Ecology and control of *Pythium* root rot in flower bulb culture

Gera van Os

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Voor mijn ouders

Ecology and control of *Pythium* root rot in flower bulb culture

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Promotor	Prof. dr. J.A. van Veen
Co-promotor	Dr. J. van Aartrijk (Koninklijke Algemeene Vereeniging voor Bloembollencultuur)
Referent	Prof. J.M. Whipps (Horticultural Research International)
Overige leden	Prof. dr. E. van der Meijden
	Prof. dr. E.J.J. Lugtenberg
	Prof. dr. A.H.C. van Bruggen (Wageningen Agricultural University)
	Dr. ir. J.M. Raaijmakers (Wageningen Agricultural University)

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CHAPTER 1

General Introduction

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Photograph Bulb field with premature die-off caused by *Pythium* infection in iris.

CHAPTER 1 General introduction

Introduction

Root rot caused by *Pythium* spp. is an important disease in ornamental bulb culture in the Netherlands. Current legislation about the registration of pesticides, the decreasing use of chemical control methods, and increasing pressures for the use of environmentally friendly methods, make it difficult to recommend chemical disease control methods. These can rapidly become out of date because of the non-availability of the chemicals, or the withdrawal of approval for the use of a chemical on a certain crop. The development and registration of new, environmentally acceptable chemicals is expensive and time consuming and the use of a limited number of specific pesticides increases the risk that pathogens develop resistance.

The long juvenile period and the slow vegetative propagation of bulb crops make them very vulnerable for diseases in general, and seriously hamper breeding. Since the 17th century, breeders selected hybrids according to flower characteristics and forcing ability, rather than to disease resistance (Le Nard & De Hertogh, 1993). Alternative control measures, therefore, have to be developed. This thesis describes research on *Pythium* root rot of ornamental bulbs, designed to obtain knowledge for the development of alternative control measures in order to reduce the use of and dependence on chemical control.

Bulb culture in the Netherlands

Ornamental bulb culture in the Netherlands comprises the cultivation of several flower crops with different kinds of perennating structures. For example, tulip, crocus and dahlia produce bulbs, corms and tubers respectively. In general, these crops are all indicated as bulb crops. Bulb cultivation, i.e. tulips, probably occurred as early as the 12th century near Persia (Botschantzeva, 1982). These bulbs originated from Central Asia (Hoog, 1973) and adjacent territories such as the Middle East and the Mediterranean. Bulbs were introduced into Europe via Turkey after the middle of the 16th century and brought to the Netherlands in 1571 (Botschantzeva, 1982). Today, the Netherlands is the centre of flower bulb production and trade in the world. Around 70% of the world production originates from the *c.* 22.000 hectares within its borders used for bulb cultivation. In 2001, the production value of the total flower bulb production was approximately 680 million Euro. The sandy soil behind the dunes in the western part of the country along the North Sea coast provides a well-suited substratum and the mild winters and summers create the optimum climate for the bulb growing “industry”. Outside the Netherlands, production areas of flower bulbs are increasing. Because the auction and trading firms still reside in the Netherlands,

most of the foreign production is sold via the Netherlands. 93% of the world trade in flower bulbs is controlled by Dutch firms. More than 75% of the Dutch production is exported. An important number of these exported bulbs is used in gardens and parks. From the bulbs that are not exported, around 90% is used for the production of cut flowers. More than 50% of the flowers thus produced are exported as well (International Flowerbulb Centre, 2002).

In the Netherlands, the majority of spring flowering bulb crops, like tulip, hyacinth, iris, crocus and narcissus, is grown on sandy soil in the provinces of Noord-Holland en Zuid-Holland, in rotations with bulb crops only. In other parts of the Netherlands and on the heavier soils in Noord-Holland, bulbs are grown in rotations with e.g. grass, potato, wheat, sugar beet and vegetables.

Bulb crops have a complex annual cycle of growth and senescence. For commercial bulb production, propagation is based on the generally slow vegetative production of daughter bulbs in the field. For example, bulbs of tulip, iris, crocus and narcissus can produce one to four new bulbs annually. These crops are planted in autumn, the bulbs root immediately and leaf emergence can be observed in early spring. The new (daughter) bulbs are harvested in June until August after senescence of the leaves. Bulbs of a sufficiently large size to produce a flower are saleable. Smaller bulbs need to grow for one or more years before they can be sold. Many cultivars of major bulb crops are sterile and do not set seed. Seeds from fertile cultivars produce a highly variable offspring and are used for breeding purposes only. Breeders depend on vegetative propagation of their seedlings in order to produce a uniform stock of sufficient quantity that can be tested for the development of new varieties. The selection of a new cultivar and the production of a reasonably large stock may take 10 to 20 years.

Diseases and pests

The vegetative propagation cycle of most bulb crops comprises several years of growth in soil. Within this period, bulbs are planted and lifted each season, stored and transported, exposing them frequently to physical damage and various contamination sources. Diseases and pests can accumulate and cause severe losses once introduced in the production cycle. Most of the ornamental bulbs and cut flowers are for export which demands high standards for quality and plant health.

Diseases can affect the whole growing plant in a range of ways. For example, pathogens affect the plant in the ‘dormant’ condition, i.e. the storage organ(s), the growing plant, such as the leaves, or the underground parts. Symptom expression does not necessarily reflect the site of the attack, as below-ground damage can result in the wilting of collapse of apparently healthy above-ground parts, or secondary infections of shoots and flowers may result from a bulb or corm infection (Rees, 1992). Important fungal diseases are caused by *Fusarium oxysporum*, *Botrytis* spp.,

Rhizoctonia solani, and *Pythium* spp. Infestation with these fungi affects both propagation in the field and flower production of several major bulb crops. *Penicillium* spp. may cause considerable damage to some bulb crops in storage. A few bacterial diseases of bulb crops are serious, 'yellow disease' (*geelziek* in Dutch) of hyacinth, caused by *Xanthomonas hyacinthi* being the most notorious one. Numerous viruses have been identified as pathogens of bulb crops. For instance, at least 14 viruses are known to infect tulip, and more than 15 are confirmed in narcissus (Rees, 1992). The vegetative propagation of bulb crops allows the spread of virus diseases throughout most bulb stocks unless specific steps are taken by the growers to establish a nucleus of virus-free material as a base for healthy stocks. Nematodes are among the most serious pests of major crops. In addition to the damage they cause directly, some, such as nematodes of the genus *Trichodorus*, are of importance as vectors of virus diseases. Other migratory nematodes (including root lesion nematodes) of the genera *Longidorus*, *Pratylenchus* and *Xiphinema* can be important for a range of bulb crops in light, sandy soils. Stem and tuber nematodes of the genus *Ditylenchus* are major pests of most commercially grown bulb crops (Rees, 1992). Also pests caused by aphids, thrips, and mites can cause severe damage directly or as vectors of viruses during bulb cultivation and storage.

Disease control

Because of the clonal nature of most bulb crops, it is important to monitor the health of stocks, and to remove and destroy diseased plants, irrespective of the severity of damage or the numbers of plants involved. The high cost of labour and increasing mechanized cultivation have decreased personal attention from the grower for his crops, which generally results in poorer crop health (Rees, 1992).

Control measures involve hygiene to prevent spread and infection, direct treatment aimed at killing the pathogen or the vector by chemical or physical means, or treatment which favours the growth of the plant rather than the pathogen, by such means as control of temperature or humidity. In many cases, knowledge of the pathogen's life history can reveal times when pathogen growth or disease spread can be reduced by simple measures. For example, spore dispersal dependent on water films can be prevented by dry conditions, and the growth of fungi requiring high temperatures can be restricted by late planting in the autumn (Rees, 1992). Methods applied in bulb cultivation on sandy soils include crop rotation, removal of crop residues, subsoil ploughing, flooding, soil treatments with chemicals, and bulb treatments including hot water treatment, rinsing and fungicide-dipping.

Currently, chemicals are essential in the control of soilborne diseases and weeds on sandy soil. Treatment with more or less specific pesticides is often combined with one or more cultural methods. Until the early nineties, at the start of the thesis work, broad spectrum methods like soil fumigation and flooding were

frequently applied for disease control. Various older reviews (Goring, 1962; Kreutzer, 1961; Wilhelm, 1966) refer to the control of plant diseases by fumigants and the resulting yield increases. In 1982, flooding was applied for the first time in ornamental bulb culture for disease control. Some fungal diseases can be controlled completely by flooding for six weeks, for example black slime caused by *Sclerotinia bulborum*, grey bulb rot caused by *Rhizoctonia tuliparum* (Muller *et al.*, 1988), and fire caused by *Botrytis tulipae* (Muller, 1987). Also, the perennial weeds way thistle (*Cirsium arvense*) and coltsfoot (*Tussilago farfara*) were effectively controlled by flooding and several species of nematodes, e.g. *Ditylenchus dipsaci* and *Trichodorus* sp., were markedly reduced after flooding (Van Zaayen *et al*., 1986). Clearly, flooding for disease control is both environmentally acceptable and effective against several important plant pathogens in ornamental bulb culture.

All disease control methods, and broad spectrum methods like fumigation and flooding in particular, can affect or eliminate nontarget microorganisms. This may have an adverse effect on the occurrence of some pathogens (Bollen, 1979; Domsch *et al*., 1983; Martin *et al.*, 1957; Mitchell & Alexander, 1962; Powlson, 1975). In the past, the consequences were insufficiently considered in ornamental bulb culture. In the nineties, the input of broad-spectrum soil disinfestation chemicals, e.g. methylbromide and dichloropropene-etridiazol, and some specific pesticides, like cyprofuram, captafol and oxamyl, were prohibited for environmental reasons in accordance with the “Multi-year Crop Protection Plan” (Netherlands Ministry of Agriculture, Nature Conservation and Fisheries, 1991). After that, regulations became even more stringent. The flower bulb industry, aware of the implications of the “Multi-year Crop Protection Plan”, joined forces and initiated research to solve the most urgent bottle necks in bulb production, breeding and forcing. The effort resulted in the Urgency Programme for Research on Diseases and Breeding of Flower Bulbs, financed jointly by the Commodity Board for Ornamental Plants (PT) and the Netherlands Ministry of Agriculture, Nature Conservation and Fisheries. The Urgency Programme intended to contribute to breeding for resistance against *Fusarium oxysporum* in gladiolus and lily, improvement of breeding techniques and the development of control measures for *Trichodoridae*/Tobacco Rattle Virus, *Pythium* and *Rhizoctonia solani*

Pythium root rot

Pathogenic species of the genus *Pythium* (Pringsh.) cause significant losses in agricultural and horticultural crops world-wide. Most pathogenic species infect seeds and seedlings, causing pre- and post-emergence damping off (Hendrix & Campbell, 1973; Martin, 1992). Several species, however, are capable of inciting diseases of mature plant tissue, e.g., root rot in sugar beets (Von Bretzel *et al.,* 1988), root rot in sugar cane (Lee & Hoy, 1992), and cavity spot in carrots (Phelps *et al.,* 1991; White,

1988). Plant residue management is an important component of disease management practices and recommended planting dates are set with consideration of soil temperature and moisture, factors that influence the pathogen directly and indirectly through effects on the antagonistic microflora. Biological control agents applied as seed or soil inoculants have been successful only in suppression of seedling disease; such agents have not reliably suppressed root rot caused by *Pythium* spp. in agricultural soils (Martin & Loper, 1999). Constraints to management of infection of mature plants by *Pythium* spp. are daunting due to differential distributions of biocontrol agents and phytopathogenic Pythium spp. in the soil and field variations in the establishment and activities of biological control agents, especially under the full spectrum of environmental conditions conducive to disease (Martin & Loper, 1999).

Flower crops, such as iris, crocus, hyacinths, tulips and lilies are also susceptible to root rot caused by *Pythium* species, whereas other flower crops like narcissus, dahlia and gladiolus are less or not susceptible to *Pythium*. In the Netherlands, a considerable area of bulb crops is cultivated on sandy soils with low organic matter contents (≤1.5%). In these soils, root rot caused by *Pythium* spp. can be a major problem resulting in considerable yield loss, up to 40% income reduction from saleable bulbs. On heavier soil types, growers report less problems caused by *Pythium*.

P. ultimum (Trow), *P. irregulare* (Buisman), *P. paroecandrum* (Drechsler), *P. intermedium* (de Bary), *P. macrosporum* (Vaartaja & Van der Plaats-Niterink), *P. sylvaticum* (Campbell & Hendrix) and *P. violae* (Chesters & Hickman) have been described as pathogens on one or more bulb crops (Plaats-Niterink, 1975, 1981; Saaltink, 1969). The first above-ground disease symptoms usually develop in spring when plants with infected roots show retarded growth, wilting, yellowing, and finally premature die-off of the crop, caused by insufficient water uptake (Saaltink, 1969; Moore, 1979). Irregular patches of premature yellowing or dead plants are seen in the field, resulting in reduction of bulb yield. No disease symptoms occur on the bulbs. However, *Pythium* propagules are transmitted via the bulbs when they are used as planting stock for the next season. Disease severity in bulb crops varies from year-to-year and from field-to-field and factors governing disease onset are not known.

Growers have been advised to avoid cultivation of susceptible crops on fields heavily infested with *Pythium* and practice broad crop rotation. Since 1950, control measures include the use of broad-spectrum soil sterilants and the application of selective fungicides on the bulbs and in the soil (Moore, 1979; Saaltink, 1969; Weststeijn & De Rooij 1974; Koster & De Rooij, 1979). There are many references to the control of *Pythium* spp. by fumigants (Goring, 1962; Kreutzer, 1961; Wilhelm, 1966). Both an indirect effect, such as the control of nematodes, preventing predisposition of

the host plant to infection (Bollen, 1979), or a direct effect on *Pythium* spp. (Koster & De Rooij, 1980) can be obtained. Under practical conditions, however, the effects of fumigants against *Pythium* spp. are inconsistent (Koster & De Rooij, 1980).

Whereas other pathogenic fungi can effectively be controlled by flooding, as are nematodes, *Pythium* root rot in bulb crops is not controlled by flooding (Muller, 1987). On the contrary, growers are confronted with enhanced damage by *Pythium* after flooding.

The fungus Pythium

The genus *Pythium* was established by Pringsheim in 1858. More than 200 described species are found world-wide. These belong to the family of Pythiaceae, order Perenosporales, and class Oomycetes. Most species live as saprophytes in various types of soil and in aquatic environments; several are known to be serious plant pathogens. Unambiguous and reliable identification of the pathogens is critical for ecological and epidemiological studies. The most widely used monographs on the genus *Pythium* are those given by Middleton (1943), Waterhouse (1968), and Van der Plaats-Niterink (1981). Species identification is based upon morphological features including, but not limited to, sporangial size and shape, production of zoospores, formation and morphological features of oospores, and mode of antheridial attachment. However, identification of distinct species at times may be difficult because of similarity in morphological features among different groups of species, and intraspecific morphological variation frequently observed in different field isolates. This may result in an inability to identify certain species or even misidentification of some species. In addition, the identification of some isolates may be difficult because some species do not produce oospores or form oospores only when paired with opposite mating types. The use of molecular criteria can alleviate some of the confusion in identification of closely related species. Comparison of restriction patterns of ribosomal DNA is one of the molecular criteria that have been utilized for taxonomic comparisons of *Pythium*. The extent of variation in restriction sites within the internal transcribed spacers (ITS) of the ribosomal RNA genes provides a reliable taxonomical tool for the identification of *Pythium* species (Chen *et al.*, 1992; Wang & White, 1997).

Pythium spp. appear to be common inhabitants of various soils in the Netherlands (Van der Plaats-Niterink, 1975). However, little is known about the variety of *Pythium* species in bulb fields and in different cultivation areas, and limited information is available concerning the comparative pathogenicity of *Pythium* spp. on bulb crops or differences in virulence among isolates within species.

It is well known that *Pythium* spp. are opportunists whose pathogenic activity is significantly influenced by other organisms of the soil microflora (Hendrix & Campbell, 1973; Chen *et al.*, 1988a). Plant pathogenic *Pythium* species are

suppressed through a mechanism known as “general suppression” (Cook & Baker, 1983), based on the numbers and activity of non-specific microorganisms competing with the saprophytic activities of *Pythium* spp. In general, suppression of *Pythium* is determined by measuring disease incidence or development in bioassays with a susceptible host, or by measuring saprophytic growth of *Pythium* through soil. Assays to estimate the size of the soil microbial biomass are based on extractible carbon (Van Ginkel *et al.*, 1994) or phospholipid phosphate (White *et al.*, 1979), and current assays to measure microbial activity include activity of dehydrogenase enzymes (Smith & Pugh, 1979) or the rate of fluorescein diacetate hydrolysis (Schurer & Rosswall, 1982). Competition for carbon can be determined by measuring glucose uptake and respiration by the soil microflora (Van Veen *et al.*, 1985). The importance of competition among coexisting microbial populations in limiting disease development, however, is difficult to quantify (Chen *et al.*, 1988a; Mandelbaum & Hadar, 1990; Hoitink *et al.*, 1996).

Outline of this thesis

In order to obtain knowledge for the development of alternative strategies to control *Pythium* root rot in flower bulb culture on sandy soil, effects of several cultural practices on disease development, on disease suppression by the soil microflora, and occurrence and pathogenicity of *Pythium* species in bulb fields were investigated. Bulbous iris and crocus were used as test crops in this study.

In the past, research on control of *Pythium* root rot in flower bulb culture was mainly concentrated on efficacy of chemical fungicides. Up to 1990, little was known of the time of infection and subsequent disease development in bulb crops. This knowledge, however, might contribute to the development of alternative control measures. Observations in field grown crops had indicated that planting date might have an effect on damage caused by *Pythium*, which might be associated with soil temperature. Therefore, the question was raised whether the onset and subsequent development of root rot in iris and crocus was affected by planting time and soil temperature (Chapter 2).

Considering the fact that fumigation and flooding are applied in ornamental bulb culture, and the fact that *Pythium* spp. can survive or be (re-)introduced after these treatments, while many other microorganisms will be reduced or eliminated, the question was raised whether these cultural practices could have adverse effects on the occurrence of *Pythium* root rot. Timing of these measures within the rotation scheme might be of great importance. In order to answer this question, the effect of fumigation and flooding on the population density of *Pythium* and on disease suppression was investigated (Chapter 3).

Maintenance of sufficient levels of organic matter contents is essential in bulb cultivation on sandy soil. In the past, this was done by application of cattle manure,

which also contributed considerably to the nutrient supply of the soil. Recently, important changes in the nutrient legislation, urged the application of nutrient-poor organic matter such as composts, in order to prevent leaching of nutrients to the groundwater. Composts are well known to suppress soilborne pathogens, including *Pythium* (Hadar & Mandelbaum, 1986; Kuter *et al.*, 1988; Lumsden *et al*, 1983; Mandelbaum *et al.*, 1988; Tuitert *et al.*, 1998). However, most reported work on compost amendments has been carried out in controlled environments, rather than in the field. Most studies were performed at relatively high temperatures (20°C-25°C) and involved high amounts of compost (up to 40-100%), unrealistic for field application. In the Netherlands, composted organic household waste is widely available. Amendment of this compost in the field is limited by law to six tons dry matter per hectare per year, or 12 tons per hectare per two years, because of heavy metal contents. In Chapter 4 of this thesis, we inquired whether amendment of soil with small quantities of composted organic household waste could suppress *Pythium* root rot in iris and crocus under controlled and under field conditions.

In order to gain insight into the nature of *Pythium* suppression in soil and the effects of treatments like fumigation, flooding and compost amendment, the changes that these treatments induce in some microbial parameters and the microbial community composition were investigated. Microbial parameters determined were biomass, nutrient competition (glucose uptake), respiration and dehydrogenase activity, in relation to suppression of *Pythium* root rot in iris and hyphal growth through soil (Chapter 5). Bacterial and fungal community profiles were made using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified ribosomal RNA genes. The profiles were analysed to determine whether specific microbial communities or populations were associated with *Pythium* suppression (Chapter 6). So, in these chapters, the hypothesis was tested that suppression of *Pythium* was linearly related to the activity of the microbial community and to the presence of specific groups.

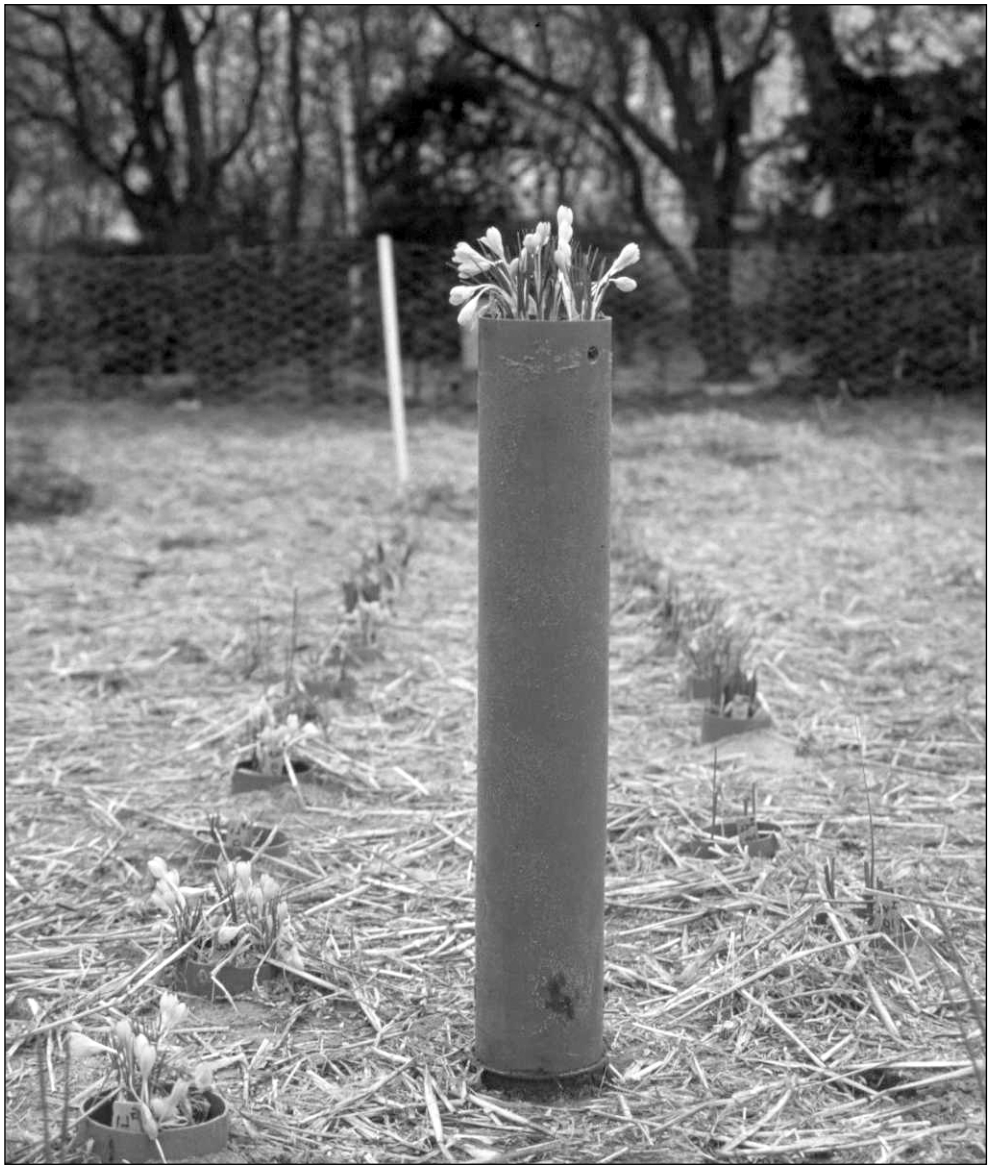
Generally, crop rotation is a method of reducing the populations of soil pathogens. Success of this procedure is determined by aspects such as the occurrence and host range of pathogenic species. In the past, little information was available on these subjects concerning *Pythium* root rot in ornamental bulb culture. Generally, short rotation with susceptible bulb crops (1:2, 1:3) resulted in increasing damage, whereas longer rotations (1:4, 1:5) revealed less problems. However, the reason for the rotation effects were unclear. The question was raised whether detection and identification of *Pythium* spp. from field soil could be used as a tool to advise on crop rotation. In order to answer this question, a survey of species occurring in bulb fields was made in the western part of the Netherlands. Field isolates were identified using restriction fragment length polymorphism (RFLP) of PCR-amplified ribosomal DNA. A selection of identified isolates was tested for pathogenicity on iris, crocus, hyacinth, tulip, and lily (Chapter 7).

Extra information on the choice of model organism

In this thesis, one of the test isolates used was an isolate of *Pythium macrosporum*. *P. macrosporum* had occasionally been isolated from diseased iris plants from field grown crops. In experiments described in Chapter 2, 3 and 5, *P. macrosporum* isolate P60 was used. This isolate had been well described and proved to be highly aggressive on iris, and mildly aggressive on crocus. However, in 1997, isolate P60 appeared to have lost its pathogenicity. *P. macrosporum* isolate 111, highly aggressive on iris and crocus, was considered as the new test isolate. Both isolates were morphologically (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands) and molecularly (Chapter 7) identified as *P. macrosporum*. In order to evaluate isolate 111 and compare its behaviour to isolate P60, some experiments, previously performed with P60, were repeated. In a pot experiment with iris, a series of inoculum concentrations was applied in untreated, fumigated, flooded, and sterilized soil, as described in Chapter 3. In a separate experiment, treated soil was amended with 0%, 0.5%, 1%, 2.5%, or 5% matured compost as described in Chapter 4. Generally, infestation with isolate 111 resulted in higher infection levels in iris compared to experiments with isolate P60. Soil treatment effects on disease development appeared to be similar for both isolates, with the same significant differences between soil treatments. Also, an *in vitro* assay of mycelial growth through soil was performed as described in Chapter 5. In this experiment growth of isolate 111 was compared to growth of the non-pathogenic isolate P60. Results showed higher growth rates for isolate 111 compared to isolate P60 in non-sterile soil treatments. Again, soil treatment effects were equivalent for both isolates. It was concluded that isolate 111 was more aggressive on iris and less susceptible to suppression of mycelial growth by the soil microflora than isolate P60. However, effects on disease suppression induced by soil treatments appeared the same for both isolates. Therefore, isolate 111 was accepted as the new test isolate, and was used in experiments described in Chapters 4 and 7.

CHAPTER 2

Disease development of *Pythium* root rot in bulbous iris and crocus



Photograph Experimental set-up of a field trial wil crocus ‘Golden Bunch’ in pipes.

CHAPTER 2 Disease development of *Pythium* root rot in bulbous iris and crocus

G.J.van Os, W.J.M. van Gulik and W.J. de Boer

Abstract

Iris bulbs and crocus corms were planted at two planting dates in sandy soil infested with *Pythium* spp. At monthly intervals during the growing season root rot infection was assessed over three consecutive years and disease development curves were predicted for both crops. The disease development was remarkably different for iris and crocus and the curve shape was determined by the crop rather than by the *Pythium* species. Planting date had a significant effect on disease development in both crops. No correlation was found between disease development and soil temperature.

Introduction

Many species of the genus *Pythium* (Pringsh.) are plant pathogens causing significant losses in agricultural and horticultural crops worldwide. Most pathogenic species are pathogens of seeds and seedlings, causing pre- and post-emergence damping off (Hendrix & Campbell, 1973; Martin, 1992). Several species, however, are capable of inciting diseases of mature plant tissue, e.g., root rot in sugar beets (Von Bretzel *et al.*, 1988), root rot in sugar cane (Lee & Hoy, 1992), and cavity spot in carrots (Phelps *et al.*, 1991; White, 1988). Flower bulb crops, such as iris, crocus, hyacinth, tulip and lily are also susceptible to root rot caused by *Pythium* species. *P. ultimum* Trow*, P. irregulare* Buisman*, P. paroecandrum* Drechsler*, P. intermedium* de Bary*, P. macrosporum* Vaartaja & Van der Plaats-Niterink*, P. sylvaticum* Campbell & Hendrix and *P. violae* Chesters & Hickman have been described as pathogens on one or more of these crops (Plaats-Niterink, 1975, 1981; Saaltink, 1969). Infected plants die off early, resulting in reduction of bulb yield. Control measures for these pathogens include soil fumigation and the application of selective fungicides (Koster & De Rooij, 1979; Saaltink, 1969). The use of fungicides is restricted by governmental regulations because of environmental and health hazards. Alternative control methods are urgently needed in order to reduce the use of, and dependence on, these fungicides.

Aspects of the ecology of *Pythium* species in field soil have been investigated in relation to factors influenced by crop production practices (Hancock, 1991; Lee & Hoy, 1992; Lewis & Filonow, 1990; Lumsden *et al.*, 1976; Phelps *et al.*, 1991; Soufi & Filonow, 1992; White, 1988). Of particular influence are soil conditions such as structure, compaction, drainage, temperature, and level of biological activity (Cook &

Baker, 1983; Fukui *et al.*, 1994; Kraft & Allmaras, 1985; Rush *et al.*, 1986). In addition, cultural practices that influence plant development, such as planting date and application of fertilizers or herbicides, may also affect plant reaction to root rot pathogens (Abawi & Pastor Corrales, 1990). For many combinations of *Pythium*, host and environment, the host is susceptible only for a limited period, e.g., in the seedling stage (Hendrix & Campbell, 1973; Martin, 1992), the pathogen is active only for a limited period, e.g., following certain temperatures (Von Bretzel *et al.*, 1988; Hancock, 1977), and environmental conditions favouring infection by the pathogen also exist for a brief period, e.g., during a cool rainy season (Bowers & Parke, 1993; Von Bretzel *et al.*, 1988). An epidemic may occur when periods of host susceptibility, pathogen activity, and suitable biotic and abiotic environment coincide (Cook & Baker, 1983).

Little is known of the disease development in flower bulb culture on sandy soils. In the Netherlands crocus corms are planted in late September/early October and harvested in June. Iris bulbs are planted in October through November and harvested in July/August. The first above-ground disease syptoms usually develop in spring when plants with infected roots grow poorly. The disease occurs in irregular patches with zones ranging from severe stunting to apparently normal plants. Leaves of infected plants die back from the tip and patches of yellowing or dead plants are seen in the field (Moore, 1979). Disease severity in bulb crops varies from year-to-year and from field-to-field and factors governing disease onset are not known. Observations in field grown crops have indicated that planting date may have an effect on damage caused by *Pythium*. In the Netherlands, soil temperatures decrease from 15°C to 5°C during the planting period. Therefore soil temperature seems to be a factor that may influence disease development. The objective of this research was to study the relation of soil temperature and planting time with the onset and subsequent development of root rot in bulbous crops of iris and crocus under field conditions in sandy soils.

Materials and Methods

Field preparation and sampling

In order to perform disease assessments without damaging the roots by harvest procedures, bulbs were planted in Polyvinyl Chloride (PVC) pipes (60 cm long and 10 cm diameter). These pipes were open at the top and covered with fine nylon mesh at the bottom. Pipes were filled with *Pythium*-infested soil or steam-sterilized soil (non-infested control treatment). After compressing the soil using a standardized procedure, pipes were dug in in the field vertically in the last week of September. Pipes were harvested from the field at monthly intervals. After harvesting the pipes, soil containing the rooted plants could be pushed out of the pipes without damaging the roots. The experiment was performed during three successive growing seasons from 1990 till 1993 at the experimental fields of the Bulb Research Centre in Lisse, the Netherlands.

