Verticillium dahliae* on eggplant roots. Plant and Soil 176: 129-137.

Fahima, T., Madi, L. and Henis, Y., 1992. Ultrastructure and germinability of *V. dahliae* microsclerotia parasitized by *Talaromyces flavus* on agar medium and in treated soil. Biocontrol Science and Technology 2: 69-78.

Faulkner, L.R., Bolander, W.J. and Skotland, D.B., 1970. Interaction of *Verticillium dahliae* and *Pratylenchus minyus* in Verticillium wilt of peppermint: influence of the nematode as determined by a double root technique. Phytopathology 60: 100-103.

Ferrandino, F.J. and Elmer, W.H., 1993. Eggplant cultivar tolerance to Verticillium wilt, 1992. Biological and Cultural Tests 8: 21.

Fitzell, R., Evans, G. and Fahy, P.C., 1980. Studies on the colonisation of plant roots by *Verticillium dahliae* with use of immunofluorescent staining. Australian Journal of Botany 28: 357-368.

Francl, L.J., Madden, L.V., Rowe, R.C. and Riedel, R.M., 1990. Correlation of growing season environmental variables and the effect of early dying on potato yield. Phytopathology 80: 425-432.

Fravel, D.R., 1989. Biocontrol of Verticillium wilt of eggplant and tomato. In: Vascular Wilt Diseases of Plants, E.C. Tjamos and C.H. Beckman (eds), pp. 487-492; Nata ASI Vol H28, Springer Verlag, Berlin.

Fravel, D.R., 1996. Interaction of biocontrol fungi with sublethal rates of metham sodium for control of *Verticillium dahliae*. Crop Protection 15: 115-119.

Fravel, D.R. and Adams, P.B., 1986. Estimation of United States and world distribution of *Talaromyces flavus*. Mycologia 78: 684-686.

Fravel, D.R. and Roberts, D.P., 1991. In situ evidence for the contribution of glucose oxidase to biocontrol of Verticillium wilt by *Talaromyces flavus*. Biocontrol Science and Technology 1: 91-99.

Fravel, D.R., Davis, J.R. and Sorenson, L.H., 1986. Effect of *Talaromyces flavus* and metham on Verticillium wilt incidence and potato yield 1984-1985. Biological and Cultural Tests for Control of Plant Diseases 1: 17.

Fravel, D.R., Lewis, J.A. and Chittams, J.L, 1995. Alginate prill formulations of *Talaromyces flavus* with organic carriers for biocontrol of *Verticillium dahliae*. Phytopathology 85: 165-168.

Fravel, D.R., Marois, J.J., Dunn, M.T. and Papavizas, G.C., 1985. Compatibility of *Talaromyces flavus* with potato seedpiece fungicides. Soil Biology & Biochemistry 17: 163-166.

Fravel, D.R., Marois J.J., Lumsden, R.D. and Connick Jr. W.J., 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75: 774-777.

Fuska, J, Fuskova, A. and Nemec, P., 1979. Vermistatin, an antibiotic with cytotoxic effects, produced form *Penicillium vermiculatum*. Biologia (Bratislava) 34: 735-739.

Gamliel, A., Grinstein, A., Peretz, Y., Klein, L., Nachmias, A., Tsror, L., Livescu, L., and Katan, J., 1997. Reduced dosage of methyl bromide for controlling Verticillium wilt of potato in experimental and commercial plots. Plant Disease 81: 469-474

Gams W., 1992. The analysis of communities of saprophytic microfungi with special reference to soil fungi. In: Fungi in Vegetation Science, W. Winterhoff (ed.), pp. 183-223; Kluwer Academic Publishers, Dordrecht.

Garibaldi, A., Guglielmone, L. and Gullino, M.L., 1990. Rhizosphere competence of antagonistic Fusaria isolated form suppressive soils. Symbiosis 9: 401-404.

Gaudreault, S.M., Powelson, M.L., Christensen, N.W. and Crowe F.J., 1995. Soil water pressure and *Verticillium dahliae* interactions on potato. Phytopathology 85: 1542-1546.

Gemrich, E.G. and Vandestreek E., 1980. Effectiveness of *Bacillus uniflagellatus* in controlling plant diseases. Plant Disease 64: 382-384.

Georgieva, O., 1992. Antagonistic characteristics of *Trichoderma koningii* towards *Verticillium dahliae* on pepper. Bulletin SROP 15: 18-20.

Gerik, J.S. and Huisman, O.C., 1988. Study of field-grown cotton roots infected with *Verticillium dahliae* using an immunoenzymatic staining technique. Phytopathology 78: 1174-1178.

Gerlagh, M., Goossen-Van de Geijn, H.M., Verdam, B. , Fokkema, N.J., 1995. Biological control of white mould (Sclerotinia sclerotiorum) in various crops by application of *Coniothyrium minitans*. IOBC/WPRS Bulletin 18: 13-17.

Gilligan, C.A., 1985. Probability models for host infection by soil borne fungi. Phytopathology 75: 61-67.

Globus, G.A. and Muromtsev, G.S., 1990. The use of *Gliocladium roseum* as antagonist for defence of cotton from fytopathogene fungi. Proceeding of the 5th International Verticillium Symposium, Leningrad, USSR, 90.

Gordon-Lennox, G., Walther, D. and Gindrat, D. , 1987. Utilisation d'antagonistes pour lénrobage des semences: efficacité et mode d'action contre les agents de la fonte des semis. Bulletin OEPP/EPPO 17: 631-637.

Green, R.J., 1969. Survival and inoculum potential of conidia and microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59: 874-876.

Green, R.J., 1980. Soil factors affecting survival of microsclerotia of *Verticillium dahliae*. Phytopathology 70: 353-355.

Green, R.J. and Papavizas, G.C., 1968. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum* in soil. Phytopathology 58: 567-570

Green, R.J. and Papavizas, G.C., 1969. The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum* in soil. Phytopathology 58: 567-570.

Gregory, G., Schreiber, L.R., Roberto, N. and Ichida, J., 1985. Antagonistic activity of *Bacillus subtilis* against three vascular wilt pathogens (Abstr.). Phytopathology 75: 963-964.

Gregory, P.H., 1948. The multiple-infection transformation. Annals of Applied Biology 35: 412-417.

Gubbins, S. and Gilligan, C., 1997. A test of heterogeneous mixing as a mechanism for ecological persistence in a disturbed environment. Proceedings Royal Society London B 264: 227-232.

Hall, T.J., Schreiber, L.R. and Leben, C., 1986. Effects of xylem-colonising *Bacillus* spp. on Verticillium wilt in maples. Plant Disease 70: 521-524.

Harman, G.E. and Hadar, Y., 1983. Biological control of *Pythium* species. Seed Science and Technology 11: 893-906.

Harris, D.C., Yang, J.R. and Ridout, M.S., 1993. The detection and estimation of *Verticillium dahliae* in naturally infested soil. Plant Pathology 42: 238-250.

Harrison, J.A.C., 1971. Association between the potato cyst nematode *Hetetodera rostochiensis* Woll. and *Verticillium dahliae* Kleb. in the early dying disease of potatoes. Annals of Applied Biology 67: 185-193.

Harrison, M.D., 1976. The effect of barley straw on the survival of *Verticillium albo-atrum* in naturally infested field soil. American Potato Journal 53: 385-394.

Hassel, M.P., 1976. The dynamics of competition and predation. Studies in biology no. 72; Camelot Press, Southampton.

Haverkort, A.J., Rouse, D.I. and Turkensteen L.J., 1990. The influence of *Verticillium dahliae* and drought on potato crop growth. 1. Effects on gas exchange and stomatal behaviour of individual leaves and crop canopies. Netherlands Journal of Plant Pathology 96: 273-289.

Haverkort, A.J., Vos, J., Groenwold, J. and Hoekstra, O., 1989. Crop characteristics and yield reduction of potato due to biotic stress in short crop rotations. In: Effects of crop rotation on potato production in the temperate zones, J. Vos, C.D. van Loon and G.J. Bollen (eds.), pp. 273-290; Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hawke, M.A. and Lazarovits, G., 1994. Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. Phytopathology 84: 883-890.