Sandy soils were used, with a low content of organic matter (1-1.5%), and a pH around 7. In 1990, naturally infested soil was collected for iris and crocus separately from severely infested areas in fields near Breezand (iris) and Julianadorp (crocus), where the disease had been observed in iris and crocus crops during the preceding season. In 1991 and 1992, soil was collected from a bulb field near Lisse and steam-sterilized (2 h at 70°C) to eliminate any (native) pathogens and left to be recolonised by microorganisms in open air during six months before further use. After this period the soil was tested for presence of pathogenic *Pythium* and none was found. In 1991, the soil was then artificially infested with 10 l/m^3 of a three week old sand-oatmeal culture of *P. macrosporum* (isolate P60) for both iris and crocus in the last week of September. In 1992, isolates used for iris and crocus were *P. macrosporum* (isolate P60) and *P. irregulare* (isolate I1683), respectively. Identification of these isolates was performed by the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands) based on the key of Plaats-Niterink (1981). In the non-infested control treatments, cyprofuram 20 kg/ha (as Tubosan 20% a.i., AgrEvo, the Netherlands) was added to prevent colonization by *Pythium*.

There were two planting dates for both crops, i.e., the first week of October and the third week of November. Prior to planting bulbs of *Iris xyphium* 'White van Vliet' (size 5-6 cm circumference) and corms of *Crocus ancyrensis* 'Golden Bunch' (size 6-7 cm circumference) were surface disinfected by immersion in a solution of 0.4% formaldehyde for 15 min, and stored at 17°C (iris) and 20°C (crocus). In each pipe, three bulbs or corms were planted at 10 cm depth. In 1990, 1991 and 1992, four infested pipes plus one non-infested pipe x two planting dates x two crops = 20 pipes were planted in randomized order within a row, with 30 cm distance between pipes. Ten rows, one for each harvest date, were randomized in the field with 50 cm between rows.

Pipes were covered with straw (15 t/ha) from November until April for frost protection. For fertilization and weed control, the field was treated according to standard cultivation practice. Soil temperatures were monitored at 10 cm depth in pipes with permanently placed temperature probes (four probes per crop) connected to a datalogger (Grant 1202 Squirrel). Groundwater level was maintained at 55-60 cm below surface level throughout the season; this level is standard in ornamental bulb culture on sandy soil and had a direct but constant influence on water tension in the top soil layer.

Root development assessment

At monthly intervals pipes were harvested from the field, starting in the third week of October with pipes from the first planting date. Pipes from the second planting date were first harvested in December. The pipes were transported to the Research Centre and processed the same day. At times of severe frost, when up to 8 cm of the topsoil was frozen, pipes were stored at room temperature for 24 h to

defrost prior to further handling and roots were washed with tap water. Root development of healthy plants was determined in non-infested pipes as total root mass (g fresh roots / pipe).

Disease severity rating

Roots were visually scored for root-rot severity using an arbitrary disease index ranging from 0-5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection, compared to the corresponding healthy root systems of the control treatments at each harvest date. Roots were scored for each plant individually and a mean root rot index for each pipe was calculated. Means of four pipes per treatment from each harvest date were used in statistical analysis.

Root segments from each plant with and without disease symptoms were plated on a *Pythium*-selective medium containing 2% corn meal agar (Oxoid) with 25 $\mu g.ml^{-1}$ pimaricin (Merck) (CMA^{+}) in Petri dishes to confirm the presence of *Pythium* spp. These roots were rinsed in sterile water but not surface sterilized, because this could restrict the recovery of *Pythium* (Denman *et al.*, 1993; Stanghellini & Kronland, 1985). After 24-48 h incubation at room temperature plates were examined for *Pythium* growth.

Data analysis

In order to investigate relations of soil temperature and planting time with disease development a model was formulated to describe the observed disease development using soil temperature and planting time. Because physiology and developmental stage of a host plant may also influence disease development, the term 'trends in time' (TT) was included in the model to represent phenomena such as plant ageing and development from planting time. The largest model studied was:

$$\text{probit}(\mu) = C + PD + ST + TT_{lin} + TT_{qua} + PD.(ST + TT_{lin} + TT_{qua}) + Y + Y.PT$$

where probit denotes the link, μ is the expected root rot rate, C is overall mean, PD is planting date (October, November) and ST is soil temperature. In addition, the linear and quadratic component, resp. TT_{lin} and TT_{qua}, represent a linear and quadratic relation to harvest date. Interactions with PD were also of interest. Y and PT refer to year and planting-time and are treated as random. Effects were considered to be non-significant at $P \geq 0.05$.

With unequal numbers of observations for planting-time the data set was unbalanced. Root rot index ratings were converted to percentages and treated as binomial observations. The model was treated as a generalized linear mixed model (GLMM) and estimation was done by a procedure referred to as iterated re-weighted residual maximum likelihood (IRREML) (Engel & Keen, 1994). This procedure is an extension of the algorithm for GLMs and is implemented within the statistical programming language Genstat 5 (Genstat 5 Committee, 1993; Goedhart & Thissen, 1992).

Results

Root development of non-infested plants of both crops is presented in Figure 1, expressed as total root mass in time. A schematic outline of the root development and infection is illustrated in Figure 2. In Figure 3 root-rot ratings of infested treatments and mean soil temperature are plotted against time for 1990-1991, 1991-1992 and 1992-1993. All non-infested pipes were free of disease when harvested.

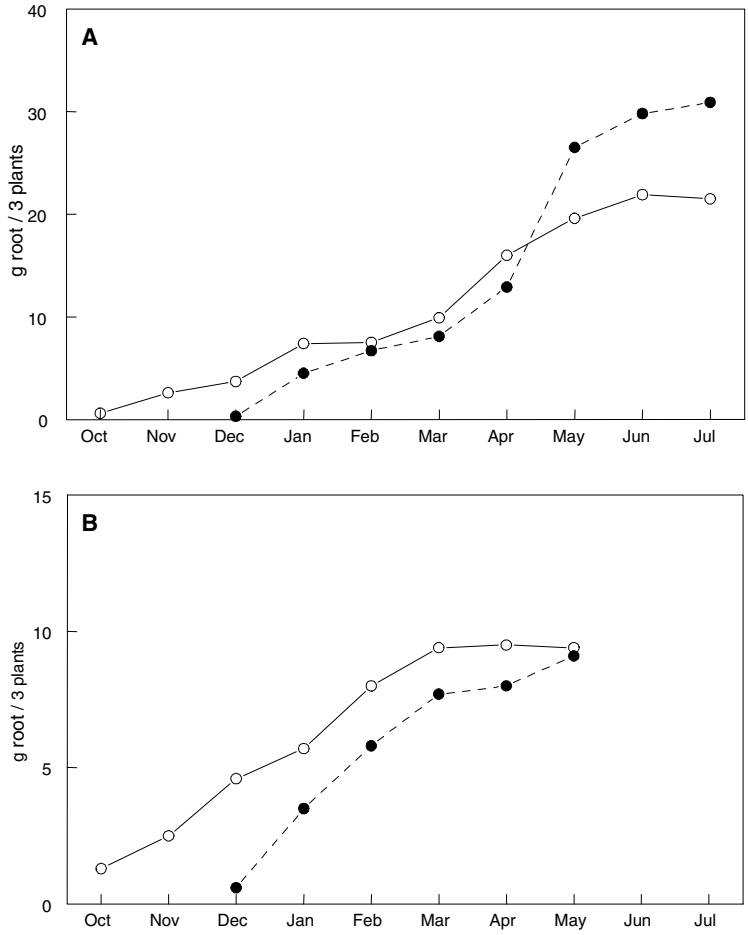


Figure 1 Total root mass (g) per three non-infested plants planted in October (———O———) and November (- - - ● - - -) plotted against time; means of three growing seasons for iris (A) and crocus (B).

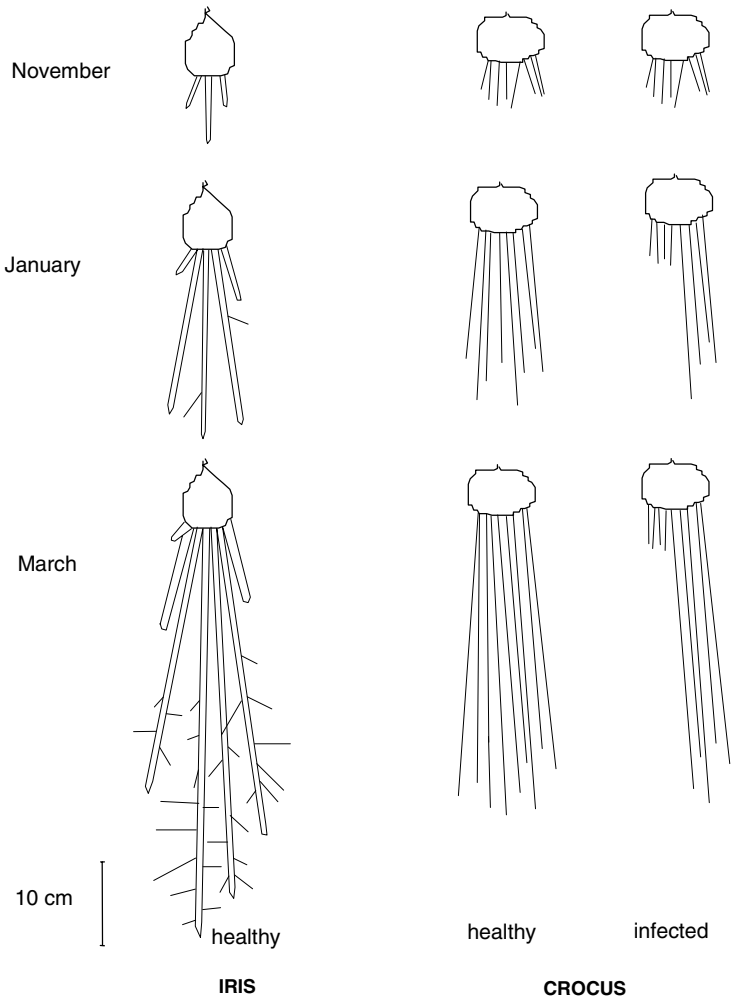


Figure 2 Schematic outline of the root development of a healthy iris plant and a healthy and infected crocus plant in time.

Iris

In iris, root development started shortly after planting with a few roots which grew and elongated until they reached the ground water at 60 cm depth in May and from January/Febuary these roots formed lateral rootlets. Throughout the season new roots emerged from the bulbs, each forming lateral roots two or three months later (Figure 2). Thin roots were produced in the first few months after planting and thick, contractile roots in the second half of the season. Natural root die-off occurred in August, therefore disease assessments were stopped after July. Root rot symptoms occurred predominantly at root tips, showing a dark grey discoloration. In

time, more root tips at various depths became diseased and the discoloration progressed upwards along the roots. Occasionally lesions occurred half-way along the roots, initially as distinct discoloured bands. Eventually complete roots were glassy and slimy, leaving a fragile hollow cylinder containing remnants of the vascular tissue. On CMA^+ growth of *Pythium* was observed from all root segments with disease symptoms and occasionally from symptomless root segments. The colony morphology of the isolates was compared. In 1990-1991, several different *Pythium* spp. (not further identified) were isolated from infected root tissue, whereas in 1991-1992 and 1992-1993 all isolates resembled isolate P60. A small selection of these latter isolates was tested for pathogenicity in a pot experiment and proved to be highly pathogenic on iris.

After fitting the statistical model, no apparent effect of soil temperature (ST) was found (P=0.55), nor any evidence of a quadratic component TT_{qua} or any interactions with these terms. In view of these results for iris, the model was reduced to:

$$\text{probit}(\mu) = C + PD + TT_{lin} + PD.TT_{lin} + Y + Y.PT$$

Table 1 shows estimates and standard errors of this fitted model for iris, with variance components σ^2_Y, $\sigma^2_{Y.PT}$ and σ^2_e, respectively for year, planting time within years and the residual variance. In Figure 4A predicted disease development curves are presented on a natural scale using this model. As more root tips were formed, more infections occurred. In particular, with the appearance of lateral rootlets, disease curves bend upward. When root growth decreased in May and eventually stopped, the curves continued to rise because the infection progressed further from the root tips upwards along the roots. Late planting resulted in a delayed root and disease

Table 1 Estimates and standard errors (in parentheses) for overall mean, main effects, interaction and variance components for iris and crocus, respectively.

	parameter	estimate iris	estimate crocus
overall mean	C	- 0.77 (0.19)	0.22 (0.59)
planting date	PD	- 0.28 (0.17)	- 0.81 (0.17)
trends in time linear	TT_{lin}	0.29 (0.02)	0.92 (0.17)
trends in time quadratic	TT_{qua}		- 0.08 (0.02)
interaction	$PD.TT_{lin}$	0.11 (0.04)	
year	σ^2_Y	0.07 (0.09)	1.01 (1.02)
planting time within year	$\sigma^2_{Y.PT}$	0.03 (0.04)	
residual variance	σ^2_e	3.50 (0.73)	10.51 (2.48)

development in iris. However, the root growth caught up with that of the early planting date within two months after planting and eventually even exceeded the root growth of plants from the first planting date (Figure 1A). For the disease development, an interaction was found between progress in time and planting date: parallel to root growth, disease progress was faster in plants from the second planting date than in plants from the first planting date, resulting in converging disease curves (Figure 4A).

Crocus

In crocus all roots developed at the same time and rate, reaching a maximum rooting depth of *c*. 40 cm in March and no lateral rootlets were formed. No disease assessments were performed later than May because natural die-off of crocus roots occurred in June. Infected root tips occurred within one month after the October planting and within two months after the November planting. At the time of infection, root length was *c.* 5 cm and infected tips showed a distinct brown discoloration. Infected roots stopped growing, whereas healthy roots continued growing normally and remained unaffected until the end of the growing season (Figure 2). On CMA^+ growth of *Pythium* was observed from all root segments with disease symptoms, but the fungus was never isolated from segments lacking symptoms. In 1990-1991, several different types of colony morphology could be distinguished among *Pythium* isolates from infected root tissue. In 1991-1992, all isolates resembled isolate P60 and in 1992-1993 all isolates resembled isolate I1683.

For crocus, after fitting the full statistical model no significant effect of soil temperature (P=0.39) or any interactions were found. The component for planting time within years, $\sigma^2_{Y.PT}$, was a very small negative value and the term was eliminated from the model. A second degree polynomial TT_{quad} was used to represent curvilinear effects in the response. The model was therefore simplified to:

$$\text{probit}(\mu) = C + PD + TT_{lin} + TT_{qua} + Y + Y.PT$$

Estimates and standard errors of this model are presented in Table 1 and predicted disease development curves are presented for crocus on a natural scale in Figure 4B.

From two months after planting, the infected root mass was more or less constant. However, the disease curves rise until three to four months after planting, because the root rot severity was determined by relative loss of healthy root mass induced by infection. As the healthy part of the root system was growing, the relative loss of root mass caused by the presence of the infected, non-growing part of the root system increased accordingly (Figure 2). The disease severity reached a constant level three to four months after planting as the infected root mass became negligible in relation to the deprivation of healthy root mass. Late planting resulted in delayed root development and disease onset.

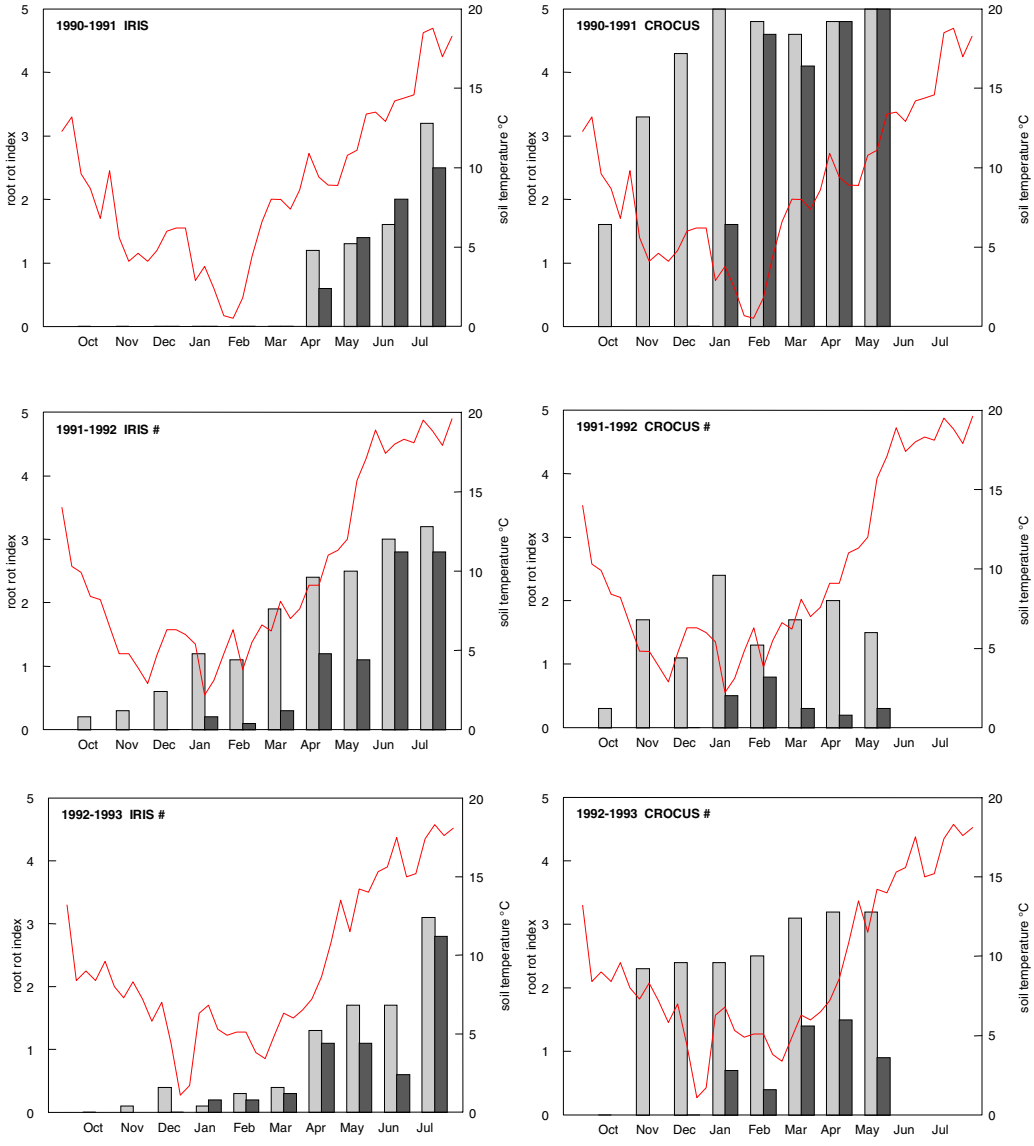


Figure 3 Root rot ratings in iris and crocus planted in October (grey bars) and November (black bars) and mean soil temperature at 10 cm depth (————) plotted against time for 1990-1991, 1991-1992 and 1992-1993; # : significant effect of planting date on infection level during the growing season.

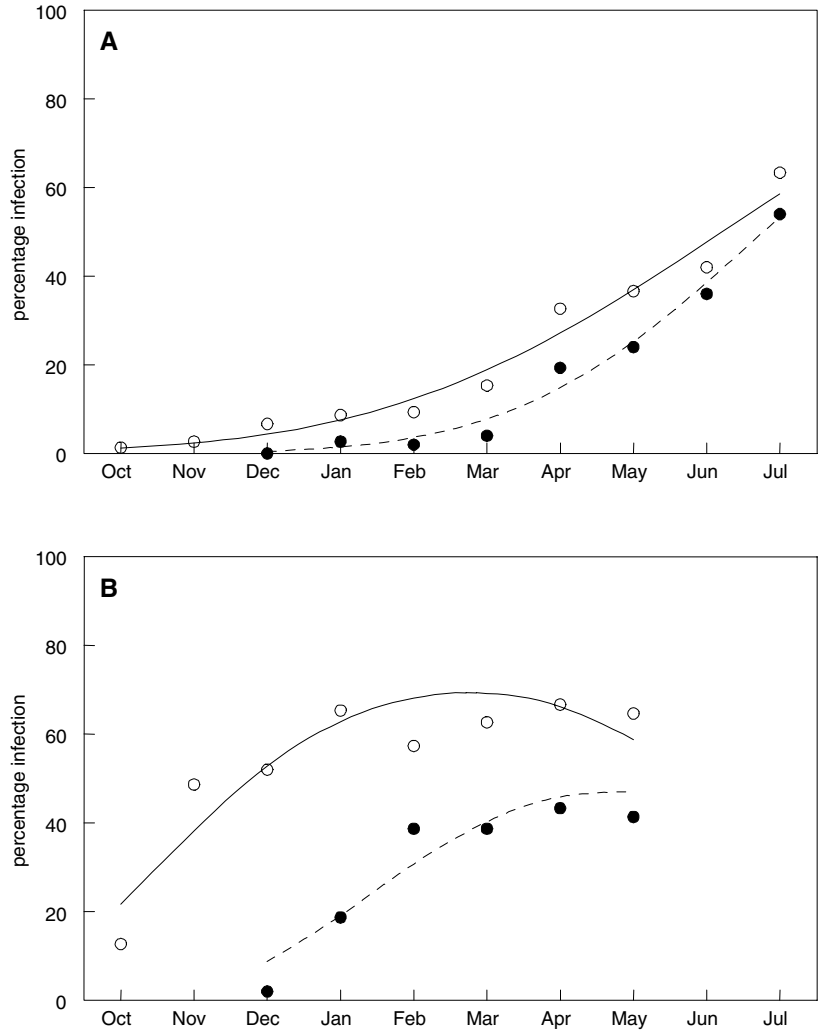


Figure 4 Mean percentages of infection from three growing seasons and predicted disease development curves after October planting (———O———) and November planting (- - ● - -), for iris (A) and crocus (B).

Discussion

In general *Pythium* is reported to be influenced by environmental factors, microbial antagonism and the presence and traits of a host. Two of the more important, or at least experimentally tested, environmental factors known to influence the activity of *Pythium* spp. are soil moisture and soil temperature. Wet soil conditions have been associated with increased incidence of disease incited by *Pythium* spp.

(Biesbrock & Hendrix 1970; Bowers & Parke, 1993; Von Bretzel *et al.*, 1988; Frank, 1974; Kraft & Roberts, 1969; Mircetich, 1971). In our experiments soil moisture was directly influenced by the ground water level, which was fixed at a level of 55-60 cm below surface level throughout the growing season; fluctuation in soil moisture at rooting depth in this situation is minimal (Valk & Stakman, 1969). Thus, soil moisture was assumed to be constant and, therefore not included in the statistical analysis of the disease development.

With regard to soil temperature, no generalization can be made for the genus as a whole; each species has an optimum for maximum activity (Stanghellini, 1974). Stimulation of *Pythium* spp. activity and/or infection is found at low temperatures (Hancock, 1977) and at high temperatures (Von Bretzel *et al.*, 1988) and cases have also been reported in which no correlation was found between soil temperature and disease severity (Lewis & Filonow, 1990). From experiments presented here, no conclusions can be drawn about a causal relationship between soil temperature and disease development. During the growing season in The Netherlands, soil temperature decreases from October to January/February and then rises again in the summer, whereas the disease severity in both crops increased continually. Obviously, in these experiments soil temperature was not an efficient term to describe disease development. Disease development curves were predicted using 'trends in time' and planting date as explanatory terms in the model. The term 'trends in time' was considered to represent phenomena such as plant aging and development. However, it may also include indirect effects of soil temperature on plant development and defence mechanisms and on the pathogen.

Disease development curves were markedly different for iris and crocus. For each crop the symptom development was consistent in consecutive years, although in crocus the final level of infection showed large variation between years (Figure 3). This variation may be caused by the different *Pythium* species used for infestation of the soil. Each species or combination of species may differ in aggressiveness (Lee & Hoy, 1992; Lewis & Filonow, 1990). On a linearized scale, in iris the disease development rate increased until the end of the growing season, whereas in crocus the disease development rate diminished. Considering the fact that each year the disease progressed similarly, regardless of the *Pythium* species used for infestation, this difference in curve shape seemed to be independent of the *Pythium* species. Moreover, in 1991-1992 *Pythium macrosporum* isolate P60 was used for both crops, causing a distinctly different disease development in each of them. Therefore, curvature seems to be determined by the crop rather than by the pathogen. It is noteworthy that, although iris and crocus belong to the family *Iridaceae*, these crops exhibit such dissimilar disease development incited by the same pathogens, a feature not reported elsewhere.

The shape of the disease development curves of both crops could be explained on the basis of root development in time and appearance of the infection. In iris, new roots were formed throughout the growing season and root tips of both main roots and lateral roots were susceptible at all times. Disease progress correlated with the number of root tips. These findings confirm reports of several other investigators who have shown that the root tip is the area most heavily attacked by *Pythium* species (Mojdehi *et al*., 1991; Napi-Acedo & Exconde, 1965; Nemec, 1972). Late planting resulted in a delayed root and disease development. However, during the productive period for bulb production of iris in spring and summer, rooting of bulbs planted in November exceeded that of bulbs planted in October, and the difference in disease level between the two planting dates diminished. Overall, the benefit of late planting may be relatively small in iris.

In crocus all roots developed simultaneously and seemed to be susceptible to *Pythium* only during a short period after planting. These findings were unexpected. Although infection started in root tips, unaffected root tips showed resistance after two months, unlike the situation with iris. Late planting resulted in delayed root development and infection. The delay in infection combined with a limited period of susceptibility, resulted in a reduced disease development. In March the disadvantage of late root development was compensated for by a significantly lower infection level. At that time the healthy root mass of plants from the later planting date could even exceed the root mass of plants from the earlier planting date. Since the productive period for corm growth of crocus is from March till May, the advantage of a lower infection level during that period may be substantial.

A disadvantage of using polynomial models, as for the analysis of the disease development in crocus, is their anomalous behaviour near the range extremes. In general, both in interpolation and extrapolation, polynomials may turn in unexpected and inappropiate directions; e.g., the predicted decrease in disease severity at the end of the growing season for corms planted in October was not observed in reality.

By planting time growers become concerned with management of the host to avoid infection. The planting date of a crop, however, also has several other effects on yield and quality. For example, late planting may lead to: enhanced calcification of crocus corms and sprout breakage by planting procedures, flower induction in iris and retarded sprout development. Effects which lead to yield reduction and/or reduced yield quality. Whether delayed planting will be profitable or not, depends on the susceptibility of the crop to *Pythium*, and also to sensitivity to the effects given above, factors which may differ for each crop, cultivar and growth season. In our experiments yield assessments were not performed because of the limited size of each experimental unit. Further field trials with larger experimental plots are needed to collect such yield data. Based on the disease severity, the benefit of planting late appeared relatively small for iris, but for crocus the advantage seemed considerable.

Many commercially important cultivars of crocus are very susceptible to *Pythium* and also sensitive to calcification, sprout breakage and growth retardation. Thus, it is not likely that planting late will be profitable for this group of cultivars.

Based on these presented results, it is recommended that the search for alternative treatments for the control of *Pythium* root rot is continued, in order to reduce the use and dependence on fungicides in ornamental bulb culture. To a certain extent the success of control treatments will depend on their efficacy in the most vulnerable stage of a susceptible crop. For iris and crocus, this efficacy may differ substantially, considering the limited period of susceptibility in crocus within two months after planting, and the increase in infection sites in iris from January/February till the end of the growing season.

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CHAPTER 3

Effects of soil fumigation and flooding on suppression of *Pythium* root rot in ornamental bulb culture

Photographs Flooded field (1) and field trial in flooding containers: flooding in August (2) and flowering iris ‘White van Vliet’ in May (3).

CHAPTER 3 Effects of soil fumigation and flooding on suppression of *Pythium* root rot in ornamental bulb culture

G.J. van Os, J.P.M. Wijnker and W.J.M. van Gulik

Abstract

The effects of flooding and soil fumigation with *cis*-dichloropropene or methylisothiocyanate on disease suppression against *Pythium* spp. were tested in pot and field experiments in sandy soil. Disease suppression was reduced by both flooding and fumigation treatments, resulting in severe infection in iris and crocus and reduction of bulb yields. It is demonstrated that the disease suppression has a biological nature, and that disease severity is more related to effects of the soil treatments on the soil microflora than to the initial inoculum density of *Pythium*. After flooding, disease suppression was restored within the experimental period of two years, whereas after fumigation, disease suppression was only partially restored. The effect of repeated fumigation in two consecutive years on the disease suppression was less severe than the effect of a single fumigation treatment prior to cultivation of a susceptible crop.

Introduction

Root rot caused by fungi of the genus *Pythium* (Pringsh.) is an important disease in bulb cultivation on sandy soils in The Netherlands. Several *Pythium* species are capable of causing root infection in iris, crocus, hyacinth and tulip (Van der Plaats-Niterink, 1981; Saaltink, 1969). Infection leads to retarded growth, wilting, yellowing, and finally premature die-off of the crop, caused by insufficient water uptake (Saaltink, 1969; Moore, 1979). In order to control *Pythium* root rot and several other soilborne diseases and weeds, soil fumigation and flooding are frequently applied. These control measures are performed at fallow in the summer period. There are many references to the control of *Pythium* spp. by fumigants and resulting yield increases (Goring, 1962; Kreutzer, 1961; Wilhelm, 1966). Since 1950, broad-spectrum soil sterilants, including 1,3-dichloropropene and sodium-N-methyldithiocarbamate (metam-Na) have been recommended for *Pythium* control in flower bulb culture (Weststeijn & De Rooij 1974). Under practical conditions, however, the effects of fumigants against *Pythium* spp. are inconsistent (Koster & De Rooij, 1980). The efficacy of soil fumigation depends on tolerance of target organisms and escape, for example in deeper soil layers. The occurrence of severe damage caused by *Pythium* after a biocidal treatment are usually explained by inadequate

application techniques, high temperatures during or shortly after application of the fumigant, or by reintroduction of the fungus with planting material. Nevertheless, inconsistent effects also occur under optimal conditions for soil fumigation combined with the use of disinfected planting material. Flooding is applied to control pathogens other than *Pythium* (Muller, 1987; Van Zaayen *et al.*, 1986). As a result of flooding, marked qualitative and quantitative microbiological changes take place in soil (Gochenaur, 1981; Mitchell & Alexander, 1962) and many species will die, illustrated by the effective control of several pathogens (Muller, 1987; Van Zaayen *et al.*, 1986). Growers are frequently confronted with enhanced damage by *Pythium* after flooding. Shokes & McCarter (1979) detected *Pythium* spp. in irrigation water sources in Georgia. Gill (1970) demonstrated that pathogenic species of *Pythium* persisted in irrigation ponds, and mentioned numerous similar studies that reinforce this point. It must be concluded that *Pythium* spp. can survive in water sources used for flooding as well as in floodwater itself, in the water-soil interface of flooded fields, and in floating or submerged plant debris (Strandberg, 1987).

Both fumigation and flooding are known to also eliminate non-target organisms. Much research has been done on this subject in the past (Bollen, 1979; Domsch *et al.*, 1983; Martin *et al.*, 1957; Mitchell & Alexander, 1962; Powlson, 1975). It is well known that *Pythium* spp. are opportunists whose activity is significantly influenced by other organisms of the soil microflora (Hendrix & Campbell, 1973; Chen *et al.*, 1988a). Plant pathogenic *Pythium* species are suppressed through a mechanism known as “general suppression”, based on the numbers of nonspecific microorganisms competing with the saprophytic activities of *Pythium* spp. (Chen *et al.*, 1988a; Mandelbaum & Hadar, 1990; Hoitink *et al.*, 1996). In soils with high propagule densities of other primary colonizing fungi and, hence, greater competition, *Pythium* spp. had a low saprophytic activity and subsequently caused less disease (Bouhot & Joannes, 1979).

Considering the fact that *Pythium* spp. can survive or be (re-)introduced after a fumigation or flooding treatment, while many other microorganisms will be reduced or eliminated, the question was raised whether these cultural practices could have an adverse effect on the occurrence of *Pythium* root rot in flower bulb culture on sandy soil. The aim of this research was to study the effect of fumigation and flooding on the population density of *Pythium* and on disease suppression against the fungus as reflected by disease severity and yield in iris and crocus.