Henis, Y., and Ben-Yephet, Y., 1970. Effect of propagule size of *Rhizoctonia solani* on saprophytic growth, infectivity and virulence on bean seedlings. Phytopathology 60: 1351-1356.

Henis, Y. and Fahima, T., 1990. Biological control of Verticillium wilt of eggplant by *Talaromyces flavus*. Proceedings of the 5th International Verticillium Symposium, Leningrad, USSR, 81.

Hoyos, G.P., Zambino, P.J. and Anderson, N.A., 1991. An assay to quantify vascular colonisation of potato by *Verticillium dahliae*. American Potato Journal 68: 727-742.

Hu, X., Nazar, R.N. and Rob, J., 1993. Quantification of Verticillium biomass in wilt disease development. Physiological and Molecular Plant Pathology 42: 23-36.

Huisman, O.C., 1982. Interrelations of root growth dynamics to epidemiology of root-invading fungi. Annual Review of Phytopathology 20: 303-327.

Huisman, O.C , 1988a. Seasonal colonisation of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus*. Phytopathology 78: 708-716.

Huisman, O.C., 1988b. Colonisation of field-grown cotton roots by pathogenic and saprophytic soilborne fungi. Phytopathology 78: 716-722.

Huisman, O.C. and Ashworth, L.J., 1974. Quantitative assessment of *Verticillium albo-atrum* in field soils: procedural and substrate improvements. Phytopathology 64: 1159-1163.

Huisman, O.C. and Gerik, J.S., 1989. Dynamics of colonisation of plant roots by *Verticillium dahliae* and other fungi. In: Vascular wilt diseases of plants, E.C. Tjamos and C. H. Beckman (eds.), pp 1-17; Springer Verlag. Berlin.

Isaac, I., 1954. Studies in the antagonism between *Blastomyces luteus* and species of *Verticillium*. Annals of Applied Biology 41: 305-310.

Jacobson, B.J., MacDonald, D.H. and Bissonette, H.L., 1979. Interaction between *Meloidogyne hapla* and *Verticillium albo-atrum* in the Verticillium wilt disease of potato. Phytopathology 69: 288-292.

Jaffee, B.A., 1993. Density-dependent parasitism in biological control of soil-borne insects, nematodes, fungi and bacteria. Biocontrol Science and Technology 3: 235-246.

Jeger, M.J., Hide, G.A., Van den Boogert, P.H.J.F, Termorshuizen, A.J. and Van Baarlen, P., 1996. Pathology and control of soil-borne fungal pathogens of potato. Potato research 39: 437-469.

Johnson, D.A. and Miliczky, E.R., 1993. Distribution and development of black dot, Verticillium wilt, and powdery scab on Russet Burbank Potatoes in Washington State. Plant Disease 77: 74-79.

Johnson, K.B., 1988. Modelling the influence of plant infection rate and temperature on potato foliage and yield losses caused by *Verticillium dahliae*. Phytopathology 78: 1198-1205.

Johnston, S.A., Lashomb, J., and Hamilton, G.C., 1994. Evaluation of the integration of soil fumigation and microbial antagonists for the control of Verticillium wilt of eggplant, 1993. Biological and Cultural Tests 9: 28.

Jordan, V.W.L., 1971. Estimation of the distribution of *Verticillium* populations in infected strawberry plants and soil. Plant Pathology 20: 21-24.

Jordan, V.W.L. and Tarr, H.S., 1978. Inoculum suppression of *Verticillium dahliae*. Annals of Applied Biology 89: 139-141.

Jordan, V.W.L., Sneh, B. and Eddy, B.P., 1972. Influence of organic soil amendments on *Verticillium dahliae* and on the microbial composition of the strawberry rhizosphere. Annals of. Applied Biology 70: 139-148.

Jorge, P.E., Green, R.J. and Chaney, W.R. Jr., 1992. Inoculation with *Fusarium* and *Verticillium* to increase resistance in *Fusarium*-resistant tomato. Plant Disease 76: 340-343.

Katan, J., 1981. Solar heating (solarization) of soil for control of soilborne pests. Annual Review of Phytopathology 19: 211-236.

Katan, T., 1985. Heat activation of dormant ascospores of *Talaromyces flavus*. Transactions British Mycological Society 84: 748-750.

Keinath, A.P. and Fravel, D.R., 1992. Induction of soil supressiveness to Verticillium wilt of potato by successive croppings. American Potato Journal 69: 503-513.

Keinath, A.P., Fravel, D.R. and Papavizas, G.C., 1990. Evaluation of formulations of *Talaromyces flavus* for biocontrol of Verticillium wilt of potato, 1989. Biological and Cultural Tests 5: 30.

Keinath, A.P., Fravel, D.R. and Papavizas, G.C., 1991a. Potential of *Gliocladium roseum* for biocontrol of *Verticillium dahliae*. Phytopathology 81: 644-648.

Keinath, A.P., Fravel, D.R. and Papavizas, G.C., 1991b. Field testing of *Gliocladium roseum* as a biocontrol for Verticillium wilt of potato, 1990. Biological and Cultural Tests 56: 27.

Kim, K.K., Fravel, D.R. and Papavizas, G.C., 1988. Identification of a metabolite produced by *Talaromyces flavus* as a glucose oxidase and its role in the biocontrol of *Verticillium dahliae*. Phytopathology 78: 488-492.

Kim, K.K., Fravel, D.R. and Papavizas, G.C., 1990. Glucose oxidase as the antifungal principal of talaron from *Talaromyces flavus*. Canadian Journal of Microbiology 36: 760-764.

Klisiewicz, J.M., 1975. Survival and dissemination of Verticillium in infected safflower seed. Phytopathology 65: 696-698.

Knudsen, G.R. and Bin, L., 1990. Effects of temperature, soil moisture, and wheat bran on growth of *Trichoderma harzianum* from alginate pellets. Phytopathology 80: 724-727.

Koike, S.T., Xiao, C.L., Hubbard, J.C, Schulbach, K.F. and Subbarao K.V., 1997. Effects of broccoli residue on the cauliflower-*Verticillium dahliae* host. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 80.

Komada, H.,1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research 8: 114-124.

Kotcon, J.B., Rouse, D.I. and Mitchell, J.E., 1984. Dynamics of root growth in potato fields affected by the early dying syndrome. Phytopathology 74: 462-467.

Kuan, T.L. and Erwin D.C., 1980. Predisposition effect of water saturation of soil on Phytophthora root rot of alfalfa. Phytopathology 70: 981-986.

Kuc, J. and Strobel N.E., 1992. Induced resistance using pathogens and nonpathogens. In: Biological control of plant diseases, E. S. Tjamos *et al.* (eds.), pp 295-303; Plenum, Press, New York, USA.

Lamers, J.G. and Termorshuizen, A.J., 1997. The influence of manual removal of mechanical destruction of potato haulms on Verticillium inoculum density of the soil. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 35.

Larson, R.E. and Hostetler, R.P., 1986. Calculus. D.C Heath and Company, Lexington.

Lazarovits, G., 1997. Organic soil amendments for the control of soilborne plant pathogens. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 73.

Leben, S.D., Wadi, J.A. and Easton, G.D., 1987. Effects of *Pseudomonas fluorescens* on potato plant growth and control of *Verticillium dahliae*. Phytopathology 77: 1592-1595.

Lemanceau, P. and Alabouvette, C., 1991. Biological control of fusarium diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. Crop Protection 10: 279-286.

Lewis, J. A. and Papavizas, G.C., 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping-off. Plant Pathology 36: 438-446.

Lewis, J.A., Papavizas, G.C. and Hollenbeck, H.D., 1993. Biological control of damping-off of snap beans caused by *Sclerotium rolfsii* in the greenhouse and field with formulations of *Gliocladium virens*. Biological Control 3: 109-115.

Lockwood, 1977. Fungistasis in soil. Biology Review 52: 1-43.

Lommen, W.J.M. and Struik, P.C., 1992. Influence of single non-destructive harvest on potato plantlets grown for minituber production. Netherlands Journal of Agricultural Science 40: 21-41.

Lumsden, R.D., 1981. A nylon fabric technique for studying the ecology of *Pythium aphanidermatum* and other fungi in soil. Phytopathology 71: 282-285.

Lungley, D.R., 1973. The growth of root systems- a numerical computer simulation model. Plant and Soil 38: 145-159.

MacGuidwin, A.E. and Rouse, D.I., 1990. Effect of *Meiloidogyne hapla*, alone and in combination with subthreshold populations of *Verticillium dahliae*, on disease symptomatology and yield of potato. Phytopathology 80, 482-486.

Madden L.V. and Campbell, C.L., 1990. Non-linear disease progress curves. In: Epidemics of plant diseases 2nd Edn, J. Kranz (ed.), pp 181-230; Springer Verlag, Berlin.

Madi, L., Katan, T. and Henis, Y., 1992. Inheritance of antagonistic properties and lytic enzyme activities in sexual crosses of *Talaromyces flavus*. Annals of Applied Biology 121: 565-576.

Madi, L., Katan, T., Katan , Y. and Henis, Y., 1997. Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. Phytopathology 87: 1054-1060.

Marois, J.J. and Fravel, D.R., 1983. Effect on respiration of addition of fungal spores to soil. Phytopathology 73: 967.

Marois, J.J., Fravel, D.R. and Papavizas, G.S., 1984. Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae*. Soil Biology & Biochemistry 16: 387-390.

Marois, J.J, Johnston, S.A, Dunn, M.T. and Papavizas, G.C., 1982. Biological control of Verticillium wilt of eggplant in the field. Plant Disease 66: 1166-1168.

Martinson, C.A. and Horner, C.E., 1962. Importance of non-hosts in maintaining the inoculum potential of Verticillium. Phytopathology 52: 742.

Matta, A. and Garibaldi, A., 1977. Control of Verticillium wilt of tomato by preinoculation with avirulent fungi. Netherlands Journal of Plant Pathology 83 (Suppl. 1): 457-462.

Maurhofer, M., Hase, C., Meuwly, P., Métraux, J.P., Défago, G., 1994. Induction of systemic resistance of tabacco to tobacco necrosis virus by the root-colonising *Pseudomonas fluorescens* strain CHAO: Influence of the *gac*A gene amd pyoverdine production. Phytopathology 84: 139-146.

McBeath, J.H., 1994. *Trichoderma atroviride*, a potential biological control agent of *Verticillium dahliae*. Phytopathology 84: 1091.

McCoy, M.L. and Powelson, R.L., 1974. A model for determining spatial distribution of soil-borne propagules. Phytopathology 64: 145-147.

McLaren, D.L., Huang, H.C. and Rimmer, S.R., 1986. Hyperparasitism of *Sclerotinia sclerotiorum* by *Talaromyces flavus*. Canadian Journal of Plant Pathology 8: 43-47.

McLaren, D.L., Huang, H.C., Kozub, G.C. and Rimmer, S.R., 1994. Biological control of Sclerotinia wilt of sunflower with *Talaromyces flavus* and *Coniothyrium minitans*. Plant Disease 78: 231-235.

Melero-Vara, J.M., Blanco-Lopez, M.A., Bejarano-Alcazar, J. and Jimenez-Diaz, R.M , 1995. Control of *Verticillium* wilt of cotton by means of soil solarization and tolerant cultivars in southern Spain. Plant Pathology 44: 250-260.

Melouk, H.A. and Horner, C.E., 1975. Cross protection in mints by *Verticillium nigrescens* against *V. dahliae*. Phytopathology 65: 767-769.

Millar, R.L., Kalb, D.W. and Keinath, A.P., 1984. Biological and chemical control of Verticillium wilt of alfalfa. (Abstr.). Phytopathology 74: 805.

Mizuno, K., Yagi, A., Takada, M., Matsura, K., Yamaguchi, K. and Asano, K., 1974. A new antibiotic, talaron. Journal of Antibiotics 7: 560-563.

Mol, L., 1995. Agronomic studies on the population dynamics of *Verticillium dahliae*. Thesis Landbouwuniversiteit Wageningen.

Mol, L. and Scholte, K., 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research 38: 143-150.

Mol, L. and Van Riesen, H.W., 1995. Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*. I. Use of root observation boxes to assess differences among crops. European Journal of Plant Pathology 101: 673-678.

Mol, L., Scholte, K., and Vos, J., 1995. Effects of crop rotation and removal of crop debris on the soil population of two isolates of *Verticillium dahliae*. Plant Pathology 44: 1070-1074.

Mol, L., Van Halteren, J.M., Scholte, K. and Struik, P.C., 1996. Effects of crop species, crop cultivars and isolates of *Verticillium dahliae* on the population of microsclerotia in the soil, and consequences for crop yield. Plant Pathology 45: 205-214.

Nadakavukaren, M.J. and Horner, C.E., 1959. An alcohol agar medium selective for determining Verticillium microsclerotia in soil. Phytopathology 49: 527-528.

Nagtzaam, M.P.M., 1995. *Talaromyces flavus* as a potential biocontrol agent for controlling *Verticillium dahliae* in potatoes. IOBC/WPRS Bulletin 18: 112-116.

Nagtzaam, M.P.M. and Bollen, G.J., 1994. Long shelf life of *Talaromyces flavus* in coating material of pelleted seed. European Journal of Plant Pathology 100: 279-282.

Nagtzaam, M.P.M. and Bollen, G.J., 1997. Colonisation of roots of eggplant and potato by *Talaromyces flavus* from coated seed. Soil Biology and Biochemistry 29,: 1499-1507.

Nagtzaam, M.P.M., Bollen, G.J. and Termorshuizen, A.J., 1998. Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiments. Journal of Phytopathology 146: 165-173.

Nagtzaam, M.P.M., Termorshuizen, A.J., Bollen, G.J., 1997. The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*. European Journal of Plant Pathology 103: 597-605.

Neate, S.M. and Berger, A.M., 1995. Nylon netting technique for the study of the three dimensional growth of *Rhizoctonia solani* in soil. Soil & Biology Biochemistry 27: 1617-1621.

Nicot, P.C. and Rouse, D.I., 1987. Relationship between soil inoculum density of *Verticillium dahliae* and systemic colonisation of potato stems in commercial fields over time. Phytopathology 77: 1346-1355.

Nnodu, E.C. and Harrison, M.D., 1979. The relationship between *Verticillium albo-atrum* inoculum density and potato yield. American Potato Journal 56: 11-25.

Ocamb, C.M. and Kommedahl, T., 1994. Rhizosphere competence of Fusarium species colonising corn roots. Phytopathology 84: 166-172.

Olsson, S. and Nordbring-Hertz, B., 1985. Microsclerotial germination of *Verticillium dahliae* as affected by rape rhizosphere. FEMS Microbial Ecology 31: 293-299.

Oostenbrink, M., 1960. Estimating nematode populations by some selected methods. In: Nematology, J.N. Sasser and W.R. Jenkins (eds.), pp. 85-102; Univ. N. Carolina Press, Chapel Hill.

Ordentlich, A., Nachmias, A. and Chet, I., 1990. Integrated control of *Verticillium dahliae* in potato by *Trichoderma harzianum* and captan. Crop Protection 9: 363-366.

Palloix, A., Pochard, E., Phaly, T. and Daubèze, A.M., 1990. Recurrent selection for resistance to *Verticillium dahliae* in pepper. Euphytica 47: 79-89.

Papavizas, G.C., 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. Annual Review of Phytopathology 23: 23-54.

Papavizas, G.C., Fravel, D.R. and Lewis, J.A., 1987. Proliferation of *Talaromyces flavus* in soil and survival in alginate pellets. Phytopathology 77: 131-136.

Paplomatas, E.J., Bassett, D.M., Broome, J.C. and DeVay, J.E., 1992. Incidence of Verticillium wilt and yield losses of cotton cultivars (*Gossypium hirsutum*) based on soil inoculum density of *Verticillium dahliae*. Phytopathology 82: 1417-1420.