Materials and Methods

Soil and plant material

Field experiments were performed in sandy soil, with a low content of organic matter (1-1.5%) and a pH 7, at the Bulb Research Centre in Lisse, The Netherlands. For pot experiments, soil was collected from the field, pasteurized (2 h at ≥70°C) to eliminate native pathogens and left to be recolonized by microorganisms in open air for six months prior to use. This soil is referred to as untreated or non-sterilized soil. Soil sterilization for pot experiments was performed by autoclaving (90 min. 121°C, twice with an interval of 48 h.).

Plant material used for pot experiments were bulbs of Dutch *Iris xyphium* 'White van Vliet' (size 7-8 cm circumference) and corms of *Crocus ancyrensis* 'Golden Bunch' (size 6-7 cm). For field experiments, *Iris xyphium* 'White van Vliet' (size 5-6 cm), *Crocus vernus* 'Flower Record' (size 5-6 cm) and *Narcissus pseudonarcissus* cv. 'Carlton' (size 14 cm) were used. All bulbs were disinfected by submerging in a solution of 0.4% formaldehyde (1% formalin, 40% a.i.) during 15 min. prior to planting. In field experiments, crops were planted in October and treated according to standard cultivation practice for bulb production. For pot experiments, bulbs and corms were planted in 1 l pots (five per pot, five pots per soil treatment, unless stated otherwise). Pots were placed in randomized blocks in a climate chamber at 18°C during eight weeks for iris, and nine weeks at 9°C followed by three weeks at 18°C for crocus, according to standard practice for flower production.

Pythium inoculum and population density assessment in pot experiments

For experiments with iris, soil was artificially infested with a three-week-old sand-oatmeal culture of *P. macrosporum* Vaartaja & Van der Plaats-Niterink sp. nov. (isolate P60) to a content of 5% (v/v) and for experiments with crocus with a sand-oatmeal culture of *P. irregulare* Buisman (isolate I1683) to a content of 2% (v/v).

Soil samples (30 g) were oven-dried at 105°C for 12 h and reweighed to determine soil moisture contents. The remainder of the samples was stored at 5°C until further processing the next day. *Pythium* population density (PD) was determined by a modified method, adapted from the soil-drop-method of Stanghellini & Hancock (1970). Dilutions of 1:1 and 1:5 (g dry soil adjusted for moisture content, ml^{-1} sterile 0.2% water agar at 35°C) were used for *P. macrosporum* and *P. irregulare* respectively, based on anticipated *Pythium* population densities. Soil dilutions were thoroughly mixed for 30 s on a Vortex tube mixer. One ml aliquots of each mixture were removed with an electronic digital pipette (Rainin EDP2) and dispensed in 40 drops of 10 µl on the surface of four Petri dishes (9 cm diameter, ten drops per dish) containing a selective medium of 2% corn meal agar (Oxoid) with 25 µg ml^{-1} pimaricin (Merck) and 1 µg ml^{-1} terramycin (oxytetracyclin HCl, Pfizer). Dishes were incubated at room temperatue for 48 h and the number of droplets with *Pythium*-outgrowth was counted. The percentage positive droplets per replicate was determined.

Fumigation experiments

Experiment 1. For pot experiments, fumigation was performed with *cis*-dichloropropene (DCP) 0.08 ml l^{-1} soil (Nematrap 1160 g l^{-1}, Cyanamid Agro, Breda, the Netherlands) or with methylisothiocyanate (MIT) 0.13 ml l^{-1} soil (Trapex 40% a.i., Schering, Boxtel, the Netherlands), the active breakdown product of sodium-N-methyldithio-carbamate (metam-Na). It was assumed that the efficacy of MIT was equivalent to metam-Na. Soil (5 l) was fumigated with MIT or DCP in closed, double plastic bags (0.1 mm thick), incubated at 20°C for five weeks and allowed to evaporate during one week. The effect of soil fumigation on disease suppression against *Pythium* was determined in sterilized and non-sterilized soil. This soil was fumigated with MIT or DCP or not fumigated and subsequently infested with *Pythium* or not infested and planted with iris or crocus. At the end of the growing period for both crops, soil samples of infested treatments were taken from each pot to determine *Pythium* PD, and disease severity was assessed. Means per treatment were calculated. The experiment was performed three times with iris and twice with crocus. Each experiment was considered as replicate.

Experiment 2. In order to determine the effect of fumigation on bulb yield caused by reduction of disease suppression and to investigate whether these effects exceed one growing season, a two-year field experiment was performed from 1995 till 1997 and repeated from 1996 till 1998 on a field plot with no or little natural *Pythium* infestation. Soil was injected by a professional injection machine at 18 cm depth with metam-Na 160 l ha^{-1} (AAMonam 510 g l^{-1}, AgrEvo, Haren, the Netherlands) in August according to standard application and covered with cellulose pulp (30 t ha^{-1}) in order to prevent evaporation. It was determined that there was no effect of fumigation on crocus yield induced by an infestation potentially present in the soil at the beginning of the experiment. Fumigation treatments were performed in the first and the second year (Table 1). In year 1, soil was fumigated or not fumigated and narcissus was cultivated as a non-susceptible fore-crop. In year 2, naturally infested soil was collected from a severely infested field where the disease had been observed in crocus during the preceding season. Six weeks after the fumigation treatment in August (year 2), plots were infested by mixing naturally infested soil (3 l m^{-2}) through the upper 30 cm. In non-infested plots furalaxyl 15 kg ha^{-1} (Fongarid 25 Wp 25% a.i., Ciba-Geigy Agro B.V., Roosendaal, the Netherlands), a fungicide with specific action against Oomycetes (Tomlin, 1994), was mixed through the soil to prevent infestation by cross-infection from infested plots. Treated field plots were 2 m x 2.25 m. Crocus corms were planted (160 m^{-2}) on 1 m x 1.25 m within the treated plots and yields were determined. Due to the impracticability to apply fumigation treatments to small units, the concept of a split plot design was used for designing the experimental layout. Each of three blocks contained four subplots, and subplots were divided into two units. Thus, for establishing treatment effects, two levels of

Table 1 Summary of soil treatments in experiment 2. Soil was fumigated or not with metam-Na in two successive years and, subsequently, infested or not with *Pythium* spp. prior to planting.

	year 1	year 2	
	fumigation	fumigation	infestation
1.	-	-	-
2.	-	-	+
3.	-	+	-
4.	-	+	+
5.	+	-	-
6.	+	-	+
7.	+	+	-
8.	+	+	+

variation were involved. Levels of fumigation in year 1 and year 2 were allocated to four subplots per block, and levels of *Pythium* infestation to units within subplots. Within each subplot, the infested treatment was performed twice and the non-infested treatment once, resulting in three units per subplot. Summarizing, each block consisted of 4 x (2 + 1) = 12 units. In order to perform disease assessments on crocus roots without disturbing the field plots and without damaging the roots by harvest procedures, a system using pipes as plant containers was used as described in Van Os *et al.* (1998, Chapter 2). Soil (5 l) was taken from the upper 30 cm soil layer of all plots from infested treatments and from one non-infested treatment (control, without fumigation). This soil was transfered into plastic pipes (one pipe for each plot), and dug in in the field. Crocus corms were planted in the pipes and roots were assessed for disease severity in April. The whole experiment was performed twice.

Flooding experiments

Experiment 3. A pot experiment was performed with iris and crocus to determine the effect of flooding on *P. macrosporum* and *P. irregulare* respectively. Flooding treatments were performed in plastic polyvinyl chloride (PVC) pipes according to Asjes *et al.* (1996). Pipes (length 70.0 cm; diameter 10.0 cm), covered with fine nylon mesh at the bottom, were filled with 5 l soil, leaving 10 cm for a water layer on top, and placed in wider and slightly shorter pipes (length 60.7 cm; diameter 12.0 cm). These outer-pipes were closed with a watertight PVC lid at the bottom. Pipes were flooded with water for eight weeks and drip irrigation was placed in inner-pipes to provide a continuous supply of water. A constant water percolation of 5.5 mm day^{-1} through the soil was established by a height difference of 3 mm between inner- and outer pipe. In flooded bulb fields percolation varies between 0.5 and 6.0 mm day^{-1},

depending on soil profile. Flooding was performed at 18°C, which resembles soil temperature under field conditions in August in the Netherlands. Pipes were filled with either sterilized soil or non-sterilized soil, infested or not infested, and subsequently flooded or not flooded, in four replicates per treatment. After the water was drained, soil contents of each pipe were divided over three pots. Soil samples of infested treatments were taken from each pot to determine *Pythium* PD and pots were planted with iris or crocus and disease severity was assessed. Means of three pots per pipe were used in statistical analysis. The experiments were performed twice with each crop.

Experiment 4. In order to determine the effect of flooding on yield reduction caused by *Pythium* a field experiment was performed with iris. Flooding was performed in buried polyester containers (1.40 m long, 0.85 m wide, 0.85 m deep). Containers were filled with untreated field soil with a natural infestation of *Pythium* spp. Each container was equipped with an individual drainage system. Flooding was established by blocking the drainage system and filling the container with water upto 5 cm above soil surface. Flooding was performed for eight weeks in August and September. At the end of the flooding period, water was drained to a level of 60 cm below soil surface, resembling standard ground water level in ornamental bulb culture on sandy soils. In non-flooded containers this water level was maintained constantly. Prior to planting, soil in all containers was tilled. Soil was flooded or not flooded and furalaxyl 20 kg ha^{-1} was added in control treatments to inactivate all *Pythium* spp. (*in vitro* tests revealed no resistance of *Pythium* spp. to furalaxyl). Bulbs were planted in four replicates per treatment (300 bulbs per container) and yields were determined.

Experiment 5. A two-year experiment was performed to examine whether effects of flooding exceed one growing season. Soil was flooded or not flooded and subsequently planted with iris or with narcissus (85 bulbs per container) as a non-susceptible fore-crop for the second year. For each crop, four replicates per treatment were planted. Following narcissus cultivation in the first year, iris bulbs were planted the second year. Bulb yields were determined. The experiment was performed twice.

Effect of inoculum density on infection

Experiment 6. A pot experiment was carried out to determine the effect of soil treatments on disease suppression against *P. macrosporum* at different inoculum densities. A dilution series of inoculum (10.0%, 5.0%, 1.0%, 0.5%, 0.0% v/v) was applied in untreated, fumigated (MIT), flooded and sterilized soil. Iris bulbs were planted (4 soil treatments x 5 inoculum levels x 5 pots = 100 pots in total) and disease severity was assessed.

Reintroduction of microflora in treated soil

Experiment 7. A pot experiment was performed to determine whether effects of soil fumigation (MIT), flooding and sterilization on disease suppression against *P. macrosporum* could be reversed by applying the original soil microflora from untreated soil. Treated soil was supplemented with 0.0%, 1.0%, or 5.0% (v/v) of untreated soil one week prior to infestation and planting of iris. In addition, three control treatments were included: untreated soil, sterilized soil supplemented with 5% (v/v) sterilized soil, and a non-infested control treatment (untreated soil). At the end of the growing period soil samples were taken from each pot to determine *Pythium* PD, and disease severity was assessed.

*Disease severity and yield assessment*s

For disease severity assessment, roots were washed with tap water at the end of the growing period, and root-rot ratings of infested treatments were related to the healthy root systems of non-infested control treatments. Roots were visually assessed for root-rot severity using a disease index ranging from 0 to 5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection (Van Os *et al.*, 1998). Root rot was determined for each plant individually and a mean root-rot index per pot was calculated. In field experiments, crocus corms and iris bulbs were harvested at the end of the growing season, in June and August respectively, and yields (bulb weight g $plot^{-1}$) were determined.

Statistical analysis

Data of *Pythium* PD were analysed as binomial proportions using a generalized linear model (GLM) and a logit link in order to determine soil-treatment effects. Root-rot ratings were converted to percentages and a binomial regression using a GLM was performed. In experiments 6 and 7, inoculum densities and quota of untreated soil respectively were log-transformed in order to achieve linearity on the linear scale. Bulb yields were analysed using analysis of variance (ANOVA). Data from repeated experiments were analysed together. All calculations were performed using the statistical programming language Genstat 5 (Genstat 5 Committee, 1993; Goedhart & Thissen, 1992). *T*-tests were used to determine all pair-wise differences of means at significant level $P \leq 0.05$.

Table 2 Effect of fumigation on disease suppression (pot experiment). Disease severity (% infection) in *Iris xyphium* 'White van Vliet' and *Crocus ancyrensis* 'Golden Bunch' and population densities (% droplets with *Pythium*-outgrowth) of *P. macrosporum* (PD_m) and *P irregulare* (PD_i) after cultivation of respectively iris and crocus in soil sterilized or not, fumigated with methylisothiocyanaat (MIT), dichloropropene (DCP) or not, and infested before planting.

soil treatment		iris		crocus	
sterilized	fumigation	% infection	PD_m	% infection	PD_i
+	-	76 a[1]	62 a	66 a	62 a
+	MIT	77 a	57 a	59 a	58 a
+	DCP	77 a	58 a	71 a	66 a
-	-	11 c	7 c	27 b	34 b
-	MIT	62 ab	27 b	55 a	58 a
-	DCP	53 b	17 bc	52 a	55 a

[1] Treatment means within columns followed by the same letter do not differ significantly ($P \leq 0.05$, Student's two-tailed *t*-test).

Results

In non-infested treatments no pathogenic *Pythium* was observed, i.e. no root infection in iris or crocus, nor outgrowth on corn meal agar in the population density assessments.

Fumigation experiments

Experiment 1. In sterilized soil, both iris and crocus were severely infected, and inoculum densities of *P. macrosporum* and *P. irregulare* after crop cultivation were high compared to non-sterilized soil (Table 2), indicating lack of disease suppression. Fumigation with MIT or DCP had no effect on disease development in sterilized soil. In non-sterilized soil, with a natural microbial population, infestation resulted in a mild infection of iris and crocus. After fumigation of this soil, both tested *Pythium* species caused significantly more infection, up to a level as high as in sterilized soil, indicating a reduction of the natural disease suppression which occurred in untreated soil. *Pythium* population densities after crop cultivation correlated with disease severity, showing similar tendencies and significant effects as a result of soil treatments.

Experiment 2. In non-infested treatments, fumigation with metam-Na had no effect on crocus yields (Table 3). Apparently, no pathogens were present in the soil which were influenced by the fumigation treatment. Infestation of non-fumigated soil resulted in a considerable yield reduction caused by *Pythium*. In soil, fumigated six

Table 3 Effect of fumigation on disease suppression (field experiment). Relative yield of *Crocus vernus* 'Flower Record' in soil fumigated or not with metam-Na in growing season 1 and/or 2, prior to infestation or not with *Pythium* and planting in growing season 2; and disease severity (% infection) in crocus in infested treatments (growing season 2).

fumigation		relative yield		%
1	2	- *Pythium*	+ *Pythium*	infection
-	-	[1]100 a[2]	89 b	12 a
-	+	98 a	76 e	28 b
+	-	101 a	84 c	22 b
+	+	101 a	80 d	26 b

[1] Bulb yield of this treatment is standardized to 100%.

[2] Treatment means followed by the same letter do not differ significantly ($P \leq 0.05$, Student's two-tailed *t*-test).

weeks prior to infestation and planting, a further yield reduction occurred compared to non-fumigated soil, indicating reduction of disease suppression as a result of the fumigation treatment. Fumigation one year prior to infestation and planting also resulted in a further yield reduction compared to the non-fumigated treatment. This indicates that disease suppression was still reduced to a certain extent one year after the treatment, although a significant recovery was observed compared to fumigation 6 weeks prior to infestation and planting. Repeated fumigation (in year 1 and 2) resulted in a further yield reduction compared to a single fumigation in year 1. However, the yield reduction was less than after a single fumigation in year 2. Root infection was enhanced in all fumigated treatments (Table 3). However, no significant differences were found between different fumigation applications because of large variation between replicates.

Flooding experiments

Experiment 3. In non-flooded treatments, PDs of both *Pythium* species were significantly higher in sterilized soil than in non-sterilized soil (Table 4). This was also evident in the percentage of root infection which was higher in sterilized soil than in non-sterilized soil for both crops, indicating the presence of a certain level of natural disease suppression in non-sterilized soil. Flooding reduced PDs in sterilized soil and this resulted in less infection in crocus, but not in iris. In non-sterilized soil, flooding had no effect on *Pythium* PDs, but the treatment induced a significant increase of infection in both crops, indicating reduction of the natural disease suppression.

Table 4 Effect of flooding on *Pythium* and disease suppression (pot experiment). Population densities (% droplets with *Pythium*-outgrowth) of *P. macrosporum* (PD_m) and *P. irregulare* (PD_i) and disease severity (% infection) in respectively *Iris xyphium* 'White van Vliet' and *Crocus ancyrensis* 'Golden Bunch' in sterilized (+) and non-sterilized (-) soil after flooding (+) or not (-).

soil treatment		iris		crocus	
sterilized	flooding	PD_m	% infection	PD_i	% infection
+	-	48 a[1]	83 a	48 a	89 a
+	+	23 b	78 a	28 b	73 b
-	-	3 c	17 c	27 b	37 c
-	+	2 c	45 b	32 b	79 b

[1] Treatment means within columns followed by the same letter do not differ significantly ($P \leq 0.05$, Student's two-tailed *t*-test).

*Experiment 4. A*ddition of furalaxyl to non-flooded soil had no effect on the bulb yield (Table 5). Apparently, no significant damage was caused by the natural *Pythium* infestation in non-flooded soil. Flooding resulted in a yield increase when *Pythium* was inhibited by furalaxyl, indicating suppression of other pathogens present in the soil. This beneficial effect of flooding may also be expected to occur in the flooded treatment without furalaxyl. However, without furalaxyl the beneficial effect of flooding was negated and, moreover, an additional yield loss occurred. Since *Pythium* caused no significant yield reduction in non-flooded soil, it can be concluded that flooding reduced disease suppression, resulting in a yield loss of 25% caused by enhanced root infection by *Pythium*.

Table 5 Effect of flooding on *Pythium* and disease suppression (field experiment). Relative yield of iris 'White van Vliet' in naturally infested soil after flooding (+) or not (-), and with (+) or without (-) addition of furalaxyl prior to planting.

	furalaxyl	
flooding	-	+
-	[1]100 b[2]	102 b
+	90 c	115 a

[1] Bulb yield of this treatment is standardized to 100%

[2] Treatment means followed by the same letter do not differ significantly ($P \leq 0.05$, Student's two-tailed *t*-test).

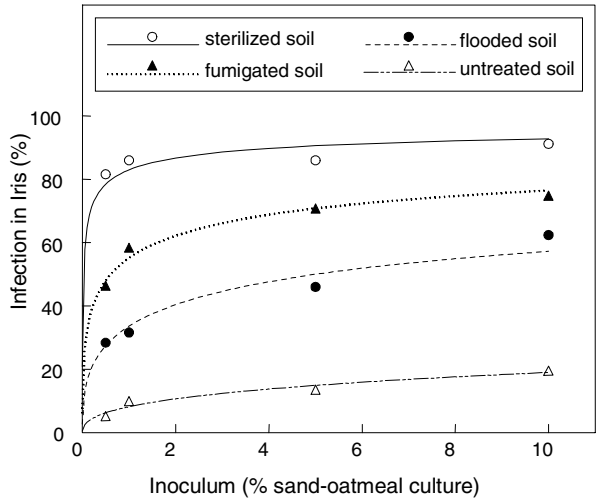

Figure 1 Effect of inoculum density on infection. Observed means (symbols) and GLM-predicted curves (lines) for disease severity (% infection) in *Iris xyphium* 'White van Vliet' in sterilized, fumigated, flooded and untreated soil after infestation with a series of inoculum densities of *Pythium macrosporum* (% sand oatmeal culture) prior to planting.

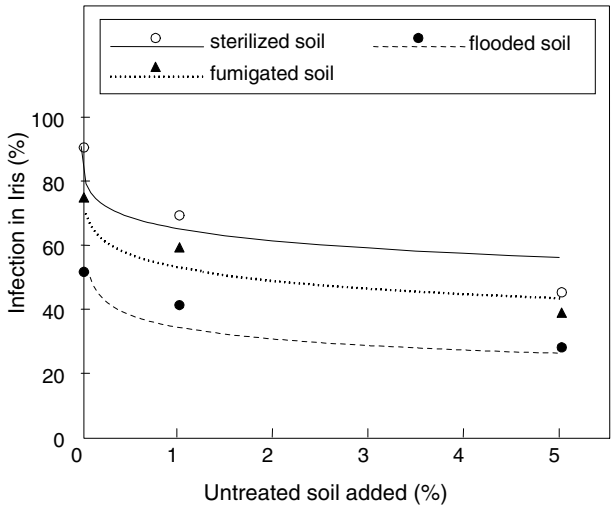

Figure 2 Reintroduction of microflora in treated soil. Observed means (symbols) and GLM-predicted curves (lines) for disease severity (% infection) in *Iris xyphium* 'White van Vliet' in sterilized, fumigated and flooded soil supplemented with 0%, 1% and 5% of untreated soil one week prior to infestation with *Pythium macrosporum* and planting.

Experiment 5. Flooding of infested soil followed by cultivation of iris resulted in a significant 15% yield reduction compared to the non-flooded treatment. Flooding had no effect on narcissus yields. When iris was cultivated succeeding narcissus, one year after the flooding treatment, no significant effects of flooding were observed. Apparently, disease suppression against *Pythium* was restored.

Effect of inoculum density on infection

Experiment 6. Disease severity in iris was significantly enhanced at higher infestation levels (Figure 1). Independent of inoculum density, soil treatments had a major effect on disease severity. Percentage of infection was lowest in untreated soil and progressively increased in flooded, fumigated and sterilized soil.

Reintroduction of microflora in treated soil

Experiment 7. Again, progressive disease development occurred in flooded, fumigated and sterilized soil. Disease severity was significantly reduced with increasing amount of untreated soil applied to treated soil (Figure 2), implying re-establishment of disease suppression. Addition of 5% sterilized soil to sterilized soil had no significant effect on disease development (85% infection, data not shown), indicating the essential role of the soil microflora in suppression of *P. macrosporum*. In neither soil treatment, the disease suppression was completely restored compared to 12% infection which was found in untreated soil (data not shown). PD of *P. macrosporum* after crop cultivation (data not shown) correlated with disease severity, with similar tendencies and significant effects as a result of soil treatments.

Discussion

The more effective a biocidal soil treatment, the smaller the population of survivors and the slower the recolonization (Powlson, 1975). This repopulation varies considerably according to the compound or method used and may lead to a typical microflora (Welvaert, 1974). The organisms which survive the treatment or those which become reestablished first reach very high numbers in a less competitive environment (Martin, 1972). Often one species becomes dominant. Of the fungi, species of *Trichoderma*, *Penicillium* spp., *Mucor* and several others usually predominate, owing to their short response time and their fast growth rate (Powlson, 1975; Welvaert, 1974; Warcup, 1952). Unfortunately, *Pythium* spp. are also rapid colonists of (partially) sterilized soil (Kreutzer, 1960).

In our experiments on the effects of fumigation and flooding on the disease suppression against *Pythium*, results of pot experiments were confirmed by the outcome of field experiments. Untreated soil had a certain level of disease suppression against *Pythium*, which was reduced after soil fumigation or flooding. In absence of the soil microflora (sterilized soil) no disease suppression was observed.

It is concluded that the disease suppression is of a biological nature, which was confirmed by the partial restoration of the disease suppression by reintroduction of the original microflora in sterilized, fumigated and flooded soil. The experimental period may have been too short for full recovery of disease suppresion, since different species of the original microflora, including possible antagonists, become reestablished at various periods after the treatment and may or may not reach numbers in the original soil (Martin, 1972). Bouhot (1979) stated that for the prediction of disease, ecological conditions favourable for the saprophytic growth of *Pythium* are more important than the initial inoculum density. This is confirmed by our experiments, in which differences in levels of disease suppression between soil treatments were independent of inoculum densities of *P. macrosporum* (Figure 2) and disease related less to the inoculum density of the pathogen than to soil treatments.

Diseases that are increased by control measures are called iatrogenic diseases (Griffiths ,1981). Whereas stimulation of antagonism can lead to integrated and indirect disease control, conversely, inhibition of antagonism can result in a change of the dominant pathogens (Bollen, 1979). The presented results imply that *Pythium* root rot can occur as an iatrogenic disease in bulb crops after both soil fumigation or flooding. This may occur particularly if a susceptible crop is grown immediately after treatment, thereby providing abundant susceptible roots for the pathogen. Under these circumstances, *Pythium* is more likely to become one of the dominant species in the soil. An adequate delay between soil treatment and cultivation of a susceptible crop might provide a greater advantage to other species as colonists of the soil.

In literature, control of *Pythium* by flooding is reported. Strandberg (1984) showed that soil populations of *Pythium* spp., that attack carrots and other vegetables, rapidly decreased during a four to six-week flooding period, and increased slowly in time after draining and cultivation of the fields. In his experiments, populations remained low long enough (30 to 90 days) to allow the production of a carrot crop under relatively low *Pythium* population levels. These findings do not correspond with the results from our experiments, where reduction of *P. macrosporum* and *P. irregulare* by flooding was not observed in non-sterilized soil, and, moreover, an enhanced infection and yield reduction occurred after draining and planting of a susceptible crop. *Pythium* species responsible for cavity spot are *P. violae* Chester & Hickman (Montfort & Rouxel, 1988) and *P. sulcatum* Pratt & Mitchell (Van der Plaats-Niterink, 1981). These species may respond in a different way to flooding as compared with *P. macrosporum*, *P. irregulare* and *Pythium* spp. in our experiments. Since the most susceptible period for iris during cultivation in the field is from early spring till the end of the growing season (Van Os *et al.*, 1998), it is possible that enhanced *Pythium* activity lasted at least during that period, i.e. ten months. In the following growing season, one year after the flooding treatment, no

effect on disease development was observed in iris. Therefore, it is concluded that the disease suppression was restored before spring in the second year after flooding.

For fumigation, contradictory experiences exist for the overall effects on *Pythium* in flower bulb culture. Fumigation often results in a yield increase, however, in some cases fumigation seems to have adverse effects. Similarly, in the literature on pesticide effects on the microflora, contradictory results are also sometimes obtained (Kreutzer, 1965). The fungal species that becomes dominant after a biocidal treatment will be determined by a combination of many factors, such as the type of treatment, the chemical and physical properties of the soil, the relative abundance of different species in the original soil, whether the soil is reinoculated either by chance or deliberately, and cultivated crop after the treatment. Because the dominant species often differ from soil to soil, it is understandable that for pesticides with selective action to the microflora, contradictory results are obtained (Bollen, 1979; Powlson, 1975). Our field experiments show that disease suppression against *Pythium* was still reduced one year after fumigation, although partial restoration had occurred. Since the susceptible period for *Pythium* in crocus is within two months after planting (Van Os *et al.*, 1998), it is concluded that recovery of the disease suppression takes longer than one year and two months after fumigation with metam-Na. Surprisingly, the damage caused by *Pythium* after repeated fumigation in two successive years was less severe than after a single application prior to cultivation of crocus. Theoretically, this phenomenon could be due to adaptation and selection of tolerant species, which, after the first fumigation, recolonized the soil and would also survive a repeated treatment. The impact would therefore be less radical the second time. If this hypothesis is assumed, repeated fumigation in consecutive years will result in disease suppression at a lower level than in untreated soil, but higher than shortly after a single fumigation treatment. Different periods for recovery of the fungal microflora after soil fumigation are reported (Martin, 1972; Reber, 1967; Welvaert, 1974). Verhagen *et al.* (1996) found recovery of the microflora within three years after application of metam-Na and requiring more than three years after application of dichloropropene.

Fumigation and flooding have different modes of action for disease control and different species of the soil microflora are killed as a result of these measures. Effects on microbial biomass, activity and diversity are subjects for further research. With respect to disease suppression, it is relevant to differentiate reversible influences and presumably persistent influences. Eventually, disease suppression seemed to recover completely after flooding. Gochenaur (1981) reported an eventual reconstruction of the microbial community both quantitatively and qualitatively within as short a period as three or four months after flooding. Apparently, the effects of flooding are reversible. For fumigation on the other hand, no complete recovery of disease suppression was observed within the experimental period. Persistent effects

can not be excluded. In order to find a way to restore disease suppression and accelerate the recovery after fumigation and flooding, possibilities of (re-) introduction of specific antagonists or an overabundance of competetive microorganisms could be an interesting option.

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CHAPTER 4

Effects of composted organic household waste on *Pythium* root rot in iris and crocus

Photographs Crocus field trial with soil fumigation and compost amendment: injection of soil fumigants (1), application of compost in field plots (2), and flowering crocus ‘Flower Record’ in Spring (3).

CHAPTER 4 Effects of composted organic household waste on *Pythium* root rot in iris and crocus

G.J. van Os and J.P.M. Wijnker

Abstract

The effects of amendment with small quantities (0.5% to 5% w/v) of matured composted vegetable, fruit, and garden waste (VFG-compost) on suppression of *Pythium* root rot in bulbous iris and crocus was evaluated in untreated, flooded, fumigated, and sterilized sandy soil under controlled and field conditions. In pot experiments with iris and crocus, flooding, fumigation, and sterilization of soil resulted in enhanced infection by *Pythium macrosporum* compared to untreated soil. In iris, compost amendment in untreated soil had no effect on infection, whereas compost amendment resulted reduction of disease development in flooded, fumigated and sterilized soil. Maturation temperature of the VFG-compost (10°C or 20°C) and temperature during iris cultivation (9°C or 18°C) had no effect on the disease suppressive effect of the compost. In crocus, compost amendment resulted in enhanced disease development in untreated, flooded, and fumigated soil. In sterilized soil, amendment of 0.5% and 1% compost appeared to reduce disease severity, which enhanced again at higher dosages of compost. In field experiments with untreated and fumigated soil, compost amendment increased disease severity in crocus and decreased corm yields in infested treatments. It is suggested that, in these experiments, the host plant is the discriminating factor between disease suppression or stimulation as a result of amendment with VFG-compost.

Introduction

In the Netherlands, the majority of spring flowering bulb crops crops is cultivated on sandy soils with low organic matter contents (≤1.5%). In these soils, root rot caused by *Pythium* spp. is a major problem resulting in considerable yield loss, up to 40% income reduction from saleable bulbs in crops such as iris, crocus and hyacinth. Soil management practices are known to influence biological processes in agricultural soils (Kennedy & Smith, 1995; Van Bruggen, 1995). For instance, disease suppression can be positively and negatively affected by several soil management practices. In the Netherlands, soil fumigation and flooding are applied in ornamental bulb culture on sandy soil to control several diseases and weeds. *Pythium*, however, cannot sufficiently be controlled by these measures. Van Os *et al*. (1999, Chapter 3) demonstrated that the natural disease suppression in soil against *Pythium* root rot was reduced by fumigation and flooding. Application of composts may induce suppression of soilborne pathogens (Hadar & Mandelbaum, 1986; Kuter

et al., 1988; Lumsden *et al.*, 1983; Tuitert *et al.*, 1998). Media amended with composts prepared from different organic wastes, such as tree barks, municipal sewage sludge, and separated cattle manure showed enhanced suppression of *Pythium* spp. (Chen *et al.,* 1988b; Mandelbaum *et al.*, 1988).