Park, C.S., Paulitz, T.C. and Baker, R., 1988. Biocontrol of fusarium wilt of cucumber resulting from interaction between *Pseudomonas putida* and non-pathogenic isolates of *Fusarium*. Phytopathology 78: 190-194.

Park, J.H., Stack, J.P. and Kenerley, C.M., 1992. Selective isolation and enumeration of *Gliocladium virens* and *Gliocladium roseum* from soil. Plant disease 76: 230-235.

Pegg, G.F. and Jonglaekha, N., 1981. Assessment of colonisation in chrysanthemum grown under different photoperiods and infected with *Verticillium dahliae*. Transactions of the British Mycological Society 76: 353-360.

Perry J.W. and Evert, R.F., 1983. Histopathology of *Verticillium dahliae* within mature roots of Russet Burbank potatoes. Canadian Journal of Botany 61: 3405-3421.

Pierson, E.A. and Weller, D.M., 1994. Use of mixtures of fluorescent Pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology 84: 940-947.

Podile, A.R., Prasad, G.S. and Dube, H.C., 1985. *Bacillus subtilis* as antagonist to vascular wilt pathogens. Current Science 54: 864-865.

Powell, K.A. and Faull, J.L., 1989. Commercial approaches to the use of biological control agents. In: Biotechnology of fungi for improving plant growth, J.M. Whipps and R.D. Lumsden (eds.), pp. 259-275; Cambridge University Press.

Powelson, R.L., 1970. Significance of population level of Verticillium in soil. In: Root diseases and soil-borne pathogens, T.A. Toussoun, R.V. Bega and P. Nelson (eds.), pp. 31-33; University of California Press, Berkeley, USA.

Price, D. and Sackston, W.E., 1983. Cross protection against Verticillium in sunflower (Abstr.). Phytopathology 73: 372-373.

Price, D. and Sackston, W.E., 1989. Cross protection among strains of *Verticillium dahliae* on sunflower. In: Vascular wilt diseases of plants, E.C. Tjamos and C.H. Beckman (eds.), pp 229-235; NATO ASI Vol. H28., Springer Verlag, Berlin, Germany.

Pullman, G.S. and DeVay, J.E., 1981. Effect of soil flooding and paddy rice culture on the survival of *Verticillium dahliae* and incidence of Verticillium wilt of cotton. Phytopathology 71: 1285-1289

Pullman, G.S. and DeVay, J.E., 1982. Epidemiology of Verticillium wilt of cotton: a relationship between inoculum density and disease progression. Phytopathology 72: 549-554.

Pullman, G.S., DeVay, J.E. and Garber, R.H., 1981. Thermal death: a logarithmic relationship between time and temperature for four soilborne pathogens. Phytopathology 71: 959-964.

Riedel, R.M, Rowe, R.C. and Martin M.J., 1985. Differential interactions of *Pratylenchus crenatus*, *P. penetrans* and *P. sribneri* with *Verticillium dahliae* in potato early dying disease. Phytopathology 75: 419-422.

Rijkers, A.J.M., Hiemstra J.A. and Bollen, G.J., 1992. Formation of microsclerotia of *Verticillium dahliae* in petioles of infected ash trees. Netherlands Journal of Plant Pathology 98: 261-264.

Ristaino, J. B., Lewis J. A. and Lumsden, R.D., 1994. Influence of isolates of *Gliocladium virens* and delivery systems on biological control of Southern Blight on carrot and tomato in the field. Plant Disease 78: 153-156.

Rodgers, P.B., 1993. Potential of biopesticides in agriculture. Pesticide Science 39: 117-129.

Rovira, A.D. and Davey, C.B., 1974. Biology of the rhizosphere. In: The plant root and its environment, E.W. Carson (ed.), pp 153-204; Univ. of Virginia Press, Charlottesville.

Rowe, R.C., Riedel, R.M. and Martin, M.J., 1985. Synergistic interactions between *Verticillium dahliae* and *Pratylenchus penetrans* in potato early dying disease. Phytopathology 75: 412-418.

Sackston, W.E. and Martens, J.W., 1959. Dissemination of *Verticillium albo-atrum* on seed of sunflower (*Helianthus annuus*). Canadian Journal of Botany 37: 759-768.

Saeed, I.A.M., MacGuidwin, A.E. and Rouse D.I., 1997. Synergism of *Pratylenchus penetrans* and *Verticillium dahliae* manifested by reduced gas exchange in potato. Phytopathology 87: 435-439.

Schnathorst, W.C., 1981. Life cycle and epidemiology of Verticillium. In: Fungal wilt diseases of plants, M.E. Mace, A.A. Bell and C.H. Beckman (eds.), pp 81-111; Academic Press, New York.

Schnathorst, W.C. and Mathre, D.E., 1966. Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology 56: 1155-1161.

Scholte, K., 1989. The effect of netted scab (Streptomyces spp.) and *Verticillium dahliae* on growth and yield of potato. Potato research 32: 65-73.

Scholte, K., 1990. Courses of differences is growth pattern, yield and quality of potatoes (*Solanum tuberosum* L.) in short rotations on sandy soil as affected by crop rotation, cultivar and application of granular nematicides. Potato Research 33:181-190

Scholte, K., Veenbaas-Rijks, J.W., Labruyère R.E., 1985. Potato growing in short rotations and the effect of Streptomyces spp., *Colletotrichum coccodes*, *Fusarium tabacinum* and *Verticillium dahliae* on plant growth and tuber yield. Potato research 28: 331-348.

Schreiber, L.R., 1992. Seasonal variations in susceptibility and internal inoculum densities in maple species inoculated with *Verticillium dahliae*. Plant Disease 76: 184-187.

Schreiber, L.R. and Green, R.J., 1962. Comparative survival of mycelium, conidia and microsclerotia of *Verticillium albo-atrum* in mineral soil. Phytopathology 52: 288-289.

Schreiber, L.R. and Green, R.J., 1963. Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. Phytopathology 53: 260-264.

Scott, E.M, Rattray, E.A.S., Prosser, J.I., Killham, K., Glover, L.A., Lynch, M. and Bazin, M.J., 1995. A mathematical model for dispersal of bacterial inoculants colonising the wheat rhizosphere. Soil Biology & Biochemistry 27: 1307-1318.

Slattery, R.J., 1981. Inoculum potential of Verticillium-infested potato cultivars. American Potato Journal 58: 135-142.

Smith, V.L. and Rowe, R.C., 1984. Characteristics and distribution of propagules of *Verticillium dahliae* in Ohio potato field soils and assessment of two assay methods. Phytopathology 74: 553-556.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G., 1986. Bergey's Manual of Systematic Bacteriology, Vol. 2. Williams & Wilkins, Baltimore.

Soesanto, L. and Termorshuizen, A.J., 1997. Formation of microsclerotia of *Verticillium dahliae*. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 33.

Sokal, R.R. and Rohlf, F.J., 1981. Biometry. W.H. Freeman and Company, New York.

Spink, D.S and Rowe, R.C., 1989. Evaluation of *Talaromyces flavus* as a biocontrol agent against *Verticillium dahliae* in potato. Plant Disease 73: 230-236.

Spuy, J.E., van der, Matthee, F.N. and Crafford, D.J.A., 1975. The heat resistance of moulds *Penicillium vermiculatum* Dangeard and *Penicillium brefeldianum* Dodge in apple juice. Phytophylactica 7: 105-108.

Stack, J.P., Kenerley, C.M., and Pettit, R.E., 1987. Influence of carbon and nitrogen sources, relative carbon and nitrogen concentrations and soil moisture on the growth in nonsterile soil of soilborne fungal antagonists. Canadian Journal of Microbiology 33: 626-631.

Stack, J.P., Knudsen, G.R., and Koch, D.O., 1988. Modelling growth of soil fungi with special reference to biocontrol of plant pathogens. Proceedings of 5th International Congress of Plant Pathology, Kyoto.

Storey, G.W. and Evans, K.,1987. Interactions between *Globodera pallida* juveniles, *Verticillium dahliae* and three potato cultivars, with descriptions of associated histopathologies. Plant pathology 36: 192-200.

Suslow, T.V. and Schroth, M.N., 1982. Rhizobacteria of sugar beets: effects of seed application and root colonisation on yield. Phytopathology 72: 199-206.

Taylor, A.G. and Harman, G.E., 1990. Concepts and technologies of selected seed treatments. Annual Review of Phytopathology 28: 321-339.

Termorshuizen, A.J., 1995. The recovery rate of microsclerotia of *Verticillium dahliae* with different detection methods (Abstr.). Phytoparasitica 23: 55-56.

Termorshuizen, A.J., 1997. Sporulation of *Verticillium dahliae*. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 34.

Termorshuizen, A.J. and Mol, L., 1995. Modelling the dynamics of *Verticillium dahliae*. In: Potato ecology and modelling of crops under conditions limiting growth, A.J. Haverkort and D.K.L. MacKerron (eds.), pp 265-280; Kluwer Academic Publishers, Dordrecht.

Tjamos, E.C. and Fravel, D.R., 1995. Agents and strategies for the biological control of *Verticillium dahliae*. IOBC/WPRS Bulletin 18: 117-122.

Tjamos, E.C. and Fravel, D.R., 1997. Distribution and establishment of the biocontrol fungus *Talaromyces flavus* in soil and on roots of solanaceous crops. Crop Protection 16: 135-139.

Tjamos, E.C. and Paplomatas, E.J., 1987. Effect of soil solarization on the survival of fungal antagonists of *Verticillium dahliae*. Bulletin OEPP/EPPO Bulletin 17: 645-653.

Tjamos, E.C. and Tsougriani, H., 1990. Formation of *Verticillium dahliae* microsclerotia in partially disintegrated leaves of Verticillium affected olive trees. Proceedings of 5th. International Verticillium Symposium, Leningrad, USSR, 20.

Tjamos, E.C., Tsitsiyiannis, D., Tjamos, S.E. and Panagopoulos, C.G., 1997. Selection and evaluation of rhizosphere bacteria as biocontrol agents against *Verticillium dahliae*. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 63.

Turchin, P., 1990. Rarity of density dependence or population regulation with lags? Nature 344: 660-663.

Wadi, J.A. and Easton, G.D., 1985. Control of *Verticillium dahliae* by coating potato seed pieces with antagonistic bacteria. In: Ecology and management of soilborne plant pathogens, C.A. Parker, A.D. Rovira, K.J. Moore, P.T.W. Wong and J.F. Kollmorgen (eds.), pp 134-136; American Phytopathological Society, St. Paul, MN, USA.

Warcup, J.H. and Baker, K.F., 1963. Occurrence of dormant ascospores in soil. Nature 197: 1317-1318.

Wheeler, T.A. and Rowe, R.C., 1995. Influence of soil characteristics and assay techniques on quantification of *Verticillium dahliae* in Ohio soils. Plant Disease 79: 29-34.

Wheeler, T.A., Madden, L.V., Riedel, R.M. and Rowe, R.C., 1994. Distribution and yield-loss relations of *Verticillium dahliae*, *Pratylenchus penetrans*, *P. scribneri*, *P. crenatus*, and *Meloidogyne hapla* in commercial potato fields. Phytopathology 84: 843-852.

Wheeler, T.A., Madden, L.V., Rowe, R.C. and Riedel, R.D., 1992. Modelling of yield loss in potato early dying caused by *Pratylenchus penetrans* and *Verticillium dahliae*. Journal of Nematology 24: 99-102.

Whipps, J.M. and Budge, S.P., 1990. Screening for sclerotial mycoparasites of Sclerotinia sclerotiorum. Mycological Research 94: 607-612.

Whipps, J.M. and Budge, S.P., 1993. Transmission of the mycoparasite *Coniothyrium minitans* by collembolan *Folsomia candida* (Collembola: Entomobryidae) and glasshouse sciarid *Bradysia* sp. (Diptera: Sciaridae). Annals of Applied Biology 123: 165-171.

Wilhelm, S., 1951. Effect of various soil amendments on the inoculum potential of the Verticillium wilt fungus. Phytopathology 41: 684-690.

Wilhelm, S., 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology 45: 180-181.

Wilkinson, H.T., Cook, R.J. and Alldredge, J.R., 1985. Relation of inoculum size and concentration to infection of wheat roots by *Gaeumannomyces graminis* var. *tritici*. Phytopathology 75: 98-103.

Williams, R.H. and Whipps, J.M., 1995. Growth and transmission of *Coniothyrium minitans* in soil. IOBC/WPRS Bulletin 18: 39-43.

Windels, C.E., 1981. Growth of *Penicillium oxalicum* as a biological seed treatment on pea seed in soil. Phytopathology 71: 929-933.

Xiao, C.L., Hao, J.J. and Subbarao, K.V., 1997. Spatial pattern of microsclerotia of *Verticillium dahliae* in soil and Verticillium wilt of cauliflower. Phytopathology 87: 325-331.

Yamaguchi, K., Kida, M., Arita, M. and Takahashi, M., 1992(a). Induction of systemic resistance by *Fusarium oxysporum* MT0062 in solanaceous crops. Annals of Phytopathology Society Japan 58: 16-22.

Yamaguchi, K., Sano, T., Arita, M. and Takahashi, M., 1992(b). Biocontrol of Fusarium wilt of tomato and Verticilium wilt of eggplant by non-pathogenic *Fusarium oxysporum* MT0062. Annals of Phytopathology Society Japan 58: 188-194.

Zaki, A.I., Keen, N.T. and Erwin, D.C., 1972. Implication of vergosin and hemigossypol in the resistance of cotton to *Verticillium albo-atrum*. Phytopathology 62: 1402-1406.

Zeise K., 1992. Gewächshaustest zur Resistenzprüfung van Winterraps (*Brassica napus* L. var. *oleifera* Metzger) gegen den Erreger der Rapswelke *Verticillium dahliae* Kleb. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 44: 125-128.

Zeise, K., 1997. The potential of *Talaromyces flavus* (Klockner) Stolk & Samson in controlling *Verticillium dahliae*. Proceedings of 7th International Verticillium Symposium, Athens, Greece, 61.

Zilberstein, Y., Chet, I. and Henis, Y., 1983a. Effect of atmosphere on germination of microsclerotia of *Verticillium dahliae*. Israel Journal of Botany 32: 33-36.

Zilberstein, Y., Chet, I. and Henis, Y., 1983b. Influence of microsclerotia source of *Verticillium dahliae* on inoculum quality. British Mycological Society 81: 613-617.

Zoon, F.C. and Van Tienderen, P.H., 1990. A rapid quantitative length and root branching by microcomputer image analysis. Plant and Soil 126: 301-308.measurement of root

Summary

Verticillium dahliae causes vascular wilt in a wide range of host plants. In The Netherlands, Verticillium wilt is a serious problem in production of potato and strawberry as field crops, in raising lime tree, elm and maple in nurseries for avenue trees, and in production of rose, chrysanthemum, eggplant and tomato in greenhouses. The pathogen causes severe losses in various other host plants, e.g. sugarbeet, common ash, and lilac. During the last decade, the significance of Verticillium wilt has increased. The success of the pathogen is due to an increased frequency of susceptible crops in present rotation cycles, the polyphagous character of the pathogen and the production of persistent resting structures by *V. dahliae* which survive in soil for many years.

Control of Verticillium wilt is by soil disinfestation and to a lesser extent by crop rotation and cultivation of resistant varieties. For environmental reasons, there is a need to develop alternatives to chemical soil disinfestation. Biological control is one of the options. The potential of *Talaromyces flavus* as a biocontrol agent in management of the pathogen is the subject of this thesis. The choice of *T. flavus* as a potentially successful antagonist was based on its high resistance to adverse conditions, its preference for the rhizosphere as a habitat, and on reports on its effectivity in biocontrol of *V. dahliae* in the USA.

The thesis deals with the development of a quantitative bioassay to study the effects of various antagonists on dynamics of *V. dahliae* (*Chapter 2*), the persistence of the spores of *T. flavus* responsible for the shelf life of a product containing the antagonist (*Chapter 3*), the dynamics of *T. flavus* on the roots of eggplant and potato and the implications of it for biocontrol of Verticillium wilt (*Chapter 4*), the performance of *T. flavus* and other antagonists *in vitro* or in growth chamber experiments (*Chapter 5*), the effect of spatial distribution of fungal antagonists on their performance as biocontrol agents (*Chapter 6*), and the performance of *T. flavus* in the field (*Chapter 7*).