In many countries, an increasing amount of compost is produced since the separate collection of organic household waste (vegetable, fruit, and garden waste [VFG]) became compulsory. Organic amendments influence physical and chemical properties of soil, as well as the composition of the biological community, which directly and indirectly affect the plant and its health (Windels, 1997). Most reports found so far indicate that composts produced in any system become consistently suppressive to *Pythium* disease, once naturally recolonized by mesophilic microorganisms after peak heating (Kuter *et al.,* 1988; Chen *et al.*, 1988a; Mandelbaum & Hadar, 1990, Hoitink *et al.*, 1993). Plant pathogenic *Pythium* species are suppressed through a mechanism known as “general suppression” (Cook & Baker, 1983). Results from numerous studies have indicated that suppressiveness of different composts to diseases caused by *Pythium* spp. can be linked to increased levels of microbial activity in the composts themselves or in soils receiving compost amendments (Boehm *et al.,* 1993; Chen *et al.* 1988a; Craft & Nelson 1996; Hadar & Mandelbaum 1986; Mandelbaum & Hadar 1990).

Most reported work on compost amendments has been carried out in controlled environments, rather than in the field. These studies were performed at relatively high temperatures (20°C-25°C) and involved high amounts of compost (up to 40%-100%), unrealistic for field application. A few references report suppression of *Pythium* diseases under field conditions (Lewis *et al*., 1992; Lumsden *et al*., 1983; Pascual *et al*., 2000). In the Netherlands, amendment of VFG-compost in the field is restricted by law to six tons dry matter per hectare per year, or twelve tons dry matter per hectare per two years (*c.* 1%), because of heavy metal contents. Such small amounts of compost make no substantial contribution to the soil, but may serve to inoculate high numbers of microorganisms. The objectives of this research were to evaluate the effects of amendment of flooded and fumigated sandy soil with small quantities of matured VFG-compost on suppression of *Pythium* root rot under controlled and under field conditions.

Materials and Methods

Compost

Composted vegetable, fruit and garden waste (VFG-compost) was obtained from a commercial composting facility (Central Afvalverwijderingsbedrijf West-Friesland, Middenmeer) in the province of Noord-Holland in the Netherlands, where the source-separated household waste was composted under standardized conditions in an enclosed environment. During the composting process, the material

was turned and moistened (when below 50% wt/wt) each week. Processing time was eleven weeks, and the fresh compost was screened through a 10 mm sieve. The fresh compost was collected and an additional curing (maturation) was carried out at the Bulb Research Centre during five weeks in a climate chamber at 20°C (unless stated otherwise), with weekly moistening and turnings of the heap of about 40 cm high. Moisture content was kept at approximately 50% (wt/wt), corresponding with moisture tensions around pF 1.7 (-7 kPa). A new batch of compost was prepared for each replicate experiment. The pH values of the composts were between 6 and 7. EC-values varied from 3 to 5 mS cm^{-1}.

Pot experiments

For pot experiments, soil (sandy soil, organic matter content 1-1.5%, pH 7) was collected from the experimental fields of the Bulb Research Centre in Lisse, the Netherlands. The soil was pasteurized (2 h at ≥70°C) to eliminate native pathogens and left to be recolonized by microorganisms in open air during six months prior to use. This soil is referred to as untreated soil. Soil-moisture content was adjusted to 20% (w/w) and treated according to Van Os *et al*. (1999) by: 1) sterilizing (autoclaving 2 x 90 min. at 121°C with 48 h in between), 2) flooding during eight weeks at 18°C (after drainage, soil moisture was readjusted to 20% w/w) , and 3) fumigating with methylisothiocyanate (MIT, 0.13 ml/l soil, as Trapex 40% a.i., Schering, the Netherlands; soil with MIT was incubated during five weeks at 20°C in sealed plastic bags and, subsequently, allowed to evaporate for one week), or 4) left untreated at 18°C. Experimental details of the flooding and fumigation treatments are described in Van Os *et al*. (1999).

Bulbs of Dutch *Iris xyphium* 'White van Vliet' (size 7-8 cm circumference) and corms of *Crocus ancyrensis* 'Golden Bunch' (size 6-7 cm) were disinfected by submerging in a solution of 0.4% formaldehyde (1% formalin, 40% a.i.) during 15 min. prior to planting.

Sterilized, flooded, fumigated and untreated soil was mixed with 0.0%, 0.5%, 1.0%, 2.5% or 5.0% (w/v) of matured VFG-compost (three replicates of 4 l soil per treatment). Amended soil was incubated at 20°C during one week prior to infestation with 1% (v/v) of a three week old sand-oatmeal culture of *Pythium macrosporum* (isolate 111) and planting. Non-infested control treatments were included with untreated soil amended with 0.0%, 2.5% and 5% compost. Bulbs and corms were planted in 1 l pots (five per pot, two pots for each of three replicate treatments per crop). Pots were placed in randomized blocks in a climate chamber at 18°C during eight weeks for iris, and nine weeks at 9°C followed by three weeks at 18°C for crocus, according to standard practice for flower production.

In a separate experiment, the effect of temperature during compost maturation and during plant growth, and time of infestation on disease development was

investigated. Sterilized, fumigated and untreated soil was amended or not with 1% VFG-compost matured at 10°C or 20°C. Two additional treatments included amendment of sterilized and fumigated soil with 1% sterilized compost (autoclaving 2 x 90 min. at 121°C with 48 h in between). One week after compost amendment, soil was infested with 0.1% or 0.01% sand-oatmeal culture of *P. macrosporum* in order to assess treatment effects on disease development. Also, untreated soil, amended or not with compost (matured at 20°), was infested with 0.1% or 0.01% inoculum one week prior to compost amendment in order to assess effects of compost amendment in soil already infested with *Pythium*. Furthermore, three non-infested control treatments were included of untreated soil, amended or not with compost matured at 10°C or 20°C. After compost amendment, the soil was incubated in a climate chamber at 9°C or 18°C, anticipating temperatures during plant growth. The whole experiment comprised twelve treatment combinations infested with two inoculum densities and three non-infested treatments, incubated at two temperatures after compost amendment and during plant growth. For all combinations, 5 l soil was treated and divided over five pots. Iris bulbs were planted one week after compost amendment and pots were replaced at 9°C or 18°C in randomized blocks during eight weeks.

Field experiment

In order to determine the effect of compost amendment in untreated and fumigated soil on bulb yield, a field experiment was performed as described by Van Os *et al*. (1999), with four treatment factors, including two fumigation treatments, compost amendment and *Pythium* infestation. Fumigation treatments with metam-Na 160 l ha^{-1} (AAMonam 510 g l^{-1}, AgrEvo, Haren, the Netherlands) were performed in August according to standard application six weeks before planting and in the preceding year. Matured VFG-compost (12 t d.w. ha^{-1}, equivalent to 1% in pot experiments) was mixed through the upper 30 cm of soil one week before planting. At the time of planting, plots were infested by incorporating naturally infested soil (3 l m^{-2}) with a mixture of unidentified *Pythium* species through the upper 30 cm. In non-infested plots furalaxyl 15 kg ha^{-1} (Fongarid 25 Wp 25% a.i., Ciba-Geigy Agro B.V., Roosendaal, the Netherlands), a fungicide with specific action against Oomycetes (Tomlin, 1994), was mixed through the soil to prevent infestation by cross-infection from infested plots. Due to the impracticability to apply fumigation treatments to small units, the concept of a split plot design was used for designing the experimental layout. In each of three blocks, the infested treatments were performed twice and the non-infested treatments once. Further details on the experimental layout are described in Van Os *et al*. (1999). *Crocus vernus* 'Flower Record' (size 5-6 cm) was planted immediately after soil infestation in October and treated according to standard cultivation practice for corm production. Soil EC was measured in non-fumigated, non-infested treatments with and without VFG-compost

amendment two weeks after planting. Corms were harvested in June and yield (weight g plot^{-1}) was determined.

In order to perform disease assessments on crocus roots without disturbing the field plots and without damaging the roots by harvest procedures, a system using pipes as plant containers was used as described in Van Os *et al*. (1998, Chapter 2). Prior to planting, soil (5 l) was taken from the upper 30 cm soil layer of all plots from infested treatments and from one non-infested control treatment (without fumigation or compost amendment). This soil was transferred into plastic pipes (one pipe for each plot), and pipes were dug in in the field. Three crocus corms were planted per pipe and roots were assessed for disease severity in April. The whole experiment was performed twice (1995-1997 and 1996-1998).

Disease severity assessment

For disease severity assessment in pot and field experiments (pipes), roots were washed with tap water and root-rot ratings of infested treatments were related to the healthy root systems of non-infested control treatments. Roots were visually assessed for root-rot severity using an arbitrary disease index ranging from 0 to 5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection (Van Os *et al*., 1998). Root rot was determined for each plant individually and a mean root-rot index per container was calculated.

Pythium population density assessment

Soil samples (30 g pot^{-1}) were taken from the pot experiment with the series of compost concentrations. *Pythium* population density was determined as described by Van Os *et al*. (1999). Dilutions of 1:5 (g dry soil adjusted for moisture content, ml^{-1} sterile 0.2% water agar at 35°C) were dispensed in drops of 10 µl on a selective medium of corn meal agar with pimaricin and terramycin. After 48 h incubation at room temperature, the number of droplets with *Pythium*-outgrowth was counted.

Statistical analysis

Root-rot ratings and number of droplets with *Pythium*-outgrowth were converted to percentages and regression was conducted on transformed data, using the logit link, in a generalized linear model (GLM) to determine soil-treatment effects. In the pot experiment with a range of compost concentrations, quota of amended compost (x + 0.1) were log-transformed in order to achieve linearity on the linear scale. Corm yields were analysed using analysis of variance (ANOVA). All calculations were performed using the statistical programming language Genstat 5 (Genstat 5 Committee, 1993; Goedhart & Thissen, 1992). *T*-tests were used to determine all pair-wise differences of means at significance level $P \leq 0.05$.

Results

Pot experiments

In non-infested control treatments, no infection, no *Pythium* outgrowth on agar, and no damage or abnormalities in root development were observed in iris or crocus, with or without amendment with VFG-compost. Fresh root weights in these treatments showed no significant differences (data not shown). In infested treatments, all *Pythium* colonies growing on the selective medium showed the same colony morphology, resembling the *P. macrosporum* isolate used for inoculation. They were considered to be *P. macrosporum*.

In iris, infested soil treatments resulted in four significantly different levels of disease severity ($P \leq 0.05$), as represented by the intercept of the curves in Figure 1A. Percentage of infection was lowest in untreated soil and progressively increased in flooded, fumigated and sterilized soil. A significant interaction ($P \leq 0.05$) was found between these soil treatments and compost amendment. In flooded, fumigated and sterilized soil, compost amendment resulted in significant reduction of disease development, as represented by the negative slope values of the predicted curves. Slope values for these treatments were not significantly different from each other. In untreated soil, compost amendment had no significant effect on infection, as the slope value of the curve was not significantly different from zero, and was significantly different from those of the other soil treatments. Similar results were found for *Pythium* population density after cultivation of iris (Figure 1B).

In crocus, regression of the complete data set revealed a complex interaction between compost amendments and soil treatments. This was due to deviant effects of compost amendments in sterilized soil (Figure 2A). As a result, the predicted curves poorly fitted the observed means for disease severity and *Pythium* population density. It was decided to exclude the data from treatments in sterilized soil from the GLM analysis in order to predict curves for untreated, flooded and fumigated soil. Infested soil treatments resulted in two different levels of disease severity ($P \leq 0.05$), as represented by the intercept of the curves in Figure 2A. Percentage of infection was lowest in untreated soil and higher in flooded and fumigated. Amendment of compost resulted in enhanced disease development in untreated, flooded and fumigated soil. No interaction was found between these soil treatments and compost amendment. Similar results were found for *Pythium* population density after cultivation of crocus, with significant different levels of population density in untreated, fumigated and flooded soil (Figure 2B).

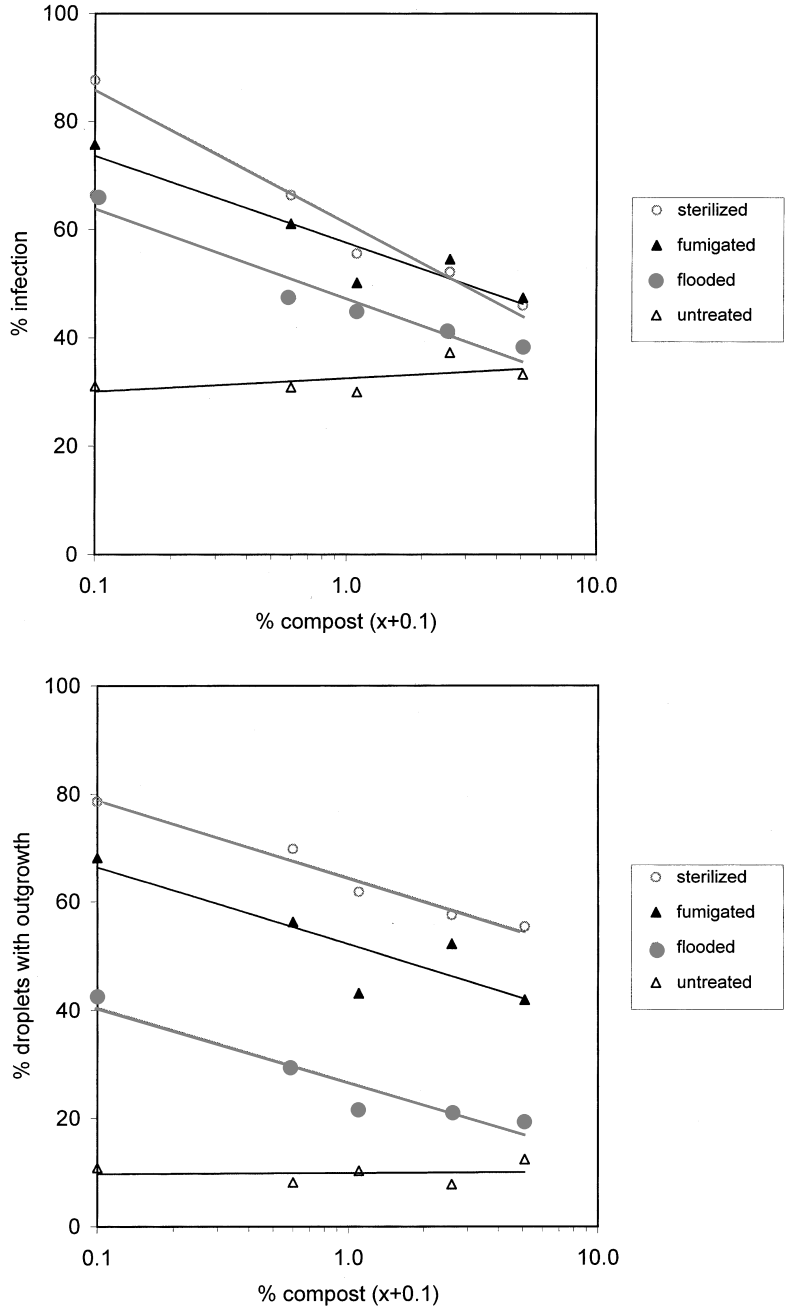

Figure 1 Effect of compost amendment on infection in *Iris xyphium* 'White van Vliet' (A) and *Pythium* population density (B) with observed means (symbols, number of replicates=3) and GLM-predicted curves (lines) in sterilized, fumigated, flooded and untreated soil after compost amendment (% compost + 0.1 on a log-scale) one week prior to infestation with *Pythium macrosporum* and planting.

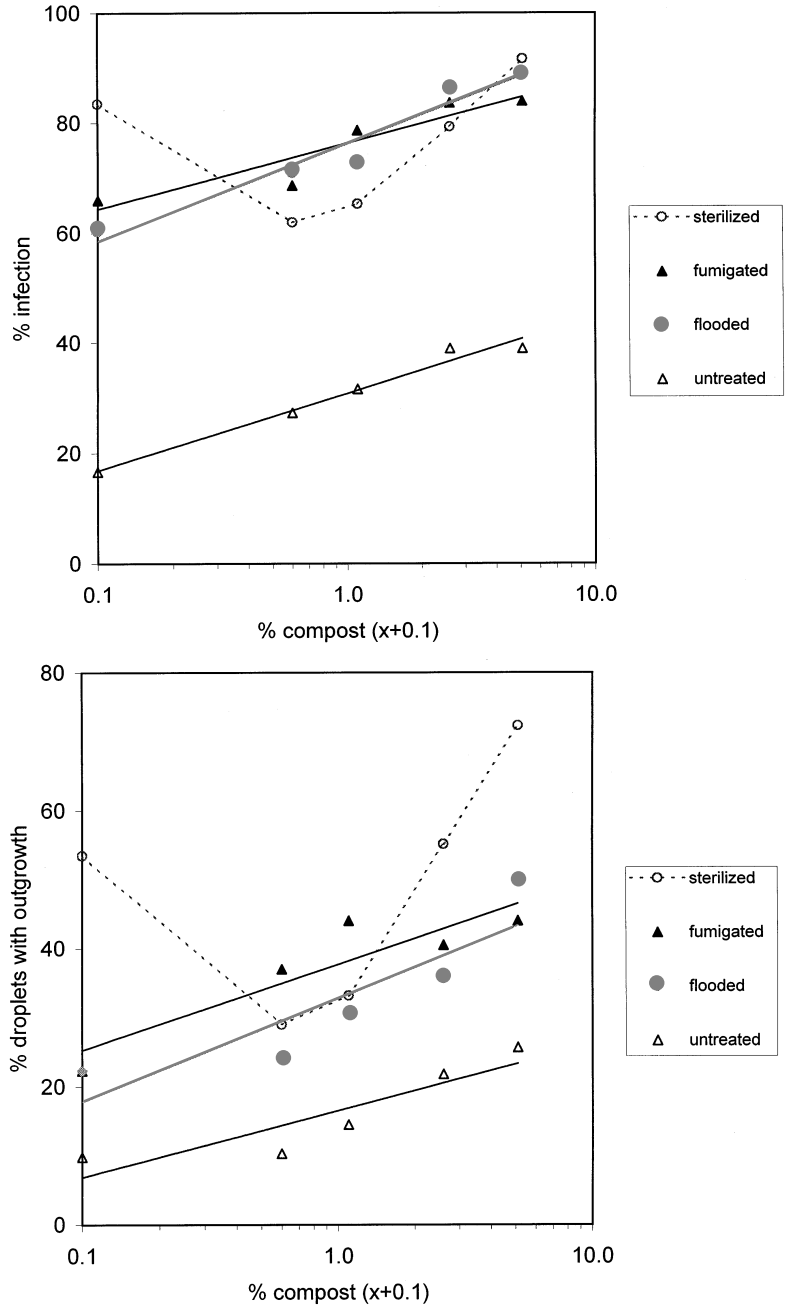

Figure 2 Effect of compost amendment on infection in *Crocus ancyrensis* 'Golden Bunch' (A) and *Pythium* population density (B) with observed means (symbols, number of replicates=3) and GLM-predicted curves (lines) in fumigated, flooded and untreated soil, and observed means (symbols with dotted line) in sterilized soil after compost amendment (% compost + 0.1 on a log-scale) one week prior to infestation with *Pythium macrosporum* and planting.

Table 1 Percentage infection in *Iris xyphium* 'White van Vliet' after compost amendment (no compost, compost matured at 10°C or 20°C, and sterilized compost) followed one week later by infestation with *Pythium macrosporum* in untreated, fumigated and sterilized soil (means of two inoculum densities and two incubation temperatures during plant growth).

compost amendment	soil treatment		
	untreated	fumigated	sterilized
none	42 ab[1]	70 e	87 f
10°C	36 a	50 bc	56 cd
20°C	43 ab	57 cd	62 de
sterilized	n.t. [2]	59 cd	82 f

[1] Treatment means followed by the same letter do not differ significantly (*P*=0.05, Student's two-tailed *t*-test, number of replicates=20).

[2] n.t. = not tested.

Table 2 Effect of compost amendment on disease suppression (field experiment). Relative yield of *Crocus vernus* 'Flower Record' in soil amended or not with 12 t ha^{-1} VFG-compost one week prior to infestation or not with *Pythium* and planting; and disease severity (% infection) in crocus in infested treatments.

compost amendment	Relative yield		% infection
	- *Pythium*	+ *Pythium*	
-	[1]100 a[2]	84 b	18 a
+	101 a	81 c	26 b

[1] Bulb yield of this treatment is standardized to 100%.

[2] Treatment means followed by the same letter do not differ significantly (*P*=0.05, Student's two-tailed *t*-test; number of replicates –*Pythium*=24, +*Pythium*=48).

Considering the contrasting results of compost amendment on disease development in iris and crocus, the effect of temperature during plant growth (18°C and 9°C respectively) was investigated in a separate pot experiment with iris. Also, two compost maturation temperatures (20°C and 10°C) were included in the experiment, because a sudden change in temperature at time of compost application to the soil might also influence the effect on activity and development of the micro-population and disease development. Main effects were found for temperature during

plant growth and for inoculum density. No interaction was found between these factors or with other treatments. Mean infection at 18°C was 61% compared to 51% at 9°C (P = 0.011). Mean infection with 0.1% inoculum was 68% compared to 43% with 0.01% inoculum (P< 0.01). In fumigated and sterilized soil, disease severity was significantly reduced by compost amendment (Table 1). No significant difference in reduction of disease development was found between compost matured at 10°C or 20°C. Amendment of sterilized soil with sterilized compost did not reduce disease severity. Whereas in fumigated soil, amendment with sterilized compost reduced disease severity to the same extent as non-sterilized compost (Table 1). In untreated soil, no effect of compost amendment occurred nor of time of infestation. Infestation one week prior to compost amendment resulted in 34% infection versus 43% infection when infested one week after compost amendment.

Field experiment

The effect of compost amendment in untreated and fumigated soil on infection and corm yield was investigated in a field experiment with crocus. In non-infested treatments, no significant effects occurred as a result of fumigation or compost amendment. Infestation resulted in a considerable infection and reduction of corm yield caused by *Pythium*. No interactions occurred between fumigation treatments and compost amendment. Effects of fumigation treatments were described in Van Os *et al*. (1999). In soil, fumigated one year or six weeks prior to infestation and planting, enhanced root infection and yield reduction occurred compared to the non-fumigated soil. Compost amendment significantly increased disease severity and decreased corm yield in infested treatments (Table 2). Compost amendment had no significant effect on soil EC two weeks after planting. Mean EC-values were 0.53 and 0.47 mS cm^{-1} in amended and non-amended soil respectively.

Discussion

Management practices are known to affect plant growth and disease development in agricultural soils (Kennedy & Smith, 1995; Van Bruggen, 1995). In our experiments, soil fumigation, flooding and sterilization reduced disease suppression against *Pythium macrosporum* in iris and crocus compared to suppression in untreated soil. These results are consistent with results from previous experiments (Van Os *et al.*, 1999) in which it was demonstrated that the disease suppression was of biological nature. In both iris and crocus experiments, *Pythium* population densities after crop cultivation correlated with observed infection levels, showing similar tendencies and significant effects as a result of soil treatments.

In pot experiments with iris, amendment of fumigated, flooded and sterilized soil with matured VFG-compost resulted in (partial) restoration of the disease suppression, concurring with several other reports on effects of compost amendment

(Lumsden *et al.*, 1983; Chen *et al.*, 1988a; Mandelbaum *et al.*, 1988). Amendment of sterilized soil with sterilized compost did not reduce disease development, indicating the essential role of the compost microflora in the disease suppression. Here, activity of antagonistic microorganisms which naturally recolonize compost during the maturation phase induces disease suppression (Craft & Nelson, 1996; Kuter *et al*., 1988; Hadar & Mandelbaum, 1992). Competition for available nutrients has been proposed as the principal mechanism (Chen *et al*., 1988a; Elad & Chet, 1987). Other proposed mechanisms include hyperparasitism (Hadar *et al*., 1983; Sivan *et al*., 1984), iron competition (Becker & Cook, 1988), production of soluble (Lockwood, 1977) and volatile (Howell *et al*., 1988) inhibitors or hydrolytic enzymes (Roberts & Lumsden, 1988) by the soil microflora, and induced plant resistance (Lynch & Crook 1992; Costa *et al.,* 1996). However, amendment of fumigated soil with sterilized compost prior to *Pythium* infestation resulted in equivalent disease development compared to amendment with non-sterilized compost, indicating that other factors than the compost microflora could also induce disease suppression. Aside from microorganisms, different elements, such as organic matter and nutrients, are introduced in the system by compost amendment. The latter may have stimulated the resident soil microflora in the fumigated soil (Van Os & Van Ginkel, 2001, Chapter 5) and thereby enhanced disease suppression. After amendment with matured compost, disease suppression may be induced by either the compost microflora, stimulation of the residential soil microflora, or a combination of these mechanisms (Pascual *et al*., 2002). An important feature of compost in any culture is its effect on pathogens already present in the soil. Available nutrients in the composts would not only serve as a food base for biological control agents but also readily support the pathogen, leading to increased disease (Hoitink & Boehm, 1999). This was not the case in our pot experiment with iris, where infestation before or after amendment of untreated soil with compost had no effect on disease development, indicating that the matured compost was fully colonized by microorganisms and nutrient availability was low.

In none of the compost-amended soils did disease suppression exceed suppression in untreated soil without compost. Apparently, addition of 5% compost could not substantially enlarge the capacity of the soil to support higher microbial numbers and activity inducing disease suppression, than the natural microflora already present in untreated soil, as suggested by Van Os and Van Ginkel (2001).

In the field, iris is cultivated from October until August of the following year, with varying temperatures (0.5°C-18°C) during the infection period from January until July (Van Os *et al.*, 1998). At planting, soil temperature is approximately 10°C and falling. Temperature is an important factor in soil biological activity (Killham, 1994) and the development of *Pythium* diseases (Hancock, 1977). Ben-Yephet and Nelson (1999) and You and Sivasipamparam (1994) reported that suppression of *Pythium* spp.

induced by composts was temperature-dependant. Drops in temperature may reduce total microbial activity in soil resulting in decreased disease suppression (Chen *et al.*,1988b). We performed a pot experiment with iris at 9°C and 18°C and amendment with compost matured at 10°C or 20°C. No differences in disease suppression were found between the composts matured at different temperatures, and no interaction occurred between compost maturation temperature and iris cultivation temperature. Thus, a temperature drop at time of compost application to the soil or low temperatures during crop cultivation do not necessarily reduce the relative effect of compost. Several field experiments with iris, including amendment of flooded and untreated soil with compost, were planted as well. Unfortunately, these experiments yielded no results because of severe infection by *Rhizoctonia tuliparum*.

In contrast to iris, compost amendment enhanced infection by *Pythium* in crocus in both pot and field experiments. Only amendment of sterilized soil with 0.5% and 1% compost (pot experiments) appeared to reduce disease severity compared to non-amended sterilized soil, whereas amendment with 2.5% and 5% compost appeared to enhance disease development. At low compost concentrations (0.5% and 1%) the beneficial effect of the compost microflora on disease suppression seemed to overrule the detrimental effects occurring at higher compost concentrations in sterilized soil. Undesirable effects of organic amendments, such as increases in disease incidence, have previously been reported. For instance, composted municipal sludge has been shown to increase incidence of *Fusarium* root rot of pea, *Pythium* damping-off of pea, *Phytophthora* root rot of pepper, and *Rhizoctonia* root rot of bean (Lumsden *et al*., 1983). Among the factors that affect populations of antagonists and disease control are the substrate, the environmental conditions, and the amendment itself. Organic materials vary in their chemical nature, in age and maturity. During the high temperature phase of composting, beneficial as well as detrimental microorganisms are killed. The maturation phase of the composting process after peak heating is essential for the recolonization by mesophiles, including biological control agents, and the development of natural disease suppression (Erhart *et al.*, 1999; Hoitink *et al*., 1996). The amount of the material added to soil, its placement and distribution in the soil profile, and time between incorporation and planting are also important (Windels, 1997). Furthermore, not all *Pythium* species are equally suppressed by compost (Ben-Yephet & Nelson, 1999). However, in our pot experiments, these variables were all identical for both crops. To our knowledge, this is the first report of compost amendment reducing infection in one crop and enhancing infection in another, using the same compost treatments, *Pythium* isolate, soil, and growing conditions. This indicates that the host plant is the discriminating factor between disease stimulation or suppression as a result of compost amendment.

Mechanisms in disease control that specifically involve the host plant include induced resistance. Using a split root system, Zhang *et al.* (1996) reported that composted pine bark and composted cow manure induced systemic resistance to *P. ultimum* in cucumber. Pharand *et al.* (2002) suggested that plant induced resistance is a key component of the increased protection conferred by suppressive systems such as composts. However, even if induced resistance is one of the mechanisms involved in disease suppression resulting from application of compost in iris, absence of induced resistance can not account for stimulation of infection in crocus. Differential sensitivity to relatively high salt contents of the VFG-compost might have damaged crocus roots and not iris roots, although no abnormalities in root development occurred and compost amendment had no effect on soil EC nor on crocus yield (non-infested treatments) in the field experiments. Differences in root development may also influence the effect of compost amendment. Iris and crocus differ conspicuously in their root development (Van Os *et al.*, 1998). Presumably, differences also exist in root exudation and rhizosphere microflora, both important factors in disease development. Hypothetically, microbial interactions in the rhizosphere may be favourably (iris) or unfavourably (crocus) affected by compost amendment. So far, the mechanism responsible for the differential plant responses remain unclear. Consequently, the effect of organic amendments on specific diseases and plant species needs to be evaluated on an individual basis under standard cultivation conditions.

Composts are gaining increased popularity not only on an ecological point of view by providing a means to recycle organic wastes, but also because their potential in suppressing soilborne plant diseases clearly meets with the current needs toward sustainable agriculture at a lower environmental cost. Because multiple mechanisms may be involved, effects of organic amendments on soil microflora and disease incidence may be longer-lasting than with other types of disease control strategies (Kokalis-Burelle & Rodriguez-Kabana,1994). Reports of long lasting effects of soil-incorporated composts on suppression of *Pythium* diseases under field conditions vary from twenty four months (Lumsden *et al*., 1983; Pascual *et al*., 2000) to four years (Lewis *et al*., 1992). Our experiments revealed contrasting effects of VFG-compost on *Pythium* infection in iris and crocus. Further testing of other compost-pathogen-crop combinations in bio-assays or controlled field experiments may yield more information on individual combinations. However, these effects may interact or be overruled by other aspects within commercial farming systems. In order to evaluate effects of compost amendment under these circumstances interdisciplinary, system-level research is needed rather than disciplinary research. Currently, the effect of repeated compost application on plant growth and disease development in general is being tested during four years in commercial bulb fields.