A quantitative bioassay was developed to study the effects of various antagonists on dynamics of *V. dahliae* (*Chapter 2*). Using potato, eggplant and thorn apple as test plants, the relationship between soil inoculum density and plant infection was studied as a basis for the development of a quantitative bioassay of *V. dahliae*. A linear relationship was demonstrated ($P<0.05$) between soil inoculum density and population density on roots for all three test plants and for soil inoculum density and population density in sap extracted from stems for eggplant. Correlation coefficients were higher with densities on or in roots (R^2 varying from 0.45 to 0.99) than with densities in stems (R^2 varying from 0.04 to 0.26).

With eggplant, population densities on/in root and in sap extracted from stems were significantly correlated at 20 and 25 °C with Pearson's correlation coefficients of 0.41 and 0.53, respectively. For potato, efficiency of root colonisation was higher at 15° than at 20 °C, whereas the reverse was observed when eggplant was used as a test plant. Stems of potato were less densely colonised than stems of eggplant. The pathozone sensu Gilligan (1985) was calculated to be <300 µm, indicating that infection was caused by microsclerotia which were located close to the roots.

Pipetting infested plant sap on solidified ethanol agar medium without salts yielded higher densities of *V. dahliae* in plant tissue than pipetting onto pectate medium or mixing the sap with molten ethanol agar. For evaluation of the effects of (a)biotic factors on development of *V. dahliae* in the plant, a bioassay is recommended; using eggplants, grown in soil infested with 300 single, viable microsclerotia g^{-1} soil at a matric potential of -6.2 kPa, and incubated at 20 °C for 8 weeks.

Availability of samples of Chinese aster and tomato seeds that had been coated with a clay product containing ascospores incorporated 17 years before, made it possible to gain information on persistence of *T. flavus* spores in a formulation product (*Chapter 3*). The seeds had been stored at room temperature. About 20% of the ascospores had retained their heat resistance and survived treatment in aqueous suspension at 60 °C for 30 min. None of the Chinese aster seeds germinated after the storage period but of the tomato seeds 90% had retained viability. Presence of *T. flavus* during storage had not affected germinability of the seeds.

The dynamics of *T. flavus* on the roots of eggplant and potato was studied to evaluate the potential to establish itself at on the site where *V. dahliae* infects the root (*Chapter 4*). *T. flavus* was shown to colonise potato and eggplant roots. In pot experiments with field soils, the fungus moved from potato seed tubers and eggplant seeds coated with ascospores to developing roots, including the root tips, albeit at a low density. The population of *T. flavus* decreased log-linearly with distance from the seed and was higher with potatoes than with eggplants. Results suggest that passive movement along the growing root is one of the main factors involved.

A monomolecular model for *V. dahliae* root colonisation and soil inoculum density (R^2=0.94), and a negative exponential model for the relationship between *T. flavus* soil inoculum density and *V. dahliae* density on the roots (R^2=0.55) were fitted.

Biocontrol of *V. dahliae* by *T. flavus* or other antagonists was tested *in vitro* and in growth chamber experiments (*Chapter 5*). *T. flavus* reduced viability of microsclerotia of

V. dahliae on senescent potato stems collected from the field when applied as ascospores in carboxymethylcellulose or in talcum powder. Incorporating an alginate wheat-bran preparation of *T. flavus* in soil at a rate of 0.5% (w/w) was followed by a decrease of >90% of the population of *V. dahliae* in soil at both 15 and 25 °C. Population densities of *V. dahliae* were negatively correlated (r=-0.50; p=0.001) with those of *T. flavus*. However, the population of *V. dahliae* was also reduced in soil with alginate wheat-bran alone. When incorporated in soil in alginate wheat-bran and simultaneously coated on seeds in talcum powder, *T. flavus* reduced colonisation of roots and infection of eggplants by *V. dahliae*. Although to a lesser extent than with the antagonist, alginate wheat-bran without *T. flavus* also reduced infection by the pathogen.

Treatment with combinations of *T. flavus* with *Bacillus subtilis*, *Fusarium oxysporum* or *Gliocadium roseum*, containing half of the inoculum of the single application of each antagonist, gave a similar reduction of root colonisation and stem infection by *V. dahliae* as application of the single antagonists. Population densities on the root of each antagonist were not or were only slightly affected by the presence of the co-inoculated antagonist suggesting that the combinations were compatible.

The type of distribution of propagules in soil is considered as an important factor in performance of mycoparasites to control soilborne pathogens. The importance of spatial distribution patterns of propagules of a mycoparasite and its fungal host were evaluated by using a simple model of omnidirectional and constant growth of the mycoparasite (*Chapter 6*). With a random distribution average distances among propagules appear to be 40-50% smaller than with a uniform distribution. and progress of infection of hosts was considerably slower. Parameters for epidemic curves were estimated from published data on the inactivation of sclerotia of *Sclerotinia minor* by different densities of macroconidia of *Sporidesmium sclerotivorum* for both types of distribution. The differences in progress of infection between the two types of distribution were quite small. The average distance among propagules of mycoparasite was better correlated than the density of the mycoparasite in soil with the area under the disease progress curve. It is argued that in studies on the dynamics of antagonists it may be epidemiologically more relevant to base different treatments on differences in average distances among propagules instead on differences in population densities.

The performance of *T. flavus* in controlling *V. dahliae*. under field conditions was studied in two independent field experiments (*Chapter 7*). To adequately interpret the effect of *T. flavus* on dynamics of *V. dahliae* in and on the plant, and on crop yield, the effect of the pathogen at different soil infestation levels on potato plants was studied. Incidence of

stem infection by *V. dahliae* and yield of tubers showed a clear dose-response relationship with the amount of pathogen inoculum applied to soil (R^2=0.69 and 0.40, respectively). Incidence of stem infection and density of stem colonisation by the pathogen gradually increased during the season. After application of a *T. flavus* preparation, stems were less densely colonised by *V. dahliae* in the treated plots than in the control plots in the first growing season of Exp. 1 and in the second growing season of Exp. 2. This reduction was not observed in the plots with the highest level of pathogen inoculum applied. The reduction in pathogen colonisation was not followed by higher tuber yields. Although population density of *T. flavus* in soil did not increase during the growing season, it remained at a higher level in *T. flavus*-treated plots than in untreated plots (P=0.05), also in the second year after introduction. In one experiment with a control treatment of alginate wheat-bran without *T. flavus*, a reduced pathogen colonisation of stems was found. The inconsistency of performance of *T. flavus* in the present field experiments, and the contradictory data reported in the literature, demonstrate the need to identify conditions required for biocontrol of *V. dahliae* by *T. flavus*.

Finally, the question whether *T. flavus* has sufficient potential for biocontrol of *V. dahliae* is discussed (*Chapter 8*). It is concluded is that the results of the experiments presented in this thesis and in the literature confirm that thes fungus has potential. However, thus far results with this antagonist have tended to be inconsistent. This means that application of *T. flavus* as a single control method has little commercial value. For the present, biocontrol should be considered as a method to be applied in combination with cultural methods, biological disinfestation, the use of tolerant cultivars or the selective application of chemical control. The next step in further research should focus on the nature and sources of the inconsistency. The control of the pathogen by inoculation of infected crop debris with antagonists before ploughing and the opportunities for control in the interior of the plant also merit further investigation.

Samenvatting

Verticillium dahliae veroorzaakt verwelkingsziekte bij een groot aantal waardplanten. In Nederland is de schimmel een belangrijke ziekteverwekker in de teelt van aardappelen en aardbeien, in boomkwekerijen met name bij de teelt van linde, iep en esdoorn en in de glastuinbouw bij de teelt van roos, chrysant, aubergine en tomaat. De schimmel veroorzaakt incidenteel ernstige schade bij andere waardplanten, zoals suikerbiet, es en lelie. Gedurende het laatste decennium is de schade door Verticillium verwelkingsziekte toegenomen. De oorzaken hiervoor zijn de opname van een toenemend deel van gevoelige gewassen in het vruchtwisselingsschema, de brede waardplantenreeks van de ziekteverwekker en de productie van persistente overlevingsstructuren door de schimmel die vele jaren in de grond kunnen overleven.

Bestrijding van Verticillium verwelkingsziekte vindt plaats door chemische grondontsmetting, vruchtwisseling en in enkele gewassen door de teelt van resistente rassen. Er wordt gestreefd naar alternatieven voor grondontsmetting. Biologische bestrijding is één van de alternatieven. Het onderwerp van dit proefschrift is na te gaan of de schimmel *Talaromyces flavus* bruikbaar is in de biologische bestrijding van *Verticillium dahliae*. De keuze voor *T. flavus* is gebaseerd op zijn hoge weerstand tegen ongunstige omstandigheden, de voorkeur van deze schimmel voor de rhizosfeer als habitat, en de gegevens in de wetenschappelijke literatuur over zijn doeltreffendheid in de biologische bestrijding van *V. dahliae* in de Verenigde Staten.

Het proefschrift behandelt de ontwikkeling van een kwantitatieve biotoets voor het bepalen van effecten van biologische bestrijdingsorganismen op de dynamiek van *V. dahliae (Hoofdstuk 2)*, de houdbaarheid van een preparaat met sporen van *T. flavus* (*Hoofdstuk 3*), de dynamiek van *T. flavus* op de wortels van aubergine en aardappel en de implicaties hiervan voor de biologische bestrijding van Verticillium verwelkingsziekte (*Hoofdstuk 4*), de resultaten van proeven met *T. flavus* en andere antagonisten tegen *V. dahliae in vitro*, in klimaatkamers en in het veld (*Hoofdstukken 5 en 7*) en het effect van de ruimtelijke verdeling van microbiële antagonisten in de grond op hun prestatie als biologische bestrijder (*Hoofdstuk 6*).

Een kwantitatieve biotoets werd ontwikkeld voor het bepalen van de effecten van biologische bestrijdingsorganismen op de dynamiek van *V. dahliae* (*Hoofdstuk 2*). Aardappel, aubergine en doornappel werden als toetsplant gebruikt om de relatie tussen de inoculumdichtheid van de ziekteverwekker in de grond en de infectie van de plant te bestuderen. Deze relatie is de basis voor de ontwikkeling van een kwantitatieve biotoets voor *V. dahliae*. Voor alle drie de toetsplanten werd een lineaire relatie aangetoond

($P<0.05$) tussen de inoculumdichtheid in de grond en de dichtheid van *V. dahliae* op de wortels. Voor aubergine werd een zelfde relatie gevonden tussen de inoculumdichtheid in de grond en de dichtheid van *V. dahliae* in sap. De dichtheid op of in de wortel (R^2 variërend van 0.45 tot 0.99) was sterker met de inoculumdichtheid gecorreleerd dan de dichtheid in de stengel (R^2 variërend van 0.04 tot 0.26). Bij aubergine waren de populatiedichtheden van *V. dahliae* op of in de wortel significant gecorreleerd met de dichtheden in stengel bij zowel 20 als 25 °C, met Pearsons correlatiecoefficiënten van 0.41 en 0.53.

Bij gelijke inoculumdichtheid van *V. dahliae* was de wortelkolonisatie bij aardappel hoger bij 15 °C dan bij 20 °C. Het tegendeel was het geval bij aubergine. Bij aardappel werden de stengels in mindere mate door *V. dahliae* gekoloniseerd dan bij aubergine. De pathozone sensu Gilligan (1985) werd berekend op <300 µm. Dit wijst erop dat de infectie van de plant wordt bewerkstelligd door microsclerotiën in de directe nabijheid van wortels. In de methode voor het bepalen van dichtheid van *V. dahliae* in plantenweefsel, geeft het uitstrijken van sap met sporen van de ziekteverwekker op ethanol agar hogere dichtheden van *V. dahliae* dan het gebruiken van pectaat medium of het mengen van het sap met gesmolten ethanol agar.

Een biotoets wordt aanbevolen om de effecten te bepalen van (a)biotische factoren op de ontwikkeling van *V. dahliae* in de plant. In de toets worden aubergineplanten geplaatst op een grond besmet met 300 afzonderlijke, vitale microsclerotieën g^{-1} met een waterpotentiaal van -6.2 kPa. Vervolgens deze planten incuberen gedurende 8 weken bij 20 °C.

De beschikbaarheid van Chinese asterzaden en tomatenzaden welke 17 jaar geleden omhuld waren met een kleiproduct met sporen van *T. flavus*, maakte het mogelijk de houdbaarheid van een biologisch preparaat te bepalen met *T. flavus* (*Hoofdstuk 3*). De zaden waren bewaard bij kamertemperatuur. Ongeveer 20% van de ascosporen hadden hun hitteresistentie behouden en overleefden een behandeling in water bij 60 °C gedurende dertig minuten. Geen van de Chinese asterzaden en 90% van de tomatenzaden kiemden na de bewaarperiode. De aanwezigheid van *T. flavus* gedurende het bewaren had geen invloed op de kieming van de zaden.

De dynamiek van *T. flavus* op de wortels van aubergine en aardappel werd bestudeerd om na te gaan of de schimmel zich vestigt op de plaats waar *V. dahliae* de wortel infecteert *(Hoofdstuk 4). T. flavus* bleek de aardappel- en auberginewortels te koloniseren. In potproeven met veldgrond verplaatste de schimmel zich van de aardappelknol en de auberginezaden waaraan ascosporen van de antagonist waren

meegegeven, naar de nieuw gevormde wortels met inbegrip van de worteluiteinden. De dichtheid op de wortel was echter betrekkelijk laag. Bij aardappel was de dichtheid hoger dan bij aubergine. Met toenemende afstand tot de knol of het zaad nam de dichtheid log-lineair af. De resultaten wijzen er op dat passief transport langs de groeiende wortel een bepalende factor is bij kolonisatie van de wortel door de schimmel. Een monomoleculair verband werd gevonden tussen wortelkolonisatie en de dichtheid van *T. flavus* in de grond (R^2=0.94) en een negatief exponentiële relatie tussen de dichtheid van *T. flavus* in de grond en de dichtheid van *V. dahliae* op de wortel (R^2=0.55).

De werkzaamheid van biologische bestrijding door *T. flavus* of andere antagonisten werd getoetst *in vitro* en in klimaatkamers (*Hoofdstuk 5*). *T. flavus* verminderde de vitaliteit van microsclerotiën van *V. dahliae* die aanwezig waren op afgestorven aardappelstengels uit het veld. De schimmel was toegediend in de vorm van ascosporen in carboxymethylcellulose of in talkpoeder. Toediening aan grond van een *T. flavus* preparaat (sporen in alginaat-tarwezemelen, 0.5% w/w) leidde tot een afname van dichtheid van *V. dahliae* met meer dan 90%. Dit werd waargenomen bij zowel 15 als 25 °C. De dichtheden van *V. dahliae* waren negatief gecorreleerd (r=-0.50; p=0.01) met die van *T. flavus*. Het was opmerkelijk dat de dichtheid van *V. dahliae* ook werd gereduceerd na toediening van alginaat-tarwezemelen zonder de antagonist. *T. flavus* verminderde de kolonisatie van de wortel en de infectie van de stengel door *V. dahliae* na toediening van *T. flavus* aan de grond en tegelijkertijd aan zaden. Alhoewel de afname minder was dan bij toepassing van het product met de antagonist, leidde de toediening van alginaat-tarwezemelen zonder *T. flavus* aan de grond ook tot een afname van infectie door het pathogeen. Behandelingen bestaande uit combinaties van *T. flavus* met *Bacillus subtilis*, *Fusarium oxysporum* of *Gliocladium roseum*, waarbij van iedere antagonist de helft van het inoculum in de afzonderlijke toepassing van iedere antagonist gegeven werd, resulteerden in een evengrote afname van zowel kolonisatie van de wortel als van infectie van de stengel door *V. dahliae* als bij toediening van afzonderlijke antagonisten. De dichtheid op de wortel van iedere antagonist afzonderlijk bleek niet of slechts in beperkte mate te zijn beïnvloed door de aanwezigheid van een andere gelijktijdig toegediende antagonist. Dit geeft aan dat de combinaties van antagonisten compatibel zijn.