Acknowledgements

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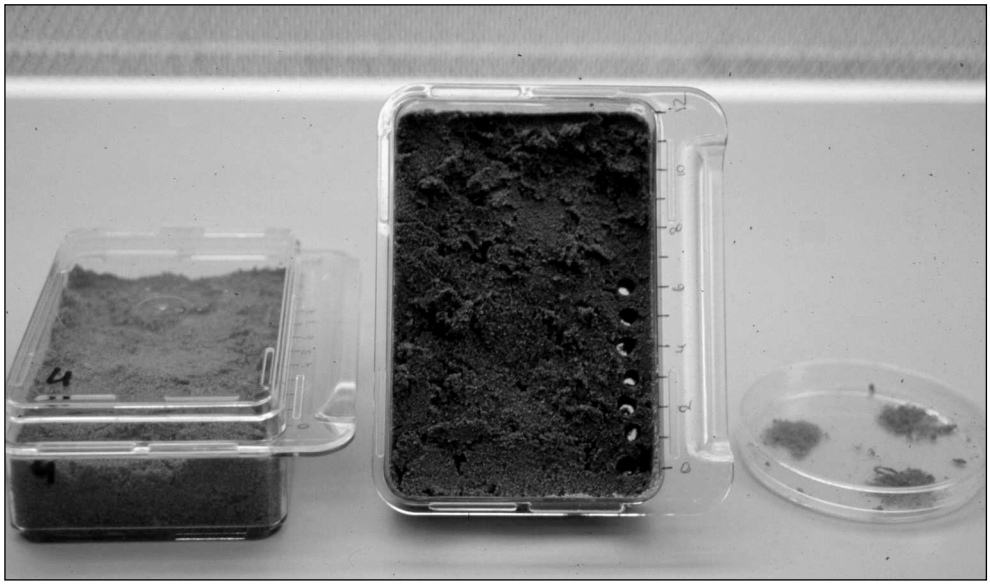
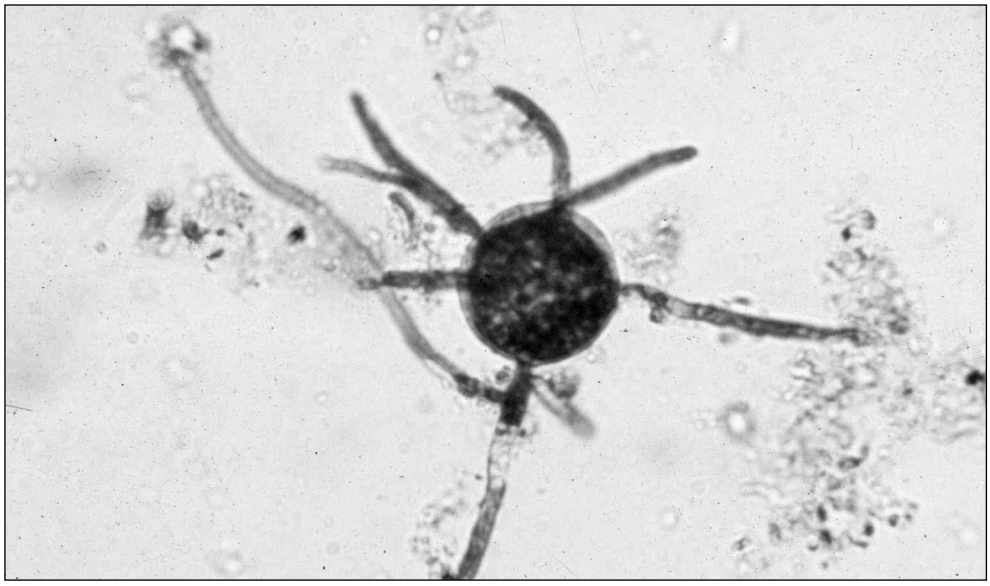
CHAPTER 5

Suppression of *Pythium* root rot in bulbous iris in relation to biomass and activity of the soil microflora





Photographs Experimental set-up to determine growth of *Pythium* through soil (1), and mycelial growth from a hypal swelling in soil (2).

CHAPTER 5 Suppression of *Pythium* root rot in bulbous iris in relation to biomass and activity of the soil microflora

G.J. van Os and J.H. van Ginkel

Abstract

Disease suppression against *Pythium* root rot can be influenced by management practices applied in ornamental bulb culture. Different levels of suppression were established in sandy soil after several soil treatments. Percentage of infection in iris caused by *Pythium macrosporum* was lowest in untreated soil and progressively increased in sterilized soil amended with 1% compost, fumigated soil (methylisothiocyanate) and flooded soil (fumigation and flooding corresponding to the same level of disease severity) and was highest in sterilized soil. The relationship between the level of disease incidence, *Pythium* growth rate through soil, and various microbial parameters was investigated. Soil microbial biomass and, after amendment of glucose as a non-selective carbon source, dehydrogenase activity, glucose uptake and respiration were determined. By using ^{14}C-labelled glucose, measurement of uptake and assimilation of amended carbon could be distuingished from soil organic matter decomposition. All microbial parameters were negatively associated with *Pythium* growth rates, indicating that high microbial biomass and activity induced suppression of *Pythium* growth through soil. However, with neither of the quantitative microbial parameters could changes in disease incidence be fully explained. It is hypothesized that competition for carbon may not be the main mechanism in disease suppression.

Introduction

Management practices are known to influence biological processes in agricultural soils (Kennedy & Smith, 1995; Van Bruggen, 1995). Among other processes, disease suppression has been demonstrated to be positively and negatively affected by several management processes. Suppressiveness is believed to be a complex phenomenon in which components of indigenous soil microflora naturally regulate the growth of pathogens (Rouxel, 1991; Kokalis-Bruelle & Rodríguez-Kabána, 1994). For instance, propagules of plant pathogenic *Pythium* species are suppressed through a mechanism known as 'general suppression' (Cook & Baker, 1983). Seed and root exudates are the principle sources of organic nutrients required for germination and growth of resting structures of *Pythium* spp. (Stanghellini & Burr, 1973; Nelson & Craft, 1989). High microbial biomass and activity reduce the amplitude and duration of the signal pulse provided by roots and seeds in

soil by depletion of that energy source (Chen *et al.*, 1988a; Mandelbaum & Hadar, 1990; Hoitink *et al.,* 1996). The importance of competition among coexisting microbial populations in limiting disease development, however, is difficult to quantify. It was demonstrated that the natural disease suppression in soil against *Pythium* root rot in bulb crops was reduced by fumigation and flooding (Van Os *et al*., 1999, Chapter 3). In the Netherlands, these management practices are applied in ornamental bulb culture on sandy soil to control diseases and weeds.

Induction of disease suppression against *Pythium* diseases has also been reported. Composts become consistently suppressive to *Pythium* disease, once naturally recolonized by mesophilic microorganisms after peak heating (Kuter *et al.,* 1988; Chen *et al.,* 1988a; Mandelbaum & Hadar, 1990). Media amended with composts prepared from different organic wastes, such as tree barks, municipal sewage sludge, and separated cattle manure showed enhanced suppression of *Pythium* spp. (Lumsden *et al.*, 1983; Chen *et al.*, 1988b; Mandelbaum *et al.,* 1988).

The objectives of this research were to evaluate effects on the suppressiveness against *Pythium* of soil fumigation, flooding, sterilization of sandy soil, and amendment of compost to sterilized soil. Parameters determined here were microbial biomass, nutrient competition (glucose uptake), respiration and dehydrogenase activity, in relation to suppression of *Pythium* root rot in iris and hyphal growth through soil.

Materials and methods

Soil treatments

Soil was collected from the experimental fields of the Bulb Research Centre in Lisse, the Netherlands. This sandy soil, with a low content of organic matter (1%) and pH 7, was heat-treated (2 h at 70°C) to eliminate pathogens and left to be recolonized by micro-organisms in open air during six months prior to further use. Soil moisture content was adjusted to 20% (w/w) and treated according to Van Os *et al*. (1999) by: sterilizing (autoclaving 2 x 90 min. at 121°C with 48 h in between), flooding during eight weeks at 18°C (after drainage soil moisture was readjusted to 20% w/w), fumigating with methylisothiocyanate (MIT, 0.13 ml l^{-1} soil, as Trapex 40% a.i., Schering, the Netherlands) soil with MIT was incubated during five weeks at 20°C in sealed plastic bags and, subsequently, allowed to evaporate for one week, or left untreated. Experimental details of the flooding and fumigation treatments are described in Van Os *et al.* (1999). Sterilized soil was supplemented or not with 1% (w/v) composted organic household waste (vegetable, fruit, and garden waste, VFG-compost), obtained from a commercial composting facility in Middenmeer, the Netherlands, which was composted and matured (five weeks) as described by Tuitert *et al.* (1998). Amended soil was stored for one week at 20°C before use.

Separate experiments were performed for assessement of root rot in a bioassay, for assessment of mycelial growth and dehydrogenase activity, and for measurement of microbial biomass, glucose-uptake and respiration.

Bioassay for disease assessment

Soil (5 l per soil treatment) was artificially infested with a three-week-old sand-oatmeal culture of *Pythium macrosporum* Vaartaja and Van der Plaats-Niterink sp. nov. (isolate P60). Non-infested, untreated soil and non-infested, sterilized soil supplemented with 1% VFG-compost were used as control treatments. For each treatment, five plastic pots (1 l) were filled with soil and five bulbs of *Iris xyphium* cv. White van Vliet (size 8 cm circumference) were planted in each pot. Bulbs were disinfected by submerging in a solution of 0.4% formaldehyde during 15 min. prior to usage. Pots were placed in randomized blocks in a climate chamber at 18°C during eight weeks, and plants were grown according to standard practice for flower production. For disease severity rating, roots were washed with tap water and root rot ratings of infested treatments were related to the healthy root systems of the non-infested control treatments. Roots were visually scored for root rot severity using an arbitrary disease index ranging from 0-5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection (Van Os *et al.*, 1998, Chapter 2). Roots were assessed for each plant individually and a mean root rot index per pot was calculated.

All soil treatments were tested as described in three experiments, which were analysed individually. Statistical analysis was performed using Genstat 5 (Genstat 5 Committee, 1993). Ratings were converted to percentages and regression was conducted on transformed data, using the logit link, in a generalized linear model (GLM) to assess the effect of soil treatment. Two-tailed Student's *t*-tests were used to determine all pair-wise differences of means at significance level $P < 0.05$.

In vitro assay of mycelial growth through soil

Growth of *P. macrosporum* through soil was determined in an in vitro assay. Soil was supplemented with sterilized oatmeal (2 g l^{-1} soil). Sterile, plastic containers of 13.5 cm by 9.0 cm by 3.5 cm (L by W by H) were filled with 350 ml soil, three containers for each of three replicates per soil treatment (five soil treatments x three replicates x three containers = 45 containers in total). A strip (9.0 x 0.5 cm) of 2% cornmeal agar (Oxoid, CMA), colonized by *P. macrosporum* was placed at one side of the container and covered with soil. Containers were closed with a lid and incubated at 24°C in the dark. During the experiment, soil moisture was maintained at 20% based on weight measurements. Hyphal growth was assessed by sequential sampling of soil after 3, 5, 7, and 10 days. Soil samples (1 ml) were taken with

sterilized, plastic straws at regular distances from the inoculum (1 cm apart). Samples were placed on a selective medium of CMA with 25 µl ml^{-1} pimaricin (Merck) and 1 ml ml^{-1} terramycine (oxytetracycline-HCl, Pfizer) and *Pythium* outgrowth was assessed after 48 h incubation at room temperature. Fungal growth through soil (cm day^{-1}) was estimated using regression analysis. Means of three containers per replicate treatment were calculated and statistically analysed using analysis of variance (ANOVA).

Measurements on the soil microflora

Dehydrogenase activity

Microbial activity was estimated by measuring the hydrolysis of 2,3,5-triphenyltetrazolium chloride (TTC) to 2,3,5-triphenyltetrazolium formazan (TTF) by dehydrogenase enzymes. In this experiment, a procedure slightly modified from Smith and Pugh (1979) was used. Soil samples (5 g) were saturated with 2 ml of a 1% solution of TTC in 0.1 M Tris buffer (pH 7.6) and 1 ml of 0.1% glucose. Six samples were taken from each of three replicates per soil treatment (five soil treatments x three replicates x six samples = 80 samples) and six samples of untreated soil without TTC were used as control. Samples were thoroughly mixed with a vortex shaker and incubated in sealed tubes at 30°C for 18 h. After incubation, 9 ml of methanol was added to each tube, the contents stirred and filtered through a S&S folded paper filter (type 595½, Schleicher & Schuell Inc., Dassel, Germany), resulting in a final volume of 12 ml filtrate per sample. The intensity of red colour in the filtrate, produced by the reduction of TTC to TTF, was determined spectrophotometrically at 485 nm. The extinction, adjusted for the control, was used as measure for dehydrogenase activity. Means of six samples per replicate were calculated. After log-transformation these data were analyzed using ANOVA to determine effect of soil treatments.

Biomass

The soil microbial biomass was determined using the fumigation-centrifugation (FC) technique: soil solutions were obtained by centrifugation of non-fumigated and chloroform fumigated soil (35 g soil, three replicates per soil treatment, 20 h fumigation), and subsequently soil solutions of the various samples were analysed for carbon content (Van Ginkel *et al.*, 1994). Carbon analyses were performed using persulfate-UV digestion (Schreurs, 1978) incorporated in an autoanalyser system (Skalar analytical mehtods 311-411, Breda, the Netherlands). The proportionality factor K_{cc}, relating the flush obtained by FC with the microbial biomass of soil can be determined by using *in situ* labelling of the microbial biomass with D(U-^{14}C)glucose, assuming that after three days the ^{14}C-label is totally incorporated into the microbial biomass (Van Veen *et al.*, 1985). In sandy soil, the proportionality factor (K_{cc}) so

obtained for the FC-method was 0.168 (Van Ginkel *et al.*, 1994). Data on microbial biomass were log-transformed before analyses using ANOVA.

Glucose-uptake and respiration

Soil samples of 70 g, three replicates per soil treatment, were amended with D[U-^{14}C]glucose (Amersham U.K., CFB 98) at a rate of 100 Bq ^{14}C and 500 µg C g^{-1} dry soil, and with NH_4Cl at 50 µg N g^{-1} dry soil. Subsequently, samples were incubated individually in 1 l glass jars containing vials with 5 ml 1 M NaOH to absorb the CO_2 evolved during 12 h at 20°C. After 6 h the NaOH solution was replaced. ^{14}C-glucose-uptake and evolved $^{14}CO_2$ were measured as per cent of total ^{14}C as described by Van Veen *et al.* (1985) using FC. In order to assess the effect of soil treatment, regression (GLM) was performed using a logit-link.

Statistical summary of data

Principal component analysis (PCA) was used to examine the interrelationships among variables. The method finds linear combinations of a set of variates that maximize the variation contained within them and reduces a multi-dimensional space to a space of fewer dimensions, preferably one or two. The scores derived this way are represented in a two-dimensional plot, called a biplot. Before analysis, scores for soil treatment were centered and standardized.

Results

In the bioassay, no infection and no damage or abnormalities in root development were observed in non-infested control treatments with or without VFG-compost. It was assumed that iris roots were not stressed by supplemented VFG-compost.

Soil treatments lead to four different levels of disease severity. Percentage of infection was lowest in untreated soil and progressively increased in sterilized soil amended with 1% compost, fumigated and flooded soil (fumigation and flooding corresponding to the same level of disease severity) and was highest in sterilized soil (Figure 1A). Thus, soil fumigation, flooding and sterilization resulted in reduction of disease suppression compared to untreated soil, and amendment of 1% VFG-compost resulted in partial restoration of disease suppression compared to unamended sterilized soil. Addition of 1% sterilized compost to sterilized soil had no effect on disease incidence (data not shown). All soil treatments were tested in several experiments with similar results. Data of one experiment are presented in Figure 1A and used for further analyses.

Soil treatments also lead to four different levels of *Pythium* growth rate through soil (Figure 1B). Again no significant difference was observed between fumigated and flooded soil. Lowest values for *Pythium* growth rate occurred in the compost-

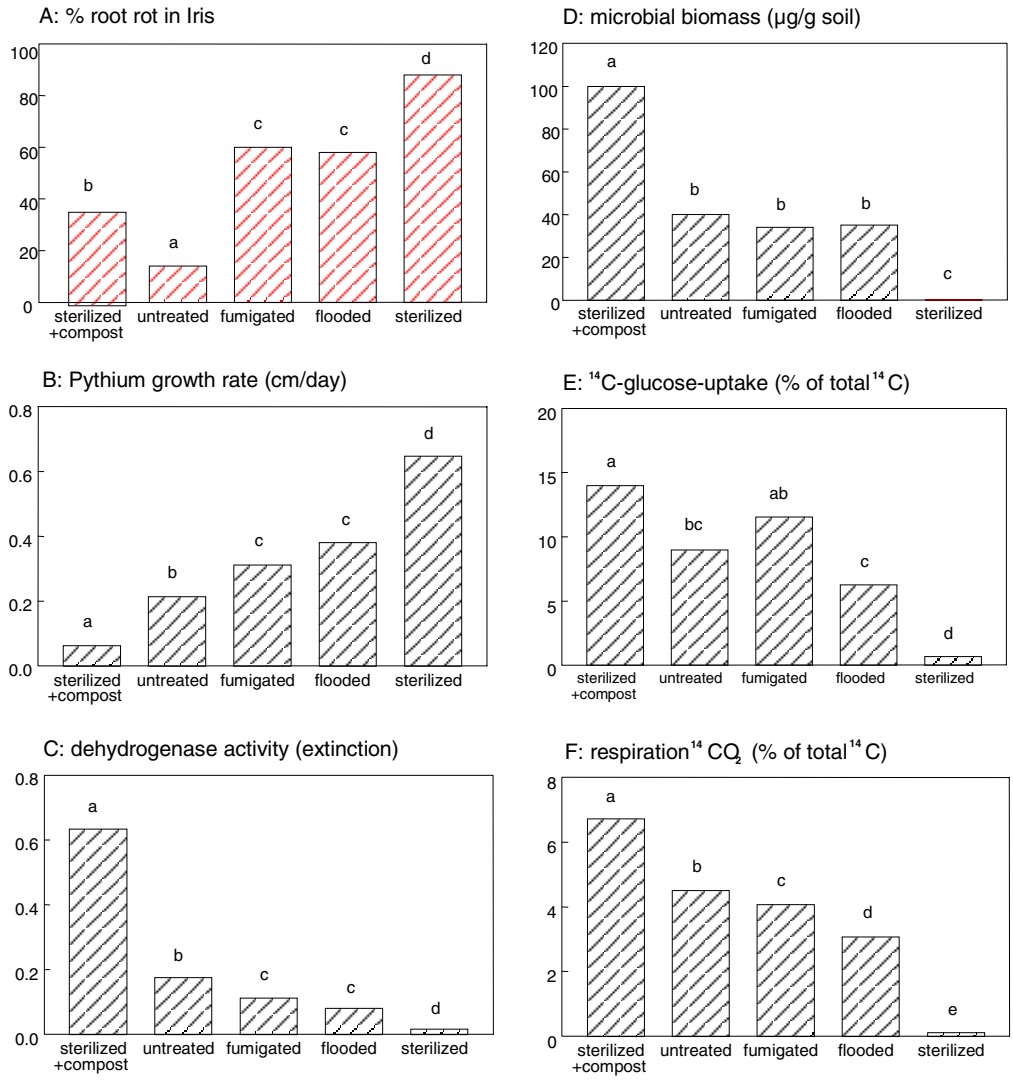


Figure 1 Effects of soil treatments on root rot infection by *Pythium macrosporum* in *Iris xyphium* (A, infested treatments), growth rate of P. macrosporum through soil (B), dehydrogenase activity (C), microbial biomass (D), glucose-uptake (E), and respiration (F); experimental data represent means of five replicates for infection and three replicates for all other parameters. Bars labeled with corresponding letter(s) do not differ significantly ($P < 0.05$, Student's two-tailed *t*-test).

amended soil and growth rates progressively increased in untreated soil, in fumigated and flooded soil, and in sterilized soil. Measurements of the microbial parameters, dehydrogenase activity, biomass, glucose-uptake and respiration, revealed highest values for the compost-amended soil and lowest values in sterilized soil without compost, with intermediate values for untreated, fumigated and flooded (Figure 1C-F). In sterilized soil, low levels of microbial activity were detected due to the fact that incubation and measurement procedures were not performed under sterile conditions. Soil fumigation and flooding induced significant reduction of dehydrogenase activity and $^{14}CO_2$-respiration compared to untreated soil, whereas microbial biomass and glucose-uptake were not significantly influenced by fumigation or flooding compared to untreated soil.

Data of all variables and soil treatments are summarized in a biplot (Figure 2A). The direction of arrows indicate direction of steepest ascent, i.e. the direction in which the score of the corresponding variable increases most. In the perpendicular direction, the fitted scores are constant. Fitted values of a variable can be read by projecting each soil treatment onto the axis of the variable. Values of soil treatments far from the origin, i.e. sterilized soil with and without compost amendment, are better represented in the biplot than values of treatments near the origin, i.e. fumigation and flooding. The angle between arrows of each pair of variables provides an approximation of their pair-wise correlation. All microbial variables were positively correlated with each other, and negatively correlated with *Pythium* growth rate. *Pythium* growth rate was positively correlated with disease severity but explained merely 53% of the variance in root infection.

Increased disease development as a result of soil fumigation and flooding corresponded with significant reduction in dehydrogenase activity and soil respiration compared to untreated soil (Figure 1). No significant effects of these treatments were observed on microbial biomass or glucose uptake. This seems in contrast with the results of the PCA-analysis (Figure 2A), which revealed no correlation of dehydrogenase activity with disease severity, and highest correlation of glucose uptake with disease incidence. This is due to the fact that microbial biomass and dehydrogenase activity were extremely high in compost-amended soil compared to the other soil treatments, and extreme values have a major influence on correlations in PCA analysis. As a result, correlations between variates in the other soil treatments may have been overshadowed (Figure 2A). Considering the above, it was decided to run PCA-analysis on our data without the compost treatment. The resulting biplot (Figure 2B) showed quite different correlations. Glucose uptake, which correlated best with disease severity (approximately 84%) when the compost-amended soil was included, showed no correlation at all when compost-amended soil was excluded from the analysis. Dehydrogenase activity, on the other hand, changed from no correlation with disease severity (approximately 17%) after analysis of the complete

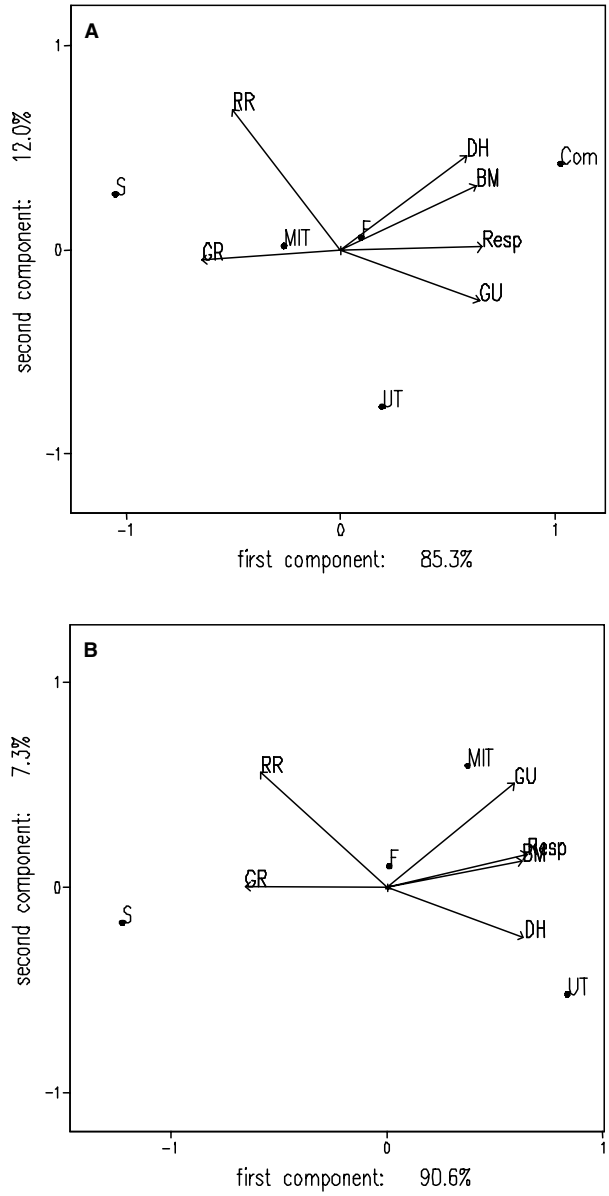


Figure 2 Covariance biplot of two-dimensional PCA ordination, including (A) and excluding (B) the compost treatment, with centralized and standardized means of variables represented by arrows (RR = root rot infection by *Pythium macrosporum* in *Iris xyphium*, GR = growth rate of *P. macrosporum* through soil, DH = dehydrogenase activity, BM = microbial biomass, GU = glucose-uptake, Resp = respiration) and with soil treatments represented by dots (S = sterilized, F = flooded, MIT = fumigated with methylisothiocyanate, UT = untreated, COM = sterilized soil supplemented with 1% VFG-compost).

data set, to approximately 93% correlation in the analysis without the compost treatment. Comparing both biplots, it is evident that the value of microbial parameters as predictors for disease suppression, is strongly influenced by the data set used for statistical analysis.

Discussion

Soil fumigation, flooding and sterilization reduced disease suppression against *Pythium macrosporum* in iris compared to suppression in untreated soil. These results are consistent with results from previous experiments (Van Os *et al.*, 1999) in which it was demonstrated that the disease suppression was of biological nature. Addition of 1% matured VFG-compost resulted in enhanced disease suppression in sterilized soil, concurring with several other reports on effects of compost amendment (Lumsden *et al.*, 1983; Chen *et al.*, 1988a; Mandelbaum *et al.*, 1988), whereas amendment of sterilized compost had no effect on the disease suppression. Thus, by these soil treatments, different levels of disease suppression were established and the relation with several microbial parameters could be investigated.

Since propagules of *Pythium* spp. depend on exogenous nutrients for germination and ultimately for successful host infection, and root exudates are the principal sources of organic carbon in nutrient impoverished soils, it has been postulated that rapid deprivation of exudates by soil microbes results in disease suppression (Chen *et al.*, 1988a; Mandelbaum & Hadar, 1990; Hoitink *et al.*, 1996). Correlations between microbial biomass, activity and suppression of *Pythium* spp. have been reported (Chen *et al.*, 1988b; Inbar *et al.*, 1991) and preliminary predictive guidelines were proposed by Chen *et al.* (1988a), based on both microbial activity and biomass, for potting mixes (containing up to 50% compost) suppressive to *Pythium* damping-off of cucumber. Coexistence of large populations of microorganisms, high microbial activity, low concentrations of available nutrients, and high degree of microbiostasis characterized container, i.e. compost, media suppressive to *Pythium* damping-off. This approach, however, does not provide information on residual effects or keeping the quality of this phenomenon. The potential for biodegradable carbon in a substrate to support an active and effective microbial biomass determines species composition and activity and, in turn, the potential for sustained biological control (Hoitink *et al.*, 1996; Hoitink *et al.*, 1997).

Alabouvette *et al.* (1985a) positively correlated initial respiratory response and suppression of *Fusarium oxysporum* Schlecht. in soil and indicated that not only microbial biomass, but also rapid consumption of amended glucose are characteristic of suppressive soils. Microbial activity developed more rapidly and more intensely in suppressive soils than in conducive soils. Similar phenomena were reported by Mandelbaum & Hadar (1990) concerning suppression of damping-off of cucumber seedlings caused by *P. aphanidermatum*. In the above mentioned reports involving

measurements of microbial activity after glucose amendments, no distinction is made between the assimilation of soil-native organic matter and amended glucose. Apart from being indirect evidence for nutrient competition, side effects as a result of glucose amendment cannot be precluded. Van Ginkel *et al.* (2000) stated that the energy-rich glucose may have a conserving effect on native-soil organic carbon decomposition, since micro-organisms may prefer the more energy-rich carbon to native-soil organic carbon. This preferential substrate use may lead to underestimation of the glucose consumption, because measured respiration activity is the result of both glucose and organic matter decompostition. On the other hand, energy-rich carbon incubated in a rather nutrient-poor soil may have a stimulating effect on soil organic carbon decomposition to satisfy the nutrient demand of the microflora, resulting in extra CO_2 release from the native organic matter. These side effects may especially be relevant when substrates or treatments with different organic matter contents, i.e. with compost-amendments, are compared. Alabouvette *et al*. (1985b) used addition of ^{14}C-labelled glucose and measurered $^{14}CO_2$ as a product of respiration to investigate nutrient competition in soils suppressive and conducive to *Fusarium* spp. By using ^{14}C-glucose, they specifically determined respiration of amended carbon. The same was done in our experiments, and, moreover, measurements were included on ^{14}C in the microbial biomass. $^{14}CO_2$-production does not necessarily correspond with ^{14}C-glucose-uptake. Part of the absorbed ^{14}C-glucose may not evolve as $^{14}CO_2$, but may accumulate in the microbial biomass or be assimilated by other anabolic pathways. $^{14}CO_2$-production and ^{14}C in the microbial biomass in total renders actual uptake of amended ^{14}C-glucose, supplying more direct evidence for the role of competition for readily available carbon as one of the mechanisms in disease suppression against *Pythium*.

Measurements of microbial parameters, such as dehydrogenase activity and respiration, in soil amended with an excess of a non-selective carbon source, reflect a potential rather than actual activity (Frankland *et al.*, 1990; Stoutmann Jensen & Sorensen, 1994). In our experiments, by nutrient amendment, rhizosphere circumstances were simulated, i.e. with readily available nutrients provided by root exudates, thus, ‘potential’ activities were measured. Sterilized soil supplemented with compost showed relatively high values for all microbial parameters and minimal *Pythium* growth rate, indicating high microbial density, activity and nutrient competition. However, disease suppression in this soil was significantly less than in untreated soil. A plausible explanation for this phenomenon is that during the bioassay, disease suppression in compost-amended soil (measured nine weeks after amendment) may be lower than the microbial data (measured one week after amendment) suggested. Microbial biomass and dehydrogenase activity may decline in course of time because the nutrient-poor sandy soil may not be able to support such high microbial numbers and activity. Periodical monitoring of these parameters

after compost amendment is necessary to elucidate this issue. Furthermore, Craft and Nelson (1996) and Erhart *et al.* (1999) reported that microbial properties of *Pythium*-suppressive compost may differ substantially, and measurements of microbial populations and activity may not be predictive of the level of disease suppression in all composts.

Microbial parameters were better correlated with *Pythium* growth than with disease severity. In both PCA-analyses, all microbial variates showed a persistent, negative correlation with *Pythium* growth rate, indicating that high microbial biomass and activity induced suppression of *Pythium* growth through soil. In pathosystems concerning pre- and post-emergance damping-off of seedlings, interaction may be strongly dependent on *Pythium* growth through soil in order to reach the host. In that case, high correlation of microbial biomass and activity with disease incidence (Chen *et al*., 1988a, 1988b; Mandelbaum & Hadar, 1990; Inbar *et al*., 1991) seems consistent. Considering root infection in iris, host and pathogen may also encounter by chance as the roots grow through the soil. In this case, root density may be an important factor, and *Pythium* growth may be less decisive for infection. *Pythium* growth rates explained approximately 53-75% (Figure 2A and 2B respectively) of the variance in root infection. It is concluded that other aspects of pathogen development also influence infection, e.g. interactions on the root surface. With neither combination of the quantitative microbial parameters evaluated in this report, variance in disease suppression could be fully explained or predicted.

Mechanisms for disease suppression are derived from correlations with microbial parameters. Chen *et al.* (1988a) found that suppression of *P. ultimum* was related to reduced germination of sporangia and reduced growth of *P. ultimum* and it occurred without a reduction in inoculum density. They proposed that antibiosis or exploitation were not the principal mechanisms responsible for suppression, but that microorganisms in the suppressive medium were actively taking up nutrients creating a nutrient sink, since addition of nutrients increased Pythium damping-off severity in cucumber. Mandelbaum and Hadar (1990) reported that hyphae of *P. aphanidermatum* lysed very rapidly in a suppressive compost-amended medium. Because the hyphal lysis was delayed by additional amendments with glucose-asparagine it was concluded that the lysis was caused by nutrient stress. Our results suggest that competition for carbon may not be the main mechanism in suppression of *Pythium* root rot in iris, as indicated by the lack of correlation between glucose uptake and disease incidence (Figure 2B). Competition for other nutrients or other forms of antagonism may play a role, as indicated by the high correlation between general microbial activity (dehydrogenase activity) and disease incidence (Figure 2B).

Experiments to distuingish habitat alteration from resource competition are difficult to conceive. In reality, resource competition as a mechanism for biocontrol exists along a continuum of microbial interactions (predation-parasitism-antibiosis-

habitat alteration-resource competition) that are not always discrete or independant (Kinkel & Lindow, 1997). In fumigated and flooded soil, disease suppression may be impaired by interference with different mechanisms. Furthermore, species composition will be altered by soil fumigation and flooding, as illustrated by the effective control of several pathogens in bulb culture (Weststeijn & De Rooy, 1974; Van Zaayen *et al.*, 1986; Muller, 1987). The composition of the microflora introduced with VFG-compost into sterilized soil may also differ considerably from untreated soil, inducing disease suppression by different mechanisms.

Microbial populations and their responses to stresses have been studied at the process level, in terms of total numbers of microorganisms, biomass, respiration rates, and enzym activities, with little attention being paid to responses at the community or the organismal levels. Although process level assessments may describe a situation, it does not indicate diversity and location of organisms responsible. These measurements, although critical to understanding the ecosystem, may be insensitive to community level changes due to the redundancy of these functions. The ability of an ecosystem to withstand extreme disturbance may depend in part on the diversity of the system (Kennedy & Smith, 1995). Further research on species composition, diversity, and relevance of specific antagonists is needed to clarify the mechanisms which bring about disease suppression against *Pythium* root rot in flower bulb culture on sandy soil.

Acknowledgements

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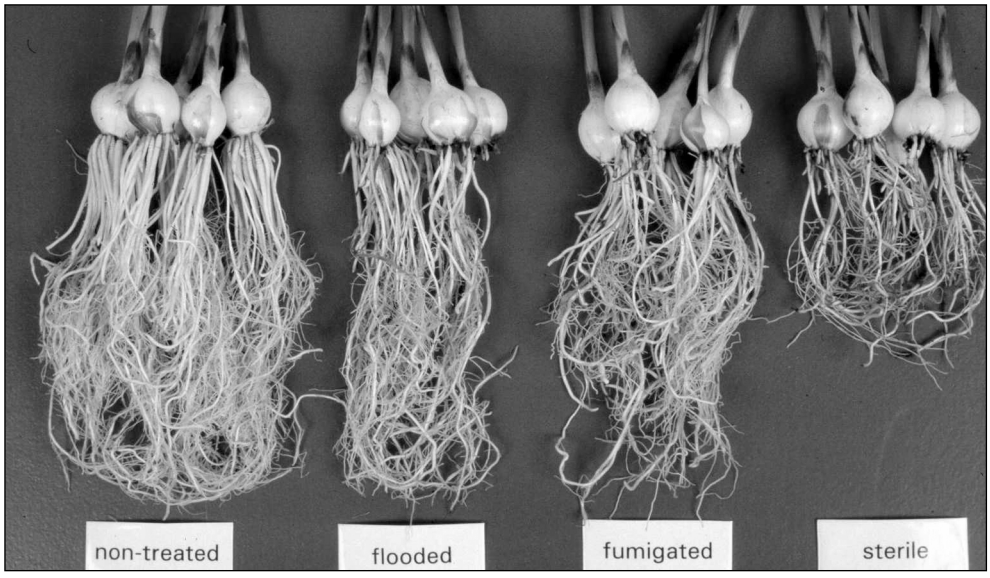
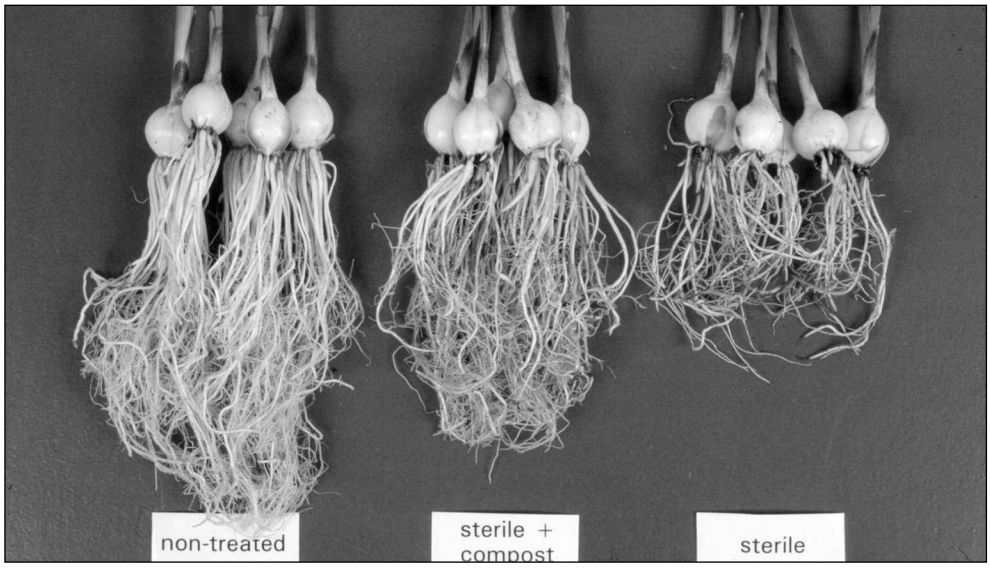
CHAPTER 6

Microbial community responses to disease management soil treatments used in flower bulb cultivation





Photographs Infected iris roots after infestation of treated soil (non-treated, flooded, fumigated with metam-Na, sterilized, and compost-amended sterlilized soil) with *Pythium macrosporum*.

CHAPTER 6 Microbial community responses to disease management soil treatments used in flower bulb cultivation

G.A. Kowalchuk, G.J. van Os, J. van Aartrijk and J.A. van Veen

Abstract

A number of management practices, such as soil fumigation and flooding, have been employed in efforts to control diseases and weeds in the cultivation of ornamental flower bulbs. However, such treatments may affect suppression of *Pythium* root rot, a serious problem in ornamental bulb culture. To gain insight into the nature of *Pythium* suppression in such soils, we sought to describe the changes that these soil treatments induce in the microbial community in order to determine if particular microbial components of the system could be associated with suppressiveness. Four PCR-DGGE strategies, two targeting bacteria and two targeting fungi, were used to compare the soil-borne microbial communities of untreated, fumigated, flooded and sterilized, compost-amended soils; the dominant community members were tentatively identified by sequencing of DGGE bands. For all profiling strategies, the compost treatment of sterilized soil appeared to have the most profound effect on the dominant microbial populations in the soil. In general, different primer sets that targeted the same microbial groups, bacteria or fungi, appeared to detect the same microbial taxa, although certain sequence types were detected in only a single profiling strategy. DNA-based microbial community profiles alone were poor predictors of *Pythium* suppression, as the dominant microbial populations remained mostly intact, even after rigorous soil treatments such as fumigation and flooding. The restoration of suppressive activity against *Pythium* in compost-amended soil was associated with a different microbial community than observed in untreated, suppressive soils. Thus, although previous studies have shown the suppression of *Pythium* to be mediated by biological agents, different microbial communities can lead to this suppression.

Introduction

A number of diseases, including *Pythium* root rot, cause serious damage in the ornamental bulb cultivation industry. In attempts to control disease levels and weeds, several soil treatments have typically been advised, such as soil fumigation and flooding (Weststeijn & De Rooij, 1974; Van Zaayen *et al.*, 1986; Muller, 1987). Although such treatments have been successful to some extent, results can be inconsistent and may even lead to increased levels of *Pythium* damage (Koster & De Rooij, 1980). Indeed, Van Os *et al.* (1999, Chapter 3) demonstrated that the natural

ability of bulb cultivation soil to suppress *Pythium* root rot was severely reduced as a result of fumigation or inundation. Furthermore, it has been demonstrated that such disease suppression is mediated by biological components of the soil (Chen *et al.*, 1988; Van Os *et al.*, 1999). However, it is not known if *Pythium* suppression is caused by a general nutrient deficiency, caused by high levels of microbial activity (Ho & Ko, 1986; Van Os & Van Ginkel, 2001, Chapter 5), or if specific microbial populations antagonize this pathogen. Microbial biomass and activity typically correlate inversely with levels of *Pythium* growth through soil, but these factors are poor predictors of actual disease development in Iris (Van Os & Van Ginkel, 2001).

The biological nature of *Pythium* suppression is further supported by the fact that compost amendments appear to provide at least partial restoration of disease suppression in sterilized soil (Van Os & Van Ginkel, 2001). However, it is not known whether the suppressive communities in sterilized, compost-amended soils are similar to those in untreated soils or if suppression is achieved via a differently structured microbial community.

PCR-based profiling methods have become powerful culture-independent tools for the examination of microbial communities, and offer the ability to monitor dominant microbial populations over space and time (Muyzer *et al.*, 1993; Muyzer & Smalla, 1998; Stephen & Kowalchuk, 2002). Such analyses typically target small sub-unit (SSU) rRNA genes, and sequence analysis of excised bands can provide information on organism identity by comparison to the database of previously determined rDNA sequences (Ferris *et al.*, 1996; Kowalchuk *et al.*, 1997b; Maidak *et al.*, 2001). PCR followed by denaturing gradient gel electrophoresis (DGGE) has become a common method for the characterization of bacterial (Muyzer *et al.* 1993; Muyzer & Smalla, 1998), and more recently fungal (Kowalchuk *et al.*, 1997a; Smit *et al.*, 1999; Vainio & Hantula, 2000; May *et al.*, 2001; Schabereiter-Gurtner *et al.*, 2001) communities. Although highly useful, such PCR-based methods are prone to biases due to preferential amplification and primer binding site mismatches, and these limitations must be kept in mind when interpreting PCR-DGGE profiling data (Chang *et al.*, 2000; Watanabe *et al.*, 2001).

To investigate the role of microbial community composition in the suppression of *Pythium* root rot, we examined the response of microbial communities in soils treated with three management strategies, fumigation, flooding, and compost treatment of sterilized soil. For comparison, untreated and sterilized soils were also examined. A total of four PCR-DGGE strategies were employed to examine the impact of these treatments on soil-borne microbial communities, two targeting bacteria and two targeting fungi. The sequencing of DGGE bands was used to identify dominant community members, allowing comparison of populations detected by the different primer sets. Community profiles were compared to determine whether specific microbial communities or populations were associated with *Pythium* suppression.

Materials and Methods

Soil Samples

Soil samples were collected and treated as described previously by Van Os *et al.* (1999) and Van Os and Van Ginkel (2001). Soil treatments, and their effects on *Pythium* root rot development, *Pythium* growth rate through soil, and microbial biomass, are summarized in Table 1.

Briefly, soil was collected from an experimental field of the Bulb Research Centre in Lisse, the Netherlands. Soils used in the experiment treatments were first pasteurized to eliminate native pathogens (2 h, 70°C), and left exposed to open air for six months, resulting in microbial recolonization. The subsequent sterilization treatment was by autoclaving (2 × 90 min. at 121°C), fumigation by application of methylisothiocyanate (MIT; 0.13 ml l^{-1} soil), and flooding was for a period of eight weeks. Some sterilized soil was inoculated with 1% (w/v) composted organic household waste (Tuitert *et al.*, 1998), which was well mixed (manual homogenization followed by 1 mm mesh sieving) into the soil and stored in sealed containers for one week at 20°C. All soils were mixed again and sieved (4 mm mesh),

Table 1 Soil treatments, root rot development, *Pythium* growth rate, soil microbial biomass and quantity of DNA extracted from soil.

Soil Treatment	% *Pythium* root rot in Iris[1, 2]	*Pythium* growth rate (cm / day) [1, 2]	Microbial biomass (µg / g dry soil) [1]	Amount of DNA extracted (µg / g dry soil)[1]
Sterilized (autoclaved)	90 d	0.66 d	0 c	1.1 ± 0.2 c
Untreated	14 a	0.22 b	40 b	12.3 ± 0.5 b
Fumigated with methyl-isothiocyanate	59 c	0.32 c	34 b	12.5 ± 0.4 b
Inundated during 8 weeks	57 c	0.38 c	35 b	11.7 ± 0.8 b
Sterilized and amended with 1% VFG-compost	35 b	0.06 a	100 a	23.2 ± 2.5 a

[1] Treatment means followed by the same letter do not differ significantly ($P < 0.05$).

[2] As determined by Van Os and Van Ginkel (2001).

and frozen at -20°C prior to DNA extraction to facilitate parallel processing. Each soil treatment was performed in triplicate.

DNA isolation

Three DNA isolations per sample (0.25 g wet soil), thus nine in total per soil treatment, were performed using the MOBIO soil DNA extraction kit (MOBIO Laboratories; Solana Beach, California). Soil samples were washed twice in 120 mM K_2HPO_4, pH 8.0, prior to DNA exaction. This treatment is designed to wash away extracellular DNA from the soil samples without the loss of intact cells (Kowalchuk *et al.*, 1997b). Extractions were as per the manufacturer's specifications except that vortex mixing was replaced with shaking 2×30 s at 5,000 r.p.m. in a mini bead beater (BioSpec Products, Techno Lab, Alkmaar, the Netherlands), and final DNA elution was in 30 µl 10 mM TRIS, pH 8.0. The concentration of DNA extracts was determined by spectrophotometric measurements at 260 nm, 280 nm, and 300 nm. Differences between amounts of DNA extracted were inspected using Tukey`s honestly significantly difference (HSD) at the 5% level (Table 1). DNA quantity and quality were also inspected by 1% agarose gel electrophoresis with standard ethidium bromide staining and ultraviolet illumination.

PCR-DGGE profiling strategies for bacteria and fungi

Three DNA extractions per treatment, each from an independently prepared soil, were chosen for molecular analysis, and all analyses applied to each sample used the same DNA extraction. In all cases 50 ng template DNA was used for the PCR amplification of bacterial or fungal SSU rRNA targets. A summary of the primer sets used is given in Table 2 and thermocycling programs for PCR or nested PCR were according to the protocols detailed in the studies cited. All amplification reactions were performed in a volume of 25 µl and consisted of 15 nM of each primer, 1 µl template DNA (≈ 50 pg), 1 U Expand High Fidelity DNA polymerase (Boehringer, Mannheim, Germany) and the manufacturer's recommended nucleotide concentrations and buffer conditions. All reactions were performed in a PTC200 thermal cycler (MJ Research; Waltham, Mass.). DGGE conditions are also summarized in Table 2 and were all performed using the D-Gene system (Bio-Rad Laboratories). One hundred percent denaturant concentration was defined as 7 M urea with 40% formamide (Muyzer *et al.*, 1993). Approximately 1 µg of PCR product was loaded per well in a final volume of 20 µl. Gels were stained with ethidium bromide and washed twice for 15 min. in deionized H_2O prior to UV transillumination and digital photography using the ImaGo system (B&L; Maarssen, the Netherlands). DGGE banding profiles were analyzed within the ImageMaster Elite Database program (version 3.0; Amersham Pharmacia Biotech), and UPGMA dendrograms were based upon Pearson's indices derived from total lane patterns as described by

Table 2 Overview of PCR-DGGE profiling strategies used.

Primer pair	Target	DGGE conditions	Reference
318f-GC / 535r	bacteria	8% acrylamide; 25-50% denaturant; 16h, 50V	Muyzer *et al.*. 1993
968f-GC / 1401r	bacteria	6% acrylamide; 45-65% denaturant; 16h, 80V	Heuer *et al.*. 1997
NS1-GC / NS2+10[1]	fungi	6% acrylamide; 25-45% denaturant; 16h, 80V	Kowalchuk 1999a; White *et al.*. 1990
FR1-GC / FF390	fungi	6% acrylamide; 40-55% denaturant; 16h, 75V	Vainio & Hantula 2000

[1] Utilized a nested PCR strategy (Kowalchuk 1999a).

Duineveld *et al.* (2001). Bootstrap analyses were based upon 100 replicates. Due to their very simple patterns (see results), fungal DGGE patterns are not presented in the cluster analysis.

Recovery and sequencing of DGGE bands

The most dominant DGGE bands from the different profiles were selected for excision and sequence analysis. For bands that appeared in multiple samples, at least two representative bands, recovered from different samples, were analyzed to confirm sequence identity. Only the innermost portion of each band was excised to avoid contamination with other DNA sequences. DNA was eluted from the polyacrylamide matrix by crushing frozen gel fragments, adding 50 µl 10 mM Tris, pH 8.0, and incubating at 37°C for 4 h. Recovered DNA was re-amplified and PCR products directly sequenced as described by Duineveld *et al.* (2001), except that 1401r was used as the reverse primer in PCR and sequence analyses. PCR products recovered after amplification of eluded DNA were also examined by DGGE to confirm product integrity, and, where necessary, a second excision and elution round was performed. DGGE bands that appeared to be present in multiple treatments were

excised and sequenced from at least two different treatments to assure that bands of the same mobility were actually identical in sequence. Band designations were given as follows: B or F to express a bacterial or fungal sequence; H, M, V, or K to designate the PCR strategy used, where the letter corresponds to the first letter of first author of the publication first describing its use; - number of band as given in figure 1, 2, or 3. Thus, sequence BM-6 would refer to the partial bacterial 16S rDNA sequence derived from band 6 of the gel using the primer set 318-GC/518 (Muyzer *et al.*, 1993).

DNA sequences from DGGE bands were compared to the available databank via the Blast-N program (Altschul *et al.*, 1997), and have been deposited in the EMBL sequence database under accession numbers AF510032-AF510056.

Results

DNA isolation

The DNA isolated from all samples was of sufficient quantity and quality for successful PCR amplification of bacterial and fungal SSU rDNA fragments. The amount of DNA isolated from each type of sample was quite consistent and was related to the microbial biomass observed for the different treatments (Table 1). A similar amount of DNA was obtained from untreated, fumigated and inundated soil, and approximately twice as much DNA was recovered from the compost-amended soil. Interestingly, DNA was also recovered from the sterilized soil, albeit at an order of magnitude decreased level compared to the other samples. Thus, background levels of extractable DNA remained in these soil samples, even after a rigorous sterilization treatment. Since two soil washing steps were performed prior to DNA isolation, this DNA may be associated with dead cells that, although killed, were not fully disrupted by the sterilization treatment, or be present as extracellular DNA that was not completely washed from the samples prior to extraction.

Bacterial community profiles

Both bacteria-directed DGGE strategies yielded complex banding patterns for all samples, suggesting a high bacterial diversity in all soil samples (Figures 1 and 2). A high level of consistency was observed within all treatments, with high levels of similarity for all samples within a single treatment. Also, analyses of replicate DNA extractions from a single sample produced highly similar patterns (not shown). For the primer pair 968-GC/1401r, all DGGE profiles were highly similar with the exception of those from the sterilized soil treated with compost (Figures 1A and 1B).

Flooding and fumigation apparently had only minor effects on the bacterial profiles obtained. Cluster analysis of these DGGE profiles typically grouped lanes within a given treatment, but only for the compost-amended soils was a major alteration in banding pattern observed with respect to the untreated control

(Figure 1B). Similar results were observed with the 318f-GC/518r primer pair, although some small, yet pronounced differences could also be observed between other treatments (Figures 2A and 2B). Although the general pattern of the DGGE profiles from the sterilized soil was highly similar to those from untreated soil, band BM-5, as well as a number of weaker bands, were absent from the sterilized soil profile.

Fungal community profiles

Fungal PCR-DGGE profiles (Figures 3A and 3B) were much simpler than those produced for bacteria. As seen for bacterial profiles, the sterilized soil amended with compost produced the most unique patterns in comparison with the other treatments. All samples, excluding the sterilized soil amended with compost, produced profiles with a common single dominant band, and this result was seen for both fungal-specific analyses used (FV-1 in Figure 3A and FK-1 in Figure 3B). In addition to this band, a number of other faint bands were visible in the analysis with the FR1-GC/EF390 primer pair (Figure 3A). The compost-amended profile for this primer set also contained a single highly dominant band, FV-7, as well as three other distinct bands. In contrast to the bacterial analyses and the fungal analysis of the other treatments, one of the compost-amended samples (sample 15) was quite different from the others. This sample contained all of the same bands as the other two profiles shown, but showed a different distribution of the signal over the detected bands. The NS1-GC/NS2+10 primer combination showed the same general result, but smearing reduced the possibility to resolve minor bands (Figure 3B). The compost-amended samples all produced one strong band as well as an additional detectable band (FK-3 and FK-2, respectively), whereas the other samples produced a single band at a slightly lower position in the gel (FK-1).

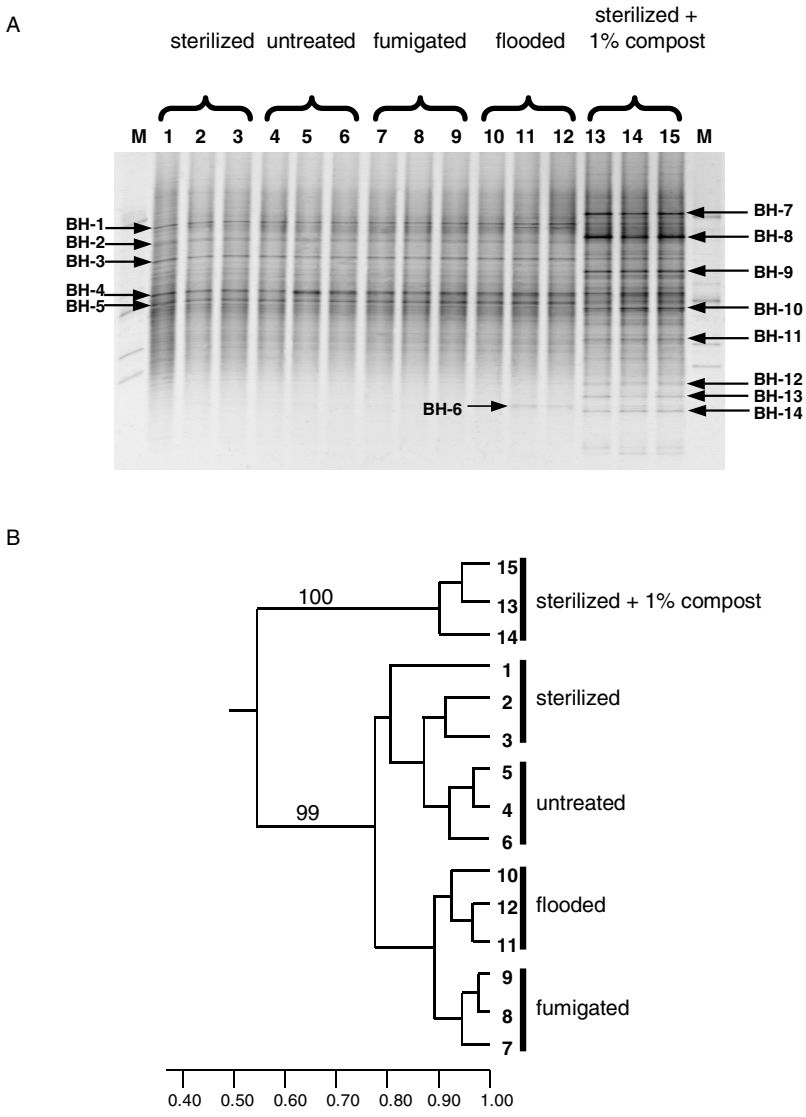


Figure 1 PCR-DGGE analysis with the bacterial primers 968f-GC and 1401r.

A. Reverse image of DGGE gel. Results for three independent samples per soil treatment are shown. The lanes designated with 'M' contain a mixture of PCR products from five bacterial pure cultures. The bands within this lane correspond to the following species, from top to bottom: *Streptomyces* sp., *Escherichia coli*, *Pseudomonas fluorescens* and *Stenotrophomonas* sp. Arrows are used to indicate band position and do not necessary point to the exact bands used for sequence determination.

B. Dendrogram derived from Pearson's indices of total lane similarity. Only bootstrap values above 70 are shown.

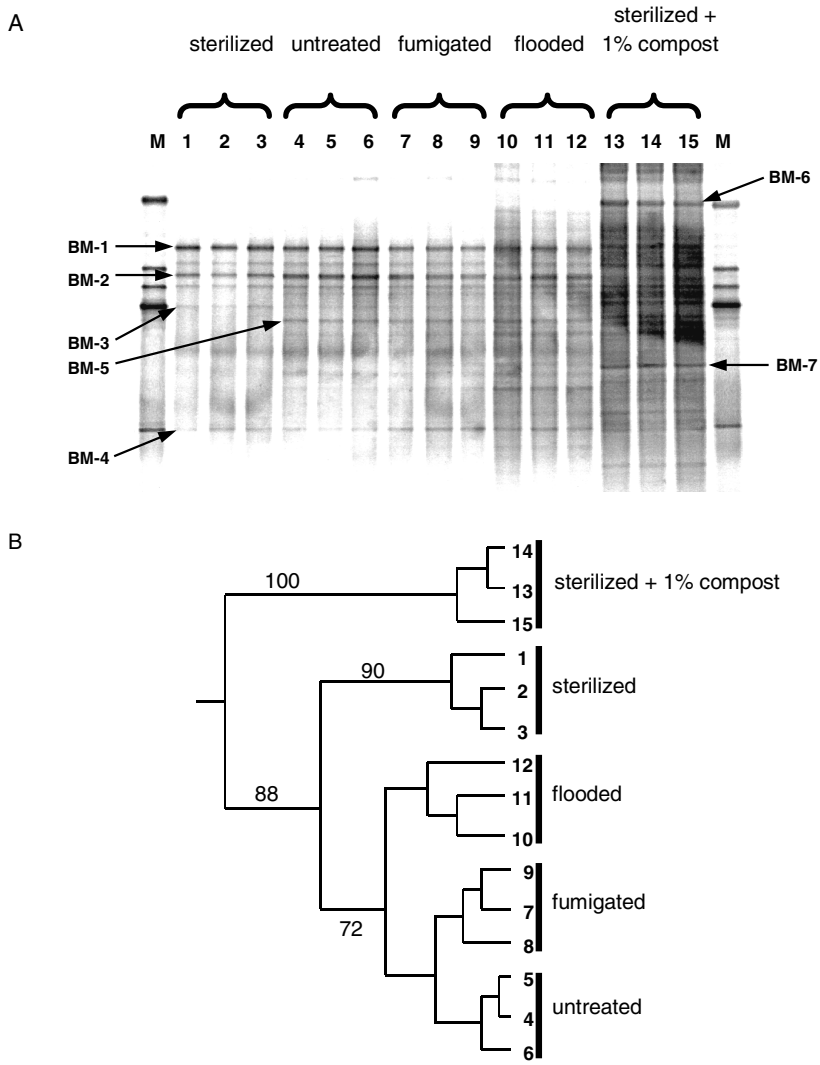


Figure 2 PCR-DGGE analysis with the bacterial primers 318f-GC and 535r.

A. Reverse image of DGGE gel. Results for three independent samples per soil treatment are shown. The lanes designated with 'M' contain a mixture of PCR products from five bacterial pure cultures. The bands within this lane correspond to the following species, from top to bottom: *Enterobacter cloaceae* BE1, *Listeria innocua* ALM105, *Rhizobium leguminosarum trifoli*, *Arthrobacter* sp. and *Pseudomonas cepacia*. Arrows are used to indicate band position and do not necessary point to the exact bands used for sequence determination.

B. Dendrogram derived from Pearson's indices of total lane similarity. Only bootstrap values above 70 are shown.

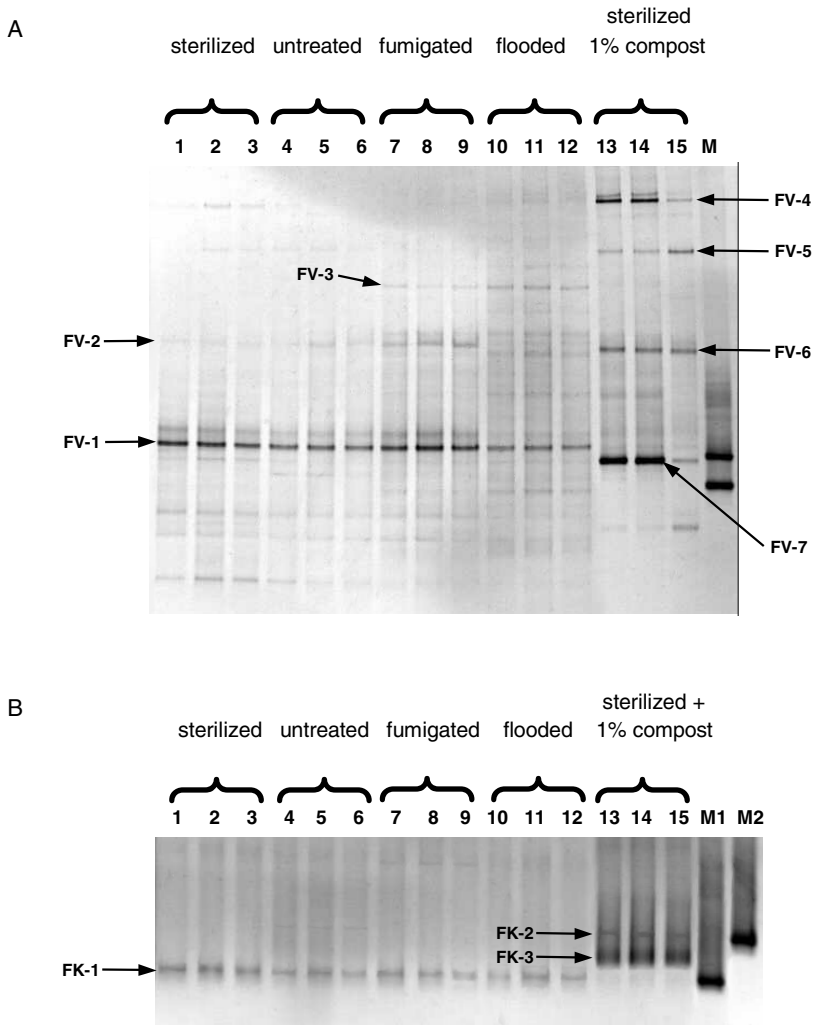


Figure 3 PCR-DGGE profiles of soil-borne fungal communities.

A. Reverse image of profile produced with the primers ff390 and fr1-GC (Vainio and Hantula, 2000). Results for three independent samples per soil treatment are shown. The lane marked M contains PCR products from two pure fungal cultures isolated from coastal sand dune soil: a *Microdochium* species (upper band) and a *Trichoderma* species (lower band).

B. Reverse image of profile produced with the primers NS1-GC and NS2+10 (Kowalchuk *et al.* 1997a). The lanes marked M1 and M2 contain the *Microdochium* and *Trichoderma* pure culture controls described above, respectively. Arrows are used to indicate band position and do not necessary point to the exact bands used for sequence determination.

Sequence analysis of DGGE bands

Dominant DGGE bands that could be excised cleanly without obvious contamination from neighboring bands were subjected to re-amplification and direct sequence analysis. The majority of these bands yielded unambiguous sequence (Table 3), however some bands failed to give clean sequence primarily due to double signals in the sequencing reactions.

No bacterial sequences were perfect matches with previously determined sequences, although a number showed a high degree of similarity with database entries (Table 3). Comparison of the bacterial sequences recovered via the two PCR-DGGE strategies, showed that some genera were detected by both primer sets, including *Bacillus, Rhodopseudomonas,* and *Pseudomonas*. The fragments produced by the two different bacterial PCR strategies do not overlap. It is therefore not possible to determine if certain bands from the two profiling methods were derived from the same bacterial populations, although this is strongly suggested by the fact that some band sequences from the different DGGE strategies share affinity with the same bacterial taxon. In addition, some DNA sequences were observed with one primer set, but not the other. For instance, sequences showing affinity with *Variovorax* and alpha subclass of the *Proteobacteria* were detected only. in the 968f-GC/1401r primer set. Similarly, *Acidobacterium*-like and *Sphingomonas*-like populations were only detected with the 318f-GC/535r primer set.

Sequence analysis of fungal DGGE bands FV-1 and FK-1 both showed near identity to sequences previously determined for a number of *Chaetomium* species. The band FK-3, which was the strongest band detected in the sterilized, compost-amended soil using the NS1-GC/NS2+10 primers (Figure 3b), was nearly identical to the sequence of *Microdochium bolleyi*. The dominant band in the sterilized, compost-amended sample, FV-7, shares greatest sequence identity with *Dicyma olivacea,* a close phylogenetic relative of *M. bolleyi,* strongly suggesting that this band was derived from the same fungal population as represented by band FK-3. It should be noted that no database entry for *M. bolleyi* exists for the region amplified by the FR1-GC/EF390 primer set. Thus, the two primer pairs used appeared to detect the same fungal populations as dominant in the different treatments analyzed, although the lack of overlap between the regions analyzed again makes this impossible to prove. Sequences obtained from faint bands detected by the FR1-GC/EF390 primer set, revealed the presence of a number of apparently minor yeast-like populations.

Table 3 Database matches for sequences derived from DGGE bands.

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DGGE band[1, 2]	Most closely related database entry (% identity)[3]	Accession # of database match[3]
	968f-GC / 1401r primer set	
BH-1 (BM-1)	uncultured *Bacillus* sp. (98.5%) / *Bacillus* sp. PL-26 (98.2%)	AY082367 / AF326369
BH-2	no legible sequence produced	-
BH-3 (BM-2)	*Rhodopseudomonas* sp. (95.8%)	D15073
BH-4	*Variovorax paradoxus* (98.5%)	AB008000
BH-5	uncultured bacterium (98.5%) / *Aquaspirillum delicatum* (98.2)	AF429108 / AF078756
BH-6	no legible sequence produced	-
BH-7 (BM-6)	*Bacillus* sp. LMG 20238 (98.8%)	AJ316309
BH-8 (BM-6)	*Bacillus* sp. LMG 20238 (99.0%)	AJ316309
BH-9	*Cytophaga* sp. (94.2%)	AB017046
BH-10 (BM-7)	*Pseudomonas putida* (99.5%)	AF094743
BH-11	*Pseudomonas jessenii* (98.8%)	AF068259
BH-12	no legible sequence produced	-
BH-13	uncultured α-Proteobacterium (98.2%) / *Pedomicrobium manganicum* (93.2%)	AF289919 / X97691
BH-14	Uncultured α-Proteobacterium (98.0%) / *Pedomicrobium manganicum* (93.5%)	AF289919 / X97691
	318f-GC / 535r primer set	
BM-1 (BH-1)	uncultured *Bacillus* sp. (98.1%) / *Bacillus infernus* (97.5%)	AY082367 / U20384
BM-2 (BH-3)	*Rhodopseudomonas palustris* (97.8%)	AF314062

BM-3	no legible sequence produced	-
BM-4	Uncultured *Halophaga-Acidobacterium* (93.8%) / *Thialkalivibrio paradoxus* (85.6%)	AJ241003 / AF151432
BM-5	*Sphingomonas* sp. ML1 (98.4%)	AY026948
BM-6 (BH-7 / BH-8)	*Bacillus* sp. LMG 21002 (98.1%)	AJ316308
BM-7 (BH-10)	*Pseudomonas putida* (98.8%)	AF094747
	FR1-GC / EF390 primer set	
FV-1 (FK-1)	*Chaetomium elatum* (99.4%)	M83257
FV-2	no legible sequence produced	-
FV-3	unidentified ascomycota (99.4%) / *Geomyces pannorum* (99.0%)	AJ301722 / AB015785
FV-4	*Kluyveromyces* sp. (99.4%)	AB016511
FV-5	*Acremonium* sp. (99.7)	AJ278754
FV-6	*Candida fermenticarens* (99.7%).	AB013525
FV-7 (FK-3)	*Ascotricha chartarum* (98.1%)	AB048283
	NS1-GC / NS2+10 primer set	
FK-1 (FV-1)	*Chaetomium globosum* (98.4%)	AB048285
FK-2	no legible sequence produced	-
FK-3 (FV-7)	*Microdochium bolleyi* (99.8%)	Z94133

[1] Bands correspond to those given in Figures 1, 2 and 3, and band designations are explained in the materials and methods.

[2] The band name in parentheses designates a possible overlap with results with a different primer set.

[3] In cases where the closest database entry was from an unidentified organism, the closest culture representative is also given.

Discussion

The most striking result of the various community profiles examined was the constancy observed between the untreated, fumigated and flooded soils. The community profiles remained very similar after these relatively rigorous treatments, despite distinct differences in *Pythium* disease suppression (Table 1). Fumigation and flooding have different modes of action for disease control, and different species of the soil microflora are presumably inhibited or killed as a result of these treatments. Soil flooding, for instance, has profound physical, chemical and biological consequences for soil habitats (Ponnamperuma 1984), principally due to the restricted entry of atmospheric oxygen into the system. This lack of oxygen alters the microbial processes carried out in the soil (Laanbroek, 1990), and has been shown to cause shifts in the metabolic diversity of the culturable portion of affected microbial communities (Bossio & Scow, 1995). However, with the data collected in this study no distinction can be made between active and inactive populations. Furthermore, the data came from soil samples as would be found at the time of planting, and thus only represent the t=0 time point with respect to a season's bulb cultivation. It may very well be that succession of microbial populations occurs during the bulb cultivation period, and this succession may be dependent on initial soil treatment. Experiments designed to follow the development of these microbial communities over time would address this issue and might provide additional information concerning the relationship between microbial community dynamics and the seasonal development of *Pythium* root rot (Van Os *et al.*, 1998, Chapter 2). The sterilized, composed amended soil had the most distinctive microbial community, presumable because the introduced microbial populations were free to develop in a system where the native populations were first eliminated.

Particularly remarkable was the similarity between profiles generated from sterilized and untreated soils. Although most of the DNA was destroyed during the sterilization procedure as evidenced by the ten-fold decrease in extractable DNA (Table 1), the DGGE profiles remained highly similar (Figures 1, 2 and 3), suggesting that some residual DNA from a broad range of the microbial community was still available for extraction and amplification. DNA from dead cells, or extracellular DNA that was not successfully washed from the samples, may still be recovered by DNA extraction method used, and subsequently amplified by PCR. This illustrates the caution that must be exercised when interpreting community profiles derived from total DNA extractions. Analyses targeting rRNA, instead of, or in addition to rDNA, are believed to provide some insight into dominantly active microbial populations, instead of just targeting intact DNA (Felske *et al.*, 1996; Teske *et al.*, 1996; Kowalchuk *et al.*, 1999; Duineveld *et al.*, 2001). We observed DNA-derived patterns to be unresponsive to environmental changes in the short-term. Community profiles based upon rRNA, or the use of other methods that focus on the active populations in a

sample (Borneman, 2000), may therefore be more useful in following the dynamics of microbial activity. Although application of rRNA-targeted strategies would be an important follow up to this study, it remains important to study all the populations present, regardless of current activity levels, as these populations can also be activated during the cultivation period or in the presence of the pathogen.

The PCR-DGGE profiling methods used only detect the most dominant microbial populations (approximately > 1% of total community; Muyzer *et al.*, 1993; Brüggemann *et al.*, 2000). Thus, although the dominant populations in the untreated, fumigated and flooded samples appear very similar, subordinate populations, which may still have an important role in disease suppression, would not be detected. Also, both bacterial primer sets used contain mismatches with a different minority subset of the bacterial 16S rDNA sequences (Watanabe *et al.*, 2001), and will therefore fail to detect some bacterial populations. Given the imperfect specificity and coverage of primers and possible effects of preferential PCR amplification for different bacterial primer sets (Chang *et al.*, 2000; Watanabe *et al.*, 2001), we employed two different primer sets for each of the microbial groups targeted. Although some differences were observed between the different PCR strategies, the results were generally consistent across different strategies, with a number of detected microbial populations probably being in common across different analyses. One notable incompatibility of the 968f-GC/1401r primer set is with strains of the genus *Sphingomonas*, which may help explain why this genus was only detected by the one of the two bacterial PCR strategies (Chang *et al.*, 2000).

Primer limitations are most evident for analyses designed to target fungi (Kowalchuk, 1999b; Schabereiter-Gurtner *et al.*, 2001). The 18S rRNA gene does not contain enough variability within the short stretches that can be analyzed by DGGE to allow for good species level identification. Clearly, a more variable marker is necessary for the identification of fungi based upon sequence information. The internal transcribed spacer regions (ITS) offer a good possibility, but lack phylogenetic robustness. Methods that combine such markers may provide the best possibilities to detect and identify fungal population via community analysis approaches. One must also consider the length of the DNA fragment targeted by the PCR, since short fragments generally provide better resolution in the DGGE, but yield less sequence information. Although some authors report high quality DGGE results using DNA fragments in excess of 1 kb (Vainio & Hantula, 2000), the selection of appropriate regions for DGGE analysis typically remains a trade-off between these factors. Also, the databank of available rDNA sequences is still rather limited for fungi, restricting the usefulness of comparative analyses.

In this study, direct sequencing of PCR products was used to determine the sequence of specific DGGE bands. Using this strategy, one recovers the sum of all sequences within a band, which carries both advantages and disadvantages. In

addition to saving time and money, direct sequencing is far less prone to PCR error or cloning artefacts (Speksnijder *et al.*, 2001). However, minority DNA species may be masked by more dominant ones, where bands actually represent a mixture of DNA species with similar migration, and mixed bands may produce ambiguous sequence, as was the case for several bands in this study. In such cases, a cloning and sequencing approach would be necessary to resolve fully the sequence information represented within a DGGE band.

This study focussed specifically on microbial community development in treated soil, and it would be of interest to see if presence of the plant or *Pythium* in the system changed the response of the microbial communities. It should be noted that the dynamics of *Pythium* populations would require methods other than those applied in this study, as this genus does not belong to the true fungi, but rather to the Oomycetes, and contains mismatches with the fungal primers used in this study. An interesting line for future research would be to monitor *Pythium* levels in the soil, and molecular methods are becoming available for this (Martin *et al.*, 2000).

The sterilized soil amended with compost was the only sample that showed a highly unique microbial community composition in comparison with the other treatments. This treatment had previously been shown to restore some of the suppression capability of the soil (Van Os & Van Ginkel, 2001), but a very different microbial community confers this suppression. The sterilized, compost-amended soil contained several *Bacillus* and *Pseudomonas*-like sequences. Some species of these genera are well-known antagonists of *Pythium* species (Elad & Chet, 1987; Becker & Cook, 1988; Weststeijn, 1990; Whipps & Lumsden, 1991) and may be involved in the disease suppression induced by compost amendment.

The dominantly recovered 18S rDNA sequences were closely related to members of the genus *Chaetomium*, members of which have previously been found to have antagonistic activities against *Pythium* (Di Pietro *et al.*, 1992). However, no conclusions as to potential antagonistic activity can be drawn from this 18S rDNA sequence data. Furthermore, it is probably premature to identify these bands as coming from a *Chaetomium* species, as a number of fungal genera are nearly identical over the regions analyzed (*e.g. Colletotrichum, Farrowia, Guanomyces,* and *Achaetomium*).

Although specific microbial populations may be responsible for *Pythium* suppression in the soil samples examined, it was not possible to correlate the presence of distinct bacteria or fungal species with the inhibition of *Pythium* growth through soil or suppression of disease development in Iris.

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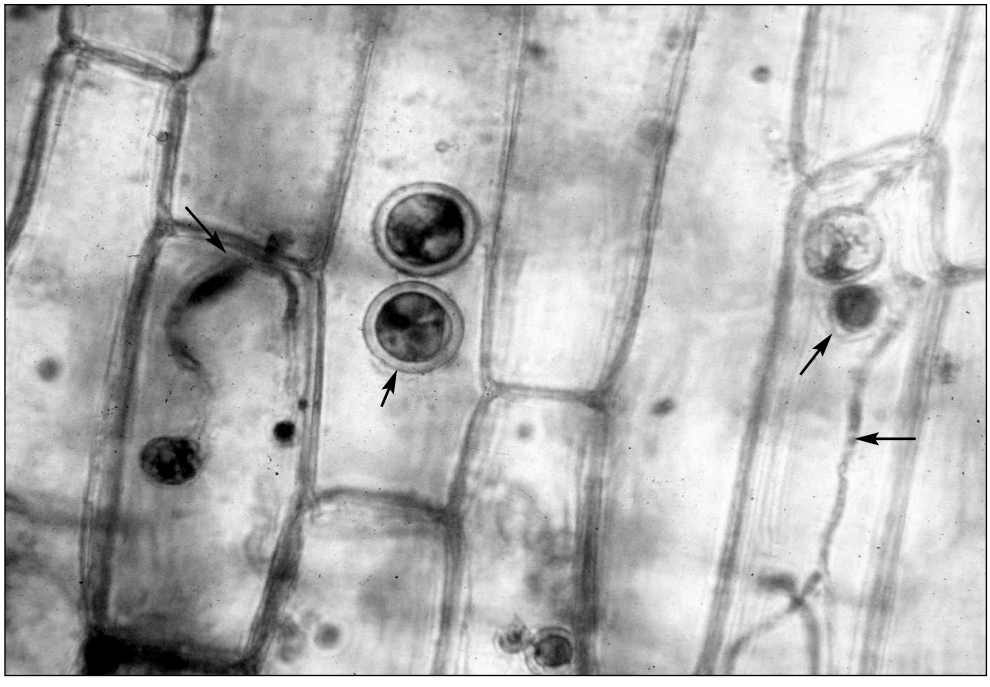
CHAPTER 7

Identification and pathogenicity of *Pythium* spp. in flower bulb culture



Photograph Iris root cells infected by *Pythium ultimum* var. *ultimum* (arrows from left to right): a cell wall penetrating appressorium, thick-walled oospores, hyphal swelling and a hyphe.

CHAPTER 7 Identification and pathogenicity of *Pythium* spp. in flower bulb culture

G.J. van Os, W.J.M. Wijnker and A.W.A.M. de Cock

Abstract

Studies were conducted to characterize the community of *Pythium* spp. occurring in bulb fields in five important areas for bulb cultivation in the western part of the Netherlands. *Pythium* isolates were identified using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. DNA regions coding for the internal transcribed spacer (ITS) were amplified and analyzed by restriction enzyme digestion. Fifteen different species were identified among the 163 isolates of *Pythium* recovered from 35 fields. The majority of sampled fields contained a mixture of species. Commonly isolated species included *P. sylvaticum*, *P. ultimum*, *P. intermedium*, and *P. heterothallicum*. Pathogenicity tests were performed on five susceptible bulb crops. Differences in host ranges and aggressiveness occurred among isolates within all tested species. These results suggest that decisions concerning crop rotation can not be based solely on the presence of certain *Pythium* species in the soil. However, crops differed in their susceptibility for individual *Pythium* isolates, indicating possibilities for crop rotation in order to reduce pathogen populations.

Introduction

Flower bulb crops, such as iris, crocus, hyacinth, tulip and lily are susceptible to root rot caused by *Pythium* species. *P. ultimum* Trow*, P. irregulare* Buisman*, P. paroecandrum* Drechsler*, P. intermedium* de Bary*, P. macrosporum* Vaartaja & Van der Plaats-Niterink*, P. sylvaticum* Campbell & Hendrix, and *P. violae* Chesters & Hickman have been described as pathogens on one or more of these crops (Van der Plaats-Niterink, 1975, 1981; Saaltink, 1969). Infected plants die off early, resulting in reduction of bulb yield. Until recently, control measures for *Pythium* root rot in ornamental bulb culture included soil fumigation and the application of selective fungicides (Koster & De Rooij, 1980; Saaltink, 1969). The use of these chemicals has been restricted by government regulations because of environmental and health hazards. Therefore, alternative control methods, such as cultural practices or application of antagonists, are currently being investigated.

Pythium spp. appear to be common inhabitants of soils in the Netherlands (Van der Plaats-Niterink, 1975). However, little is known about the variety of *Pythium* species in bulb fields and in different cultivation areas, and limited information is available concerning the comparative pathogenicity of *Pythium* spp. on bulb crops or

differences in virulence among isolates of each species. Saaltink (1969) indicated that in fields in which hyacinths became seriously diseased, iris and crocus remained healthy. In fields in which crocus or iris were infected by *Pythium*, hyacinth did not show root rot. If specificity in host range exists for pathogenic *Pythium* spp., crop rotation could be a method of reducing the population densities of these pathogens (Hendrix & Campbell, 1973).

Unambiguous and reliable identification of the pathogens is critical for ecological and epidemiological studies. Presently there are more than hundred and twenty species within the genus *Pythium* (Van der Plaats-Niterink, 1981; Dick, 1990). It is often difficult to identify *Pythium* isolates to the species level on the basis of morphological traits, because these features are frequently variable and are influenced by environmental conditions which may result in an inability to identify certain species or even misidentification of some species. The use of molecular criteria can alleviate some of the confusion in identification of closely related species (Martin *et al*., 2000).

Comparison of restriction patterns of ribosomal DNA is one of the molecular criteria that have been utilized for taxonomic comparisons of *Pythium* (Chen *et al*., 1992; Wang & White, 1997). The extent of variation in restriction sites within the internal transcribed spacers (ITS) of the ribosomal RNA genes was determined for thirty six important plant pathogenic *Pythium* species by Wang and White (1997), for five homothallic species by Chen *et al*. (1992) and for three heterothallic species by Chen (1992). The ITS regions showed variations in length and restriction sites among species, but variation was minimal within species. With a small number of exceptions, species could reliably be distinguished by restriction fragment length polymorphism (RFLP) analysis (Wang & White, 1997). The authors suggested that the species-specific banding would provide a reliable taxonomical tool for the identification of *Pythium* species (Chen *et al*., 1992; Wang & White, 1997).

In the present study, PCR-RFLP was used to identify field isolates from soil and diseased plants in order to make up a survey of species occurring in flower bulb fields in the western part of the Netherlands. Subsequently, a selection of identified isolates was tested for pathogenicity on iris, crocus, hyacinth, tulip, and lily. The objectives of this research were to compare the pathogenicity of *Pythium* spp. on susceptible bulb crops, to determine host ranges and the amount of intraspecific variation in virulence. The specificity of the interaction caused by different *Pythium* isolates will be designated with the term ‘virulence’ throughout this paper, whereas the term ‘aggressiveness’ refers to the overall rate of disease development (Van der Plank, 1984). Combined with an inventory of *Pythium* spp. in field soil, prospects could be made for crop rotation and the use of sampling and identification of *Pythium* spp. in field soil in order to advise on crop rotation.

Materials and Methods

Fungal isolates.

Thirty two isolates of known identity, representing twenty five different *Pythium* species, were obtained from the collection of the Centraalbureau voor Schimmelcultures (CBS, Utrecht, the Netherlands). These isolates were selected to represent a broad spectrum of species common to Dutch soils. The isolates were confirmed to species level according to the descriptions and key of Van der Plaats-Niterink (1981). Twenty five strains were used as reference strains for molecular identification; they were mostly ex-type or ex-neotype strains or those strains that were used by Van der Plaats-Niterink (1981) for the species description in her Monograph of the genus *Pythium* (Table 1).

During 1993 to 1998, thirty five bulb fields, with and without visible root rot symptoms, in five important areas for bulb cultivation in the western part of the Netherlands were sampled according to a random sampling strategy, dividing each field in twenty four or twenty five evenly proportioned plots of which five were sampled. From each plot a soil sample of 10 l was taken from the upper 20 cm soil. In area 'De Noord' (sandy soil, 0.5-2.0% OM) twelve bulb fields were sampled, in area 'De Zuid' (sandy soil, 0.5-2.0% OM) eleven fields, in area 'Kennemerland' (sandy soil, 0.5-2.0% OM) eight fields, in area 'Wieringermeer' (loamy soil, 1-5% OM) three fields, and in area 'West-Friesland' (loamy soil, 5% OM) one field. Soil samples were stored at 5°C for one day. In order to isolate *Pythium* spp., each soil sample was thoroughly mixed, a subsample of 10 g was suspended (1 g ml^{-1} soil in sterile water), and 0.4 ml suspension was plated on 2% cornmeal agar (Oxoid) (CMA) with 25 μg ml^{-1} pimaricin (Merck) and 1 μg ml^{-1} terramycin (oxytetracycline HCl, Pfizer) (CMA^{+}), using a soil drop method as previously described by Van Os *et al*. (1999, Chapter 3). From each field three to seven representative isolates were selected based on differences in colony morphology, hundred sixty three isolates in total. Furthermore, thirty three *Pythium* isolates were obtained from diseased plants originating from various locations. Stock cultures of all isolates were maintained on CMA at 5°C.

Molecular identification.

For DNA isolation, isolates were grown in pea-glucose broth according to Hwang *et al*. (1991). After four to seven days incubating at room temperature (18°C-23°C), mycelia were collected on Whatman No. 2 filter paper and washed with sterile, distilled water. Total DNA was extracted directly from mycelia using the procedures of Goodwin and Lee (1993). Briefly, mycelium in 10xPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl) was treated in the microwave (three times 30 s at 750 W) and centrifuged at 13000 rpm during 5 min. 1 μl of the supernatant was directly used for PCR.

Table 1 Morphologically identified *Pythium* isolates and their accession numbers, of which the RFLP banding patterns were used as reference for the molecular identification of field isolates.

Species	Accession numbers[1]
P. acanthicum	284.31 T[2]
P. aphanidermatum	118.80 NT
P. aquatile	215.80 NT
P. aristosporum	263.38 T
P. arrhenomanes	324.62 T
P. deliense	314.33 NT
P. dissotocum	166.68
P. graminicola	327.62 NT
P. heterothallicum	450.67 T+
P. intermedium	266.38 T- PN
P. irregulare	250.28 NT
P. macrosporum	574.80 T+
P. mamillatum	251.28 NT
P. myriotylum	254.70 PN
P. oligandrum	382.34 PN
P. paroecandrum	157.64 PN
P. spinosum	276.67 PN
P. splendens	462.48 PN
P. sylvaticum	452.67 T+
P. torulosum	316.33 PN
P. ultimum var. *sporangiiferum*	219.65 T
P. ultimum var. *ultimum*	398.51 NT
P. vanterpoolii	295.37
P. vexans	119.80
P. violae	178.86

[1] All accession numbers refer to isolates obtained from the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands

[2] T = ex-type strain; T+ = male mating type; T- = female mating type; NT = ex-neotype strain; PN = used by Van der Plaats-Niterink (1981) for species description.

Thirty two morphologically identified *Pythium* isolates and hundred ninety six unidentified isolates from diseased plants and field soil were characterized based on RFLP analysis of the ITS region of rDNA, according to the principle as described by Wang and White (1997). Amplifications of the ITS regions of *Pythium* species were performed in a programmable thermocycler (Perkin-Elmer, GeneAmp PCR System 9600), using the primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') from 28S and ITS5 (5'-GGAAGTAAAAGTCGTAA CAAGG-3') from 18S rDNA (White *et al*., 1990). A 50 µl reaction mixture contained 20 pmol of each primer, one unit SuperTaq DNA polymerase (SphaeroQ, HT Technologies, Cambridge), 1 mM each of the four deoxynucleotides, dATP, dCTP, dGTP, and dTTP in a PCR reaction buffer (SphaeroQ, HT Technologies, Cambridge), and 1 µl of the supernatant with template DNA. Reactions were amplified for thirty five cycles, DNA denaturation was at 95°C, 5 min. for the first cycle and 1 min. for subsequent cycles, 1 min. at 50°C for primer annealing, and 2 min. 72°C for primer extension. PCR amplifications containing no DNA template were carried out in every experiment as controls to test for the presence of contamination in reagents and reaction mixtures by non-sample DNA.

In order to assess sequence variation in the ITS regions, the amplified DNA (5 µl) was digested with the restriction endonucleases *Alu*I, *Cfo*I, *Dra*I, *Rsa*I, and *Taq*I, using conditions specified by the manufacturer (Roche, Germany). The digested DNA fragments were separated by electrophoresis in horizontal gels containing 1.6% agarose (Pronase 1-D, Hispanagar) with Tris-borate EDTA buffer (0.44 M boric acid, 0.44 M Tris base, and 10 mM EDTA) at 120 V during 3 h. A 100-bp molecular weight ladder (Promega) was used as the size standard. Gels were stained with ethidium bromide (0.05 µl ml^{-1}) and photographed using ultraviolet light. Band positions were determined by comparison of migration distances of fragments to those of the marker fragments. Banding patterns of unidentified isolates were visually compared to the patterns of twenty five morphologically identified isolates from Table 1, most of which were used by Van der Plaats-Niterink (1981) for species identification. Isolates were positively identified in case of identical banding patterns with one of these twenty five reference isolates for each of five restriction enzymes.

Pathogenicity tests

Fifty one *Pythium* isolates from Dutch origin, representing ten species, potentially pathogenic (isolated from diseased plants) or frequently isolated from field soil, were screened for pathogenicity on five bulb crops (Table 2). In this experiment, bulbs were planted in pots with river sand that was inoculated with one of the isolates. Inoculum was prepared by inoculating a sterilized sand-oatmeal mixture (6 g l^{-1} oatmeal) with agar plugs taken from CMA plates with the required isolate and incubating the mixture for three weeks at 20°C. River sand was infested by mixing with 25 ml l^{-1} sand-oatmeal culture. In each of three experiments, a different set of

isolates was screened on five crops. For each crop one pathogenic reference isolate was included in each experiment, in order to estimate variance between experiments due to variability in susceptibility of the planting stock. Reference isolates were: isolate 10 *P. ultimum var. ultimum* for tulip and lily, isolate 11 *P. intermedium* for hyacinth, isolate 24 *P. irregulare* for crocus, and isolate 111 *P. macrosporum* for iris. These isolates were confirmed to species level according to the descriptions and key of Van der Plaats-Niterink (1981).

A cultivar of each crop known to be susceptible was used: Dutch *Iris xyphium* 'White van Vliet', *Crocus ancyrensis* 'Flower Record', *Hyacinthus orientalis* 'Pink Pearl', *Tulipa* 'Gander', and *Lilium* 'Enchantment'. Bulbs and corms were disinfected by submerging in a solution of 0.4% formaldehyde (1% formalin, 40% a.i.) for 15 min. prior to storage in order to eliminate native pathogens. Crops were prepared, planted, and incubated according to standard practice for flower production, at 16°C-18°C for iris, tulip and lily, and at 9°C for crocus and hyacinth followed by 18°C after eight weeks. Infested sand was divided over twenty five pots, five pots for each crop. Five bulbs or corms were planted per pot. For each crop, pots were placed in five randomized blocks. Blocks contained pots of each isolate and a pot with uninfested sand. At flowering, roots were washed with tap water for disease severity assessment. Root-rot ratings of infested treatments were related to the healthy root systems of the non-infested control treatments. Roots were visually scored for root-rot severity using an arbitrary disease index ranging from 0-5, where 0 = no root rot, 1 = 1-20%, 2 = 21-40%, 3 = 41-60%, 4 = 61-80%, and 5 = >80% root rot, i.e., relative loss of healthy root mass induced by infection (Van Os *et al.*, 1998, Chapter 2). Roots were assessed for each plant individually and a mean root rot index per pot was calculated.

Data analysis

Statistical analysis was performed using Genstat 5 (Genstat 5 Committee, 1993). Root-rot ratings were converted to percentages and regression was conducted on transformed data, using the logit link, in a generalized linear model (GLM) to assess the pathogenicity of tested isolates compared to the non-infested control treatment for each crop. 1-tailed Student's *t* tests were used to determine pair-wise differences of means at significance level $P \leq 0.05$. Non-pathogenic isolates were discarded in further analyses.

In order to identify groups of different *Pythium* spp. isolates showing virulence on susceptible bulb crops, an agglomerative hierarchical clustering procedure was performed as described by Corsten and Denis (1990). The appropriate data format for the proposed clustering is a two-way table, rows representing different isolates and columns representing different crops. For this purpose, transformed (logit) root rot percentages were analysed using Restricted Maximum Likelihood (REML),

according to an incomplete block design with five replicates per experiment. The analyses were performed per crop, yielding tables with (transformed) mean root rot ratings for each isolate and their accompanying standard errors. The observations were considered mutually independent normal random means with common variance σ^2. The residual variance estimate was calculated as the average of the five squared standard errors accompanying the corresponding tables of means: s^2=0.46 with 810 degrees of freedom. It was assumed that between isolates and crops interactions had been established. Grouping of isolates and crops was performed, such that a minimum number of groups was identified, which accounted for the overall interaction, but were internally homogeneous. The clustering was stopped just before the step where the cumulated sums of squares of selected interactions exceeded the critical value c(α)=71.1 at significance level α = 0.05.

Results

Molecular identification

The amplified ITS region ranged from 850 to 1050 bp among the twenty five morphologically identified *Pythium* species from Table 1. Analysis of the RFLP patterns, obtained by restriction of the ITS amplicons with each of five restriction enzymes *Alu*I, *Cfo*I, *Dra*I, *Rsa*I, and *Taq*I, revealed that these species could be distinguished, except *P. arrhenomanes* and *P. aristosporum*, which could not be discriminated from each other. PCR-RFLP was applied to identify hundred ninety six unidentified *Pythium* isolates from field soil and diseased plants. One hundred and seventy three isolates showed identical banding patterns with one of the reference isolates and thus could successfully be identified (Table 2 and 3). Isolates from soil samples obtained from thirty five bulb fields represented thirteen species and two groups. In most soil samples, some species were represented by more than one isolate. Each field contained one or more potentially pathogenic *Pythium* species. Mixtures of two to five different species were detected in thirty two fields (Table 3). *P. sylvaticum* (69%), *P. ultimum* var. *ultimum* (60%), *P. intermedium* (48%), and *P. heterothallicum* (40%) were most frequently isolated.

Molecular identification was not successful for twenty three isolates with deviating RFLP banding patterns. These isolates were sent to the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands) for morphological identification according to the key of Van der Plaats-Niterink (1981). Nine isolates were identified as *P. paroecandrum*, revealing two different banding patterns, not identical to the reference isolate CBS 157.64. The separation of isolates in two groups with different banding patterns coincided with the origin of the isolates: patterns of five isolates from diseased plants were dissimilar to those of four isolates from soil. Two isolates were identified as *P. rostratum*, six isolates as *Pythium* Group G, and one isolate as *Pythium* Group HS. These species had not been included in the list of reference

Table 2 *Pythium* isolates used in the pathogenicity test, collection accession numbers, method of identification (morphological or molecular; - = not submitted to morphological identification; + = positively identified; ? = RFLP banding pattern not identical to any of the reference isolates), and cluster numbers of pathogenic isolates derived from Figure 1 (- = non-pathogenic, not included in the cluster analysis).

Species	Origin	Accession Number CBS	PPO	Identification morph.	mol.	Cluster
P. dissotocum	soil		29.6	-	+	-
P. dissotocum	iris		103	-	+	-
P. dissotocum	crocus		113	-	+	-
P. dissotocum	crocus		117	-	+	-
P. dissotocum	crocus		119	-	+	-
P. heterothallicum	soil		21.7	-	+	-
P. heterothallicum	soil		22.2	-	+	-
P. heterothallicum	soil		23.8	-	+	-
P. heterothallicum	soil		26.8	-	+	-
P. heterothallicum	soil		28.6	-	+	-
P. intermedium	hyacinth		11	+	+	5
P. intermedium	hyacinth		32	-	+	5
P. intermedium	soil	221.68 T+	46	+	+	7
P. intermedium	iris		105	-	+	4
P. intermedium	hyacinth		294	-	+	5
P. intermedium	tulip		328	-	+	3
P. irregulare	crocus		8	-	+	4
P. irregulare	crocus		24	+	+	7
P. irregulare	beet	265.38	49	+	+	-
P. macrosporum	lily	577.8	23	+	+	-
P. macrosporum	flower bulb	574.80 T+	51	+	+	-
P. macrosporum	iris		111	+	+	2
P. macrosporum	iris		265	-	+	2
P. macrosporum	lily		288	-	+	2
P. macrosporum	tulip		343	-	+	2
P. mamillatum	*Chionodoxa*	213.68	55	+	+	2
P. mamillatum	soil	212.68	54	+	+	7
P. paroecandrum	soil		11.4	+	?	-
P. paroecandrum	soil		12.4	+	?	-
P. paroecandrum	soil		27.5	+	?	-
P. paroecandrum	soil		35.5	+	?	-
P. paroecandrum	tulip		280	+	?	1
P. paroecandrum	tulip		281	+	?	1
P. paroecandrum	tulip		283	+	?	1
P. paroecandrum	*Fritilaria*		291	+	?	1
P. paroecandrum	tulip		295	+	?	1
P. sylvaticum	crocus	226.68 T+	2	+	+	-
P. sylvaticum	*Prunus*	453.67 T-	3	+	+	-
P. sylvaticum	crocus		116	-	+	4
P. sylvaticum	iris		284	-	+	4
P. sylvaticum	freesia		286	-	+	4
P. sylvaticum	*Ornithogalum*		290	-	+	7
P. ultimum var. *ultimum*	tulip		10	+	+	1
P. ultimum var. *ultimum*	lily		30	-	+	4
P. ultimum var. *ultimum*	iris		266	-	+	3
P. ultimum var. *ultimum*	crocus		268	-	+	3
P. ultimum var. *ultimum*	lily		289	-	+	3
P. ultimum var. *ultimum*	tulip		335	-	+	3
P. violae	iris		99	-	+	6
P. violae	iris		110	-	+	6
P. violae	iris		112	-	+	2

Table 3

Pythium species isolated from soil from bulb fields (indicated by number) in five different cultivation areas (De Noord, De Zuid, Kennemerland, Wieringermeer [WM] and West Friesland [WF]).

	De Noord												De Zuid										Kennemerland								WM			WF	
Species	1	5	6	13	14	23	24	25	26	27	28	35	2	3	4	15	16	17	18	19	20	29	30	7	8	9	11	12	21	22	34	31	32	33	10	No. of fields
P. dissotocum																						+														1
P. heterothallicum			+		+	+	+		+		+	+														+	+	+	+	+				+	14
P. intermedium		+	+	+			+			+	+		+			+				+	+		+		+	+	+				+	+		+	17
P. macrosporum		+																																		1
P. mamillatum			+											+			+	+	+	+				+												7
P. oligandrum					+			+																												2
P. paroecandrum										+		+															+	+								4
P. spinosum													+																							1
P. sylvaticum	+	+	+		+	+	+	+	+		+	+	+					+		+		+		+	+	+			+	+	+	+	+	+	+	24
P. species										+													+				+	+						+		5
P. rostratum																						+	+													2
P. torulosum																				+																1
P. ultimum	+	+				+	+	+	+		+	+	+	+	+		+		+		+	+	+	+				+		+	+				+	21
P. violae	+				+																			+												3
Group G						+																+	+							+			+			6
Group HS							+																													1
total no. species/field	3	4	4	1	4	4	5	3	3	3	4	4	4	2	1	1	2	2	2	4	2	5	5	4	2	3	4	4	2	4	3	3	3	2	4	

isolates (Table 1). Five isolates, originating from five different fields and revealing four distinctive RFLP banding patterns, could not be identified with either molecular or morphological method.

Pathogenicity tests

A selection of fifty one isolates representing ten species was used in the pathogenicity tests. Isolates differed considerably in virulence and aggressiveness on iris, crocus, hyacinth, tulip, and lily, as did the crops in susceptibility to *Pythium* (Table 4). Nineteen isolates which caused no significant infection on either crop were considered non-pathogenic (Table 2). This was the case for all tested isolates of *P. dissocotum*, *P. heterothallicum*, and one or more isolates from *P. irregulare, P. macrosporum*, *P. paroecandrum*, and *P. sylvaticum*. These isolates were discarded in further statistical analysis. Scores of thirty two pathogenic isolates and five crops were clustered according to a minimal contribution to non-additive variance. This approach groups isolates with relatively deviant scores on one or more crops compared to other isolates. If significant differences in host range and aggressiveness occur between groups of isolates, crop rotation could be a method of reducing the population densities. Seven groups of pathogenic isolates were distinguished (Figure 1) with significant differences in virulence and aggressiveness between groups. The majority of isolates of *P. paroecandrum*, *P. ultimum*, and *P. macrosporum* clustered together in one group for each species. However, five of seven groups contained isolates of more than one species, indicating that isolates of different species displayed similar host ranges and aggressiveness. All five pathogenic isolates of *P. paroecandrum*, originally isolated from diseased tulips and Fritilaria, clustered together in group 1, primarily pathogenic on tulip and lily. Four other isolates of the same species, isolated from soil, were non-pathogenic. Four isolates of *P. macrosporum* were pathogenic on all crops and clustered together in group 2. Two other *P. macrosporum* isolates were non-pathogenic. All tested isolates of *P. ultimum* var. *ultimum* were pathogenic on 1 or more crops. Four of six isolates clustered in group 3, causing severest infection in crocus, tulip and lily. Three of four pathogenic isolates of *P. sylvaticum* grouped together in group 4, causing moderate infection in all crops except iris. Three other isolates of the same species were non-pathogenic. Group 5 contained three isolates of *P. intermedium*, predominantly pathogenic on hyacinth. Three other isolates of *P. intermedium*, clustered in three different groups. Group 6 contained two isolates of *P. violae*, pathogenic on crocus. One other isolate of *P. violae* clustered in group 2. Group 7 contained four isolates of four different species, causing severest infection on crocus and lily.

Crops were clustered with relatively deviant susceptibility for one or more isolates compared to the other crops. The crops showed no significant similarity in their susceptibility for individual isolates (Figure 1). However, tulip and lily were

closely related, and could primarily be distinguished from the other crops by their susceptibility for isolates of *P. paroecandrum* and *P. ultimum* in group 1. Crocus was distinguished from the other crops by high susceptibility for *P. violae* isolates in group 6, and, to a lesser extent, to isolates from group 7. Hyacinth revealed relatively high susceptibility for *P. intermedium* isolates in group 5. Iris was least susceptible for *Pythium*, showing little infection, except for the isolates in group 2 which caused severe infection on all crops including iris.

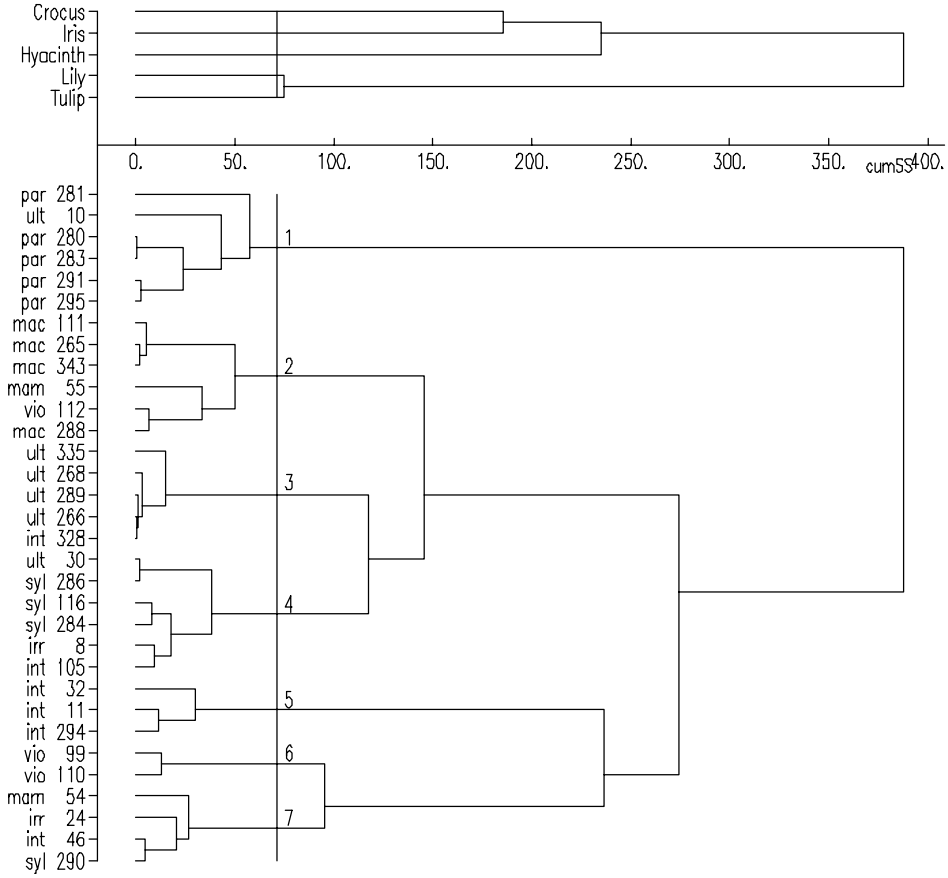


Figure 1 Dendrogram produced by cluster analysis of host crops and *Pythium* isolates, based on mean % infection from the pathogenicity experiments. Crops and isolates are clustered succesively according to similarity in terms of minimal contribution to variance for interaction. Numbers 1 to 7 refer to significant different groups of isolates, as indicated by the significance level (vertical line at cumSS 71.1).

Table 4 Mean % infection in five bulb crops inoculated with thirty two pathogenic *Pythium* isolates, sorted towards increasing aggressiveness of isolates (vertically) and increasing susceptibility of the crops (horizontally). Results from three pathogenicity tests combined.

No.	isolate	iris	hyacinth	crocus	tulip	lily	mean
112	*P. violae*	0.5	0.9	4.0	0.5	19.8	5.1
55	*P. mamillatum*	7.2	1.4	4.5	0.8	15.6	5.9
54	*P. mamillatum*	0.9	2.5	39.8	0.6	20.8	12.9
290	*P. sylvaticum*	0.5	0.9	35.3	2.0	41.3	16.0
8	*P. irregulare*	0.5	4.9	6.7	5.5	65.3	16.6
46	*P. intermedium*	0.5	2.7	21.7	2.7	57.4	17.0
116	*P. sylvaticum*	1.5	5.4	5.5	24.8	64.0	20.2
11	*P. intermedium*	0.8	53.7	3.4	4.3	47.4	21.9
286	*P. sylvaticum*	0.5	2.6	13.5	30.3	64.6	22.3
32	*P. intermedium*	0.5	43.3	19.4	1.9	54.7	24.0
30	*P. ultimum*	0.5	1.7	15.5	26.0	80.5	24.8
24	*P. irregulare*	0.5	1.4	48.7	0.6	74.2	25.1
284	*P. sylvaticum*	0.5	7.1	4.7	36.5	82.1	26.2
105	*P. intermedium*	0.5	29.7	10.1	22.2	79.3	28.4
291	*P. paroecandrum*	0.5	0.6	1.8	48.2	94.8	29.2
295	*P. paroecandrum*	0.5	0.4	3.5	65.9	92.1	32.5
280	*P. paroecandrum*	0.8	0.2	7.5	59.2	96.8	32.9
283	*P. paroecandrum*	0.5	0.1	6.0	67.4	96.6	34.1
99	*P. violae*	0.6	18.7	96.8	0.5	61.2	35.6
289	*P. ultimum*	2.4	0.9	49.9	29.5	96.5	35.8
110	*P. violae*	1.3	9.2	99.4	0.8	83.2	38.8
281	*P. paroecandrum*	0.5	7.0	4.0	84.2	99.1	39.0
294	*P. intermedium*	0.5	84.0	16.9	23.4	78.8	40.7
335	*P. ultimum*	20.5	7.1	43.3	46.5	99.1	43.3
268	*P. ultimum*	16.5	2.4	60.8	42.7	98.2	44.1
10	*P. ultimum*	0.7	0.9	59.9	87.0	97.9	49.3
328	*P. intermedium*	15.6	4.5	74.9	65.3	99.0	51.9
266	*P. ultimum*	14.0	9.5	76.5	69.2	99.0	53.7
343	*P. macrosporum*	67.0	23.3	38.7	64.2	98.3	58.3
288	*P. macrosporum*	39.0	64.2	75.3	70.8	95.5	69.0
111	*P. macrosporum*	81.4	32.8	63.7	72.8	97.2	69.6
265	*P. macrosporum*	70.4	40.8	75.8	78.2	99.4	72.9
	mean	10.9	14.5	34.0	35.4	76.6	34.3

Discussion

Molecular analyses are now commonly used for fungal identification. Usually, these analyses are performed using pure cultures (Martin *et al*., 2000). Many methods and media for isolation of *Pythium* spp. from soil have been tested (Jeffers & Martin, 1986; Lumsden *et al*., 1975; Mircetich & Kraft, 1973; Tsao, 1970). The choice of basal medium and anti-microbial agents play an important role in the efficacy of selective media in isolating *Pythium* spp. Mycelia and spores of *Pythium* spp. possess differential sensitivity to antibiotics (Tsao, 1970; Mircetich & Kraft, 1973). Also, *Pythium* spp. show differential responses to incubation temperatures and the pH of the isolation media (Lumsden *et al*., 1975). Of fifteen selective media recommended for isolation of *Pythium* spp. directly from soil, corn meal agar (CMA) supplemented with several additives and antibiotics, was most efficient (Mircetich & Kraft, 1973). In our research, only one selective medium, CMA containing pimaricin and terramycin, was used for isolation of *Pythium* spp. Undoubtedly, the best medium to isolate a given *Pythium* species under the diverse biological and environmental conditions that exist will not always be the same (Jeffers & Martin, 1986; Mihail *et al*., 2002). However, since species with different optimum growth temperatures as well as fast growing species (e.g. *P. intermedium, P. sylvaticum*, *P. ultimum*) and slow growing species (e.g. *P. dissotocum*, *P. rostratum*, *P. torulosum*, *P. violae*) were isolated on this medium, it was assumed that the isolated species were representative for each soil sample.

The twenty five *Pythium* species included as reference in the molecular identification represented a broad spectrum common to Dutch soils (Van der Plaats-Niterink,1975; 1981). Variations in the ITS rDNA regions allowed differentiation of these species, except for *P. arrhenomanes* and *P. aristosporum*. This is consistent with the results from Chen *et al*. (1992) and Wang and White (1997), who reported that most species could be distinguished by this method. The ITS regions appeared to be variable between, but largely conserved within *Pythium* species. However, Wang and White (1997) found that *P. aphanidermatum* and *P. butleri* were indistinguishable from each other, whereas several other species could only be distinguished by using seven restriction enzymes. Molecular identification and characterization of *Pythium* communities was also performed by Mazzola *et al*. (2002) and Moorman *et al*. (2002). They compared base pair sequences of ITS1, 5.8S, and ITS2 regions of rDNA, which effectively differentiated the species they encountered, except for sequences from *P. ultimum* and *P. irregulare* isolates, which exhibited some variation within each of the species. The intraspecific differences were insufficient to differentiate populations (Moorman *et al*., 2002).

In our survey, nine isolates, morphologically identified as *P. paroecandrum*, revealed two different banding patterns, both not identical to the banding pattern of the isolate used for species description by Van der Plaats-Niterink (1981). The

separation of the isolates in two groups with different RFLP banding patterns coincided with the origin of the isolates: patterns of five isolates from diseased plants were dissimilar to those of four isolates from soil. Also, the distinctive banding patterns of the isolates corresponded with the division in pathogenic and non-pathogenic isolates as determined in the bioassay. Martin (1990) also found differences in the ITS region of *P. paroecandrum* isolates for numbers and locations of restriction sites and he distinguished four groups. This raises the question whether the species description of *P. paroecandrum* might need adjustment and perhaps should be divided into two or more different species. Three percent of the *Pythium* isolates could not be identified by either molecular or morphological methods. This was not unexpected, because a restricted number of twenty five reference species was used for molecular identification, and morphological identification can be severely hindered by variable morphological traits and absence of reproductive structures (Van der Plaats-Niterink, 1975; Mihail *et al*., 2002). Mazzola *et al*. (2002) and Moorman *et al.* (2002) also encountered some isolates which showed significant differences in ITS rDNA base pair sequences with their closest match among the referent strains. They suggested that these isolates could be new species. Clearly, the use of molecular criteria can alleviate some of the confusion in identification of closely related species. However, molecular features also reveal differences within and between species, which make classification more complex.

The majority of sampled bulb fields contained a mixture of up to five different *Pythium* species and each field contained one or more potentially pathogenic species. Commonly isolated species included *P. sylvaticum*, *P. ultimum*, *P intermedium*, and *P. heterothallicum*. In general, Dutch arable fields seem to contain mixtures of a variety of different *Pythium* species. Van der Plaats-Niterink (1975) examined samples from soil and diseased plants originating from flax fields in the provinces of Noord-Holland and Friesland. She also found *Pythium* in each sampled field, with up to seven different species per field. In total, nine species occurred in flax fields in Noord-Holland, being *P. intermedium*, *P. irregulare*, *P. oligandrum*, *P paroecandrum*, *P. rostratum*, *P. sylvaticum*, *P. ultimum*, *Pythium* group HS and *Pythium* group P. *Pythium* populations in Friesland were different from those in Noord-Holland, the latter showing greater variation in species. All species found by Van der Plaats-Niterink, except *P. rostratum* and *Pythium* group P, were also detected in bulb fields in Noord-Holland. Based on her results from 1966 to 1972, Van der Plaats-Niterink concluded that *P. heterothallicum* was not common in the Netherlands and occurred only in the Friesland samples. More than twenty years later, our results show that *P. heterothallicum* was common in ‘De Noord’ and ‘Kennemerland’ in Noord-Holland, but could not be detected in ‘De Zuid’ in Zuid-Holland. In the sampled bulb fields, species composition varied within and between cultivation areas, consistent with the findings of Van der Plaats-Niterink (1975). So

far, this variation in species composition can not be explained. Differences in soil type, soil management practices and crop rotation might have some influence. Some care should also be exercised in judging the relative importance of the *Pythium* species presented here. In many sampled fields no obvious root rot symptoms occurred in the bulb crops at time of sampling, and, in those days, application of fungicides, like dichloropropene-etridiazol, fosetyl-Al and metalaxyl, was common practice in susceptible crops. Differential sensitivity to these chemicals may have influenced isolation of *Pythium* species. Historical data on species isolated and hosts infected by particular species may not reflect the present situation because of the significant cultural changes that have occurred over time in agriculture, like the use of pesticides, mechanization and crop rotation.

Pathogenic isolates from *P. irregulare* and *P. macrosporum* have regularly been isolated from diseased plants. For example, Saaltink (1969) isolated *P. irregulare* from diseased hyacinth plants in seven out of eleven fields. Remarkably, these species were not or only once isolated from field soil. Van der Plaats-Niterink (1975) also observed that some species were isolated from diseased plants and were not detected in soil samples from corresponding fields. Possibly, these species occurred in very low concentrations in the soil, sufficient for infection, but not for detection as described.

The level of infection caused by *Pythium* in the field will be greatly influenced by soil physical, chemical and biological factors, by management practices (Mazzola *et al*., 2002; Van Os *et al*., 1999), and by the weather conditions, which may influence both the host and the pathogen. Infection levels may vary between years and fields. In order to exclude these sources of variation, our pathogenicity tests were performed in the greenhouse, under standardized optimum conditions for flower production, in a substrate low in microbial biomass and activity (river sand). Because of this, it was possible to compare results from isolates tested in separate experiments. However, as a consequence, the results may not reflect disease severity levels as they occur in the field. Tulip and lily, for example, are very susceptible to *Pythium* under conditions for flower production, and less susceptible in bulb cultivation under field conditions. Also, different crops had different optimum incubation temperatures, and, considering infection levels, interaction between *Pythium* isolates and incubation temperatures cannot be excluded. For example, infection levels by *P. macrosporum* in iris can be 10% higher at 18°C than at 9°C (Chapter 4).

Not all isolates from diseased plants displayed pathogenicity in the pathogenicity test. It is usually assumed that any *Pythium* found in a sample is a pathogen, however, isolates may have been the primary cause of disease or merely associated with plants whose health was weakened by other factors. In the current study, in order to catagorize the isolates on pathogenicity, they were clustered based on interaction between host and disease severity. A considerable diversity in species

composition was found between groups. Groups contained up to four different *Pythium* species, indicating that isolates of these species displayed similar host ranges and aggressiveness. Considering non-pathogenic isolates as a separate group, isolates of each species clustered in more than one group (Figure 1; Table 2), indicating differences in host range and aggressiveness between isolates within all tested species. Saaltink (1969) repeatedly isolated *Pythium* species from rotting roots of iris and crocus, but never *P. violae*. He found *P. violae* to be pathogenic on hyacinth and suggested that this species determined the specificity of the root rot complex for hyacinth. In our experiments, however, three isolates of *P. violae* were included, originating from iris roots. Two of these isolates were mildly pathogenic on hyacinth and highly pathogenic on crocus. Considering the intraspecific variation in pathogenicity observed for all *Pythium* species, including *P. violae*, it is conceivable that other *P. violae* isolates could display different host ranges. Other reports also describe variation in pathogenicity within species. McCarter and Littrell (1970) reported that individual isolates of *P. aphanidermatum* and *P. myriotylum* varied markedly in pathogenicity on susceptible crops. Yanar *et al*. (1997) detected a range in aggressiveness among field isolates of *P. arrhenomanes* from maize. Similar differences were reported among isolates from maize and sugarcane (Deep & Lipps, 1996; Hoy *et al*., 1988).

Traditionally, bulb crops are grown in the western part of the Netherlands on sandy soil. Rotation with bulb crops only is common practice in these areas, and *Pythium* root rot is a serious threat. Generally, crop rotation is a method of reducing the populations of soil pathogens. Because the more common and destructive pathogenic *Pythium* spp. have wide host ranges, this procedure is not usually successful (Hendrix & Campbell, 1973). In the past, bulb growers have been advised to avoid cultivation of susceptible crops on fields heavily infested with *Pythium*, and practice broad crop rotation. Generally, short rotation with susceptible bulb crops (once in every two or three years) resulted in increasing damage, whereas longer rotations (once in every four or five years) revealed less problems. However, rotation effects were not consistent and the working mechanism remained unclear. Based on the presented results, sampling and identification of *Pythium* species from soil samples or diseased plants would not be sufficient in order to advise on crop rotation with bulbous crops, because the majority of sampled fields contained a mixture of species and all tested species showed intraspecific variation in pathogenicity. The results also revealed that iris, crocus, hyacinth, tulip, and lily showed no significant similarity in their susceptibility for individual *Pythium* isolates, although tulip and lily are closely related. Therefore, crop rotation with iris, crocus, hyacinth, and tulip or lily could still be effective in reducing populations of *Pythium*. The efficacy may vary between fields depending on the pathogenicity of the *Pythium*s present.

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CHAPTER 8

Summary and concluding remarks

Photograph Iris ‘White van Vliet’.

CHAPTER 8 Summary and concluding remarks

Summary

In the Netherlands, the majority of spring flowering bulb crops is grown on sandy soil, in rotations with other bulb crops only. Root rot caused by *Pythium* spp. is a major disease on these soils resulting in yield loss up to 40% income reduction from saleable bulbs. This thesis describes the research carried out on *Pythium* root rot, designed to obtain knowledge for the development of alternative control measures in order to reduce the use of and dependence on chemical control. Effects of several cultural practices on disease development, on disease suppression by the soil microflora, and occurrence and pathogenicity of *Pythium* species in bulb fields were investigated. Bulbous iris and crocus were used as target crops in this study.

Up to 1990, little was known of the time of infection and subsequent disease development in bulb crops. Observations in field grown crops had indicated that planting date might have an effect on damage caused by *Pythium*, which might be associated with soil temperature. In order to answer the question whether the onset and subsequent development of root rot in iris and crocus is affected by planting time and soil temperature, these crops were planted at two planting dates in soil infested with *Pythium* spp. (Chapter 2). The disease development was remarkably different for iris and crocus. Major increase of disease severity occurred shortly after planting in crocus, and during spring and summer in iris. For each crop the disease progress was similar every year, regardless of the *Pythium* species used for infestation. The distinct difference in disease development between iris and crocus seemed to be determined by the crop rather than by the pathogen. The shape of the disease development curves of both crops could be explained on the basis of root development in time and appearance of the infection. In iris, new roots were formed throughout the growing season and root tips of both main roots and lateral roots were susceptible at all times along the whole rooting system. Disease progress correlated with the number of root tips, which increased during the growing season. In crocus, all roots developed simultaneously and seemed to be susceptible to *Pythium* only during a short period after planting, at *c.* 5 cm root length. Although infection started in root tips, unaffected root tips showed resistance after two months. No correlation was found between disease development and soil temperatures occurring during the cultivation periods. Late planting resulted in delayed root development, infection and subsequent disease development in both crops. In crocus, the delay in infection combined with a limited period of susceptibility resulted in a reduced disease development. Based on the disease severity, the benefit of planting late appeared considerable for crocus, but seemed relatively small for iris. Whether delayed planting will be profitable or not, depends on the susceptibility of the crop to *Pythium*,

but also on sensitivity to several physiological effects of late planting on bulb yield and quality.

Soil management practices are known to affect plant growth and disease development in agricultural soils (Kennedy & Smith, 1995; Van Bruggen, 1995). In ornamental bulb culture on sandy soil, fumigation and floodin are applied in order to control several diseases and weeds. *Pythium* spp. can survive or be (re-)introduced after these treatments, while many other microorganisms will be reduced or eliminated (Bollen, 1979; Mitchell & Alexander, 1962). Fumigation and flooding have different modes of action and different species of the soil microflora are killed as a result of these measures. In order to determine whether these cultural practices could have adverse effects on the occurrence of *Pythium* root rot, the effects of flooding and soil fumigation with *cis*-dichloropropene or methylisothiocyanate (metam-Na) on disease suppression against *Pythium* spp. was tested in pot and field experiments (Chapter 3). Disease suppression was reduced by both flooding and fumigation treatments, resulting in enhanced infection in iris and crocus and reduction of bulb yields. It was demonstrated that the disease suppression is of a biological nature, and that disease severity is more related to effects of soil treatments on the soil microflora than to the initial inoculum density of *Pythium*. After flooding, disease suppression was restored within the experimental period of two years. Whereas after fumigation, disease suppression was only partially restored and persistent effects cannot be excluded. Timing of these measures within the crop rotation might be of great importance. An adequate delay between soil treatment and cultivation of a susceptible crop would provide a greater advantage to other species as colonists of the soil and reduce the opportunity for *Pythium*.

In order to find a way to restore disease suppression and accelerate the recovery of the soil microflora after fumigation and flooding, (re-)introduction of specific antagonists or an abundance of competitive microorganisms by compost amendment was considered to be an interesting option (Chapter 4). Composts are well known to suppress soilborne pathogens, including *Pythium* (Hadar & Mandelbaum, 1986; Kuter *et al.*, 1988; Lumsden *et al*, 1983; Mandelbaum *et al.*, 1988; Tuitert *et al.*, 1998). In general, application of composts can affect plant health directly and indirectly through alterations in physical, chemical and biological properties of the soil (Windels, 1997). In bulb cultivation on sandy soil, application of nutrient-poor organic matter, such as compost, is essential in order to maintain organic matter contents. In the Netherlands, composted organic household waste is widely available. Amendment of this compost in the field is limited by law to six tons dry matter per hectare per year, or twelve tons per hectare per two years (*c*. 1% w/v), because of heavy metal contents. I investigated whether amendment of soil with small quantities (0.5% to 5% w/v) of matured, composted vegetable, fruit, and garden waste (VFG-compost) could suppress *Pythium* root rot in iris and crocus under

controlled and under field conditions. Again, the target crops reacted differently. In pot experiments with iris, compost amendment resulted in reduction of disease development in flooded and fumigated soil, whereas compost amendment in untreated soil had no effect on infection. The disease suppressive effect of the compost was not affected by the maturation temperature of the VFG-compost (10°C or 20°C) nor by the temperature during iris cultivation (9°C or 18°C). In crocus, compost amendment resulted in enhanced disease development in untreated, flooded, and fumigated soil. In field experiments with untreated and fumigated soil, compost amendment increased disease severity in crocus and decreased corm yields in infested treatments. Thus, the host plant is the discriminating factor between disease suppression or stimulation as a result of amendment with VFG-compost. So far, the mechanism responsible for the differential plant responses remain unclear. Factors which could be of influence include presence or absence of induced resistance by the compost microflora, and differences in root development, exudation and rhizosphere microflora between iris and crocus. Within the scope of this thesis, further experiments were focused on the mechanism of disease suppression rather than on the differential plant responses.

In order to gain insight into the nature of *Pythium* suppression and the effects of soil treatments like fumigation, flooding and compost amendment, I studied the changes that these treatments induce in microbial parameters (Chapter 5) and the microbial community composition (Chapter 6). The hypothesis was tested that suppression of *Pythium* was linearly related to the activity of the microbial community and to the presence of specific groups. Different levels of suppression were established in sandy soil by application of several soil treatments. Percentage of infection in iris caused by *Pythium macrosporum* was lowest in untreated soil and progressively increased in sterilized soil amended with 1% compost, fumigated soil and flooded soil (fumigation and flooding with equal levels of disease severity) and was highest in sterilized soil. When assessing the relationship between the level of disease incidence, *Pythium* growth rate through soil, and various microbial parameters were investigated, I found that the value of microbial parameters as predictors for disease suppression was strongly influenced by the data set (soil treatments) used for statistical analysis. Compost amendment in particular, had a major influence on correlations between microbial parameters and disease suppression. Microbial parameters, such as biomass, dehydrogenase activity, glucose uptake and respiration, were negatively associated with *Pythium* growth rates, indicating that high microbial biomass and activity induced suppression of *Pythium* growth through soil. Neither combination of the quantitative microbial parameters could fully explain the disease suppression. So, other aspects of pathogen development must influence infection. These could include interactions on the root surface. Moreover, it seems that competition for carbon may not be the main

mechanism in disease suppression, as indicated by the lack of correlation between glucose uptake and disease incidence. Competition for other nutrients or other forms of antagonism may play a role, as indicated by the relatively high correlation between general microbial activity and disease incidence. Furthermore, different soil treatments may affect disease suppression by interference with different mechanisms. Changes in species composition and diversity of the microflora as a result of the soil treatments might clarify mechanisms which bring about the disease suppression.

In order to analyse the microbial community independent of the applied treatment and related to *Pythium* suppression, bacterial and fungal community profiles were made using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified ribosomal RNA genes. Four PCR-DGGE strategies, two targeting bacteria and two targeting fungi, were used to compare the microbial communities of untreated, fumigated, flooded, and sterilized compost-amended soils, and the dominant community members were tentatively identified by sequencing of DGGE bands. DNA-based microbial community profiles alone were poor predictors of *Pythium* suppression, as the dominant microbial populations consistently remained present in the profiles, even after rigorous soil treatments such as fumigation and flooding. The compost treatment of sterilized soil appeared to have the most profound effect on the dominant microbial populations in the soil. The restoration of suppressive activity against *Pythium* in compost-amended soil was associated with a different microbial community than observed in untreated soil. Thus, different microbial communities can induce this suppression of *Pythium*.

Generally, crop rotation is a method of reducing the populations of soil pathogens (Hendrix & Campbell, 1973). Success of this procedure is determined by aspects such as the occurrence and host range of pathogenic species. In order to determine whether detection and identification of *Pythium* spp. from field soil could be used as a tool to advise on crop rotation, a survey of species occurring in bulb fields was made in five important areas for bulb cultivation in the western part of the Netherlands (Chapter 7). *Pythium* isolates were identified using PCR-RFLP analysis. Sixteen different species were identified among the 163 isolates of *Pythium* recovered from thrity five fields. The majority of sampled fields contained a mixture of species. Each field contained one or more potentially pathogenic species. Commonly isolated species included *P. sylvaticum*, *P. ultimum*, *P. intermedium*, and *P. heterothallicum*. A selection of identified isolates was tested for pathogenicity on five susceptible bulb crops (iris, crocus, hyacinth, tulip, and lily). Differences in host ranges and aggressiveness occurred between isolates within all tested species. Based on these results, sampling and identification of *Pythium* spp. in field soil would not be sufficient in order to advise