De wijze van verdeling van schimmelkiemen in de bodem wordt beschouwd als een bepalende factor voor de mate waarin schimmelparasieten effectief zijn tegen bodempathogenen. Om de invloed te bepalen van het ruimtelijke verspreidingspatroon van de kiemen van een parasiet en zijn schimmelgastheer, werd een modelstudie uitgevoerd waarin een regelmatige verspreiding vergeleken werd met een willekeurige verspreiding (*Hoofdstuk 6*). In het model werd een constante groei van de parasiet in alle

richtingen verondersteld. De gemiddelde afstand tussen kiemen bleek bij de willekeurige verdeling 40-50% kleiner te zijn. De willekeurige verspreiding leidde tot een lagere infectiesnelheid van de waardschimmel dan een regelmatige verdeling. Parameters voor epidemiologische curven voor beide typen van verdeling werden geschat aan de hand van literatuurgegevens over de aantasting van de ziekteverwekker *Sclerotinia minor* bij verschillende dichtheden van de mycoparasiet *Sporidesmium sclerotivorum*. De verschillen in infectiesnelheid van de waardschimmel tussen de twee typen van verdeling bleken vrij gering te zijn. De gemiddelde afstand tussen de kiemen van de mycoparasiet was sterker gecorreleerd dan de dichtheid van de parasiet in de grond met de oppervlakte onder de ziekteontwikkelingscurve. Bij het bestuderen van de dynamiek van antagonisten wordt een grotere epidemiologische betekenis toegekend aan de verschillen tussen de behandelingen te baseren op verschillen in gemiddelde afstanden tussen kiemen van de mycoparasiet in de grond dan van op verschillen in dichtheden per eenheid volume.

Om na te gaan in welke mate *V. dahliae* in het veld met *T. flavus* kan worden beheerst, werden twee onafhankelijke veldproeven uitgevoerd (*Hoofdstuk 7*). Om op een juiste wijze het effect van *T. flavus* op de infectie van de plant door *V. dahliae* of op de opbrengst van het gewas te kunnen interpreteren, werd eveneens de samenhang bepaald tussen de dichtheid van de ziekteverwekker in de grond en de mate van infectie van de plant en de knolopbrengst. Het aantal door *V. dahliae* geïnfecteerde stengels en de knolopbrengst vertoonde een duidelijke dosis-respons relatie met de hoeveelheid pathogeen inoculum toegediend aan de grond (R^2=0.69 en 0.40, respectievelijk). Zowel het aantal geïnfecteerde stengels als de dichtheid van *V. dahliae* in de stengel namen geleidelijk toe gedurende het seizoen.

Na toediening van het *T. flavus*-preparaat was in het eerste groeiseizoen van Exp. 1 en in het tweede groeiseizoen van Exp. 2 de dichtheid van *V. dahliae* in de plant lager in percelen met de antagonist dan in die zonder de antagonist. Deze afname werd niet waargenomen in de percelen met de hoogste dichtheid van pathogeeninoculum. De gevonden afname in kolonisatie door het pathogeen ging niet samen met een hogere knolopbrengst. Alhoewel de dichtheid van *T. flavus* in de grond na de toediening niet toenam gedurende het seizoen, bleef de dichtheid in de behandelde percelen hoger dan in onbehandelde percelen (P=0.05). Dit werd ook in het tweede jaar na toediening van de antagonist gevonden. In één van beide experimenten werd ook een verminderde kolonisatie van de stengel door *V. dahliae* waargenomen in de controle met alginaat-tarwezemelen zonder de antagonist. De wisselende resultaten van *T. flavus* in de veldproeven en de tegenstrijdige gegevens in de wetenschappelijke literatuur wijzen op

de noodzaak om in vervolgonderzoek de aandacht allereerst te richten op het leren kennen van de omstandigheden waaronder *T. flavus* de beheersing van de ziekte tot stand brengt.

Tenslotte wordt in de algemene discussie besproken of *T. flavus* voldoende perspectief heeft voor bestrijding van *V. dahliae* (*Hoofdstuk 8*). Geconcludeerd wordt dat de resultaten in dit proefschrift en de wetenschappelijke literatuur de potentie van *T. flavus* aangeven. Echter, de resultaten met deze antagonist zijn wisselend. Daardoor is *T. flavus* op dit moment zeker niet rijp voor de praktijk.
Voorlopig moet biologische bestrijding daarom toegepast worden in combinatie met andere methoden, zoals vruchtwisseling, biologische grondontsmetting, het gebruik van resistente rassen en chemische bestrijding. In vervolgonderzoek zullen de perspectieven bestudeerd moeten worden van de toediening van antagonisten aan geïnfecteerde gewasresten voordat deze in de grond geploegd worden. Ook moeten de mogelijkheden onderzocht worden voor biologische bestrijding in de plant.

List of publications

Nagtzaam, M.P.M., 1995. *Talaromyces flavus* as a potential biocontrol agent for controlling *Verticillium dahliae* in potatoes. IOBC wprs Bulletin 18:112-116.

Nagtzaam, M.P.M., 1995. *Talaromyces flavus* as a potential biocontrol agent against *Verticillium dahliae* in potatoes. Phytoparasitica 23: 69.

Nagtzaam, M.P.M., 1997. Zoeken naar een natuurlijke vijand. Inheemse bodemschimmel biedt perspectief. In: *Verticillium dahliae*. Een gevreesde verwelkingsschimmel, Landbouwkundige Uitgeverij G.C. van den Berg, Waddinxveen, p.21-25..

Nagtzaam, M.P.M. and G.J. Bollen, 1994. Long shelf life of *Talaromyces flavus* in coating material of pelleted seed. European Journal of Plant Pathology 100: 279-282.

Nagtzaam, M.P.M. and G.J. Bollen, 1997. Colonization of roots of eggplant and potato by *Talaromyces flavus* from coated seed. Soil Biology and Biochemistry 29: 1499-1507.

Nagtzaam, M.P.M., Bollen, G.J. and A.J. Termorshuizen, 1998. Efficacy of *Talaromyces flavus* alone or in combination with other antagonists in controlling *Verticillium dahliae* in growth chamber experiments. Journal of Phytopathology 146: 165-173.

Nagtzaam, M.P.M., Termorshuizen, A.J. and G.J. Bollen, 1997. The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*. European Journal of Plant Pathology 103: 597-605.

Nagtzaam, M.P.M. and F. van den Bosch, 1994. Distance distribution between soilborne fungal propagules and the implications for biocontrol by mycoparasites. In: Abstracts of the 7th International Workshop on Epidemiology of Plant Diseases. Arnhem, 1994, p. 47.

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Mario Nagtzaam

Curriculum vitae

Mario Nagtzaam werd geboren op 17 mei 1966 te Roosendaal. Na het behalen van het Vwo-diploma aan het St. Norbertuscollege te Roosendaal begon hij in 1984 met de studie Plantenziektenkunde aan de Landbouwuniversiteit te Wageningen. In juni 1989 studeerde hij af met de specialisaties entomologie en fytopathologie. In de periode van 1989 tot februari 1990 voerde hij als onderzoeker twee projecten uit bij Jonker Fris en was korte tijd werkzaam bij de Suiker Unie als laborant. Vanaf 1990 was hij werkzaam als beleidsmedewerker bij de Gewestelijke Raad voor Zeeland van het Landbouwschap. September 1991 trad hij in dienst van de vakgroep Fytopathologie van de Landbouwuniversiteit als assistent in opleiding en verrichtte het onderzoek dat beschreven is in dit proefschrift. Vanaf 1 april 1996 werkt hij als beleidsmedewerker kwaliteit en milieu bij de Directie Landbouw van het Ministerie van Landbouw, Natuurbeheer en Visserij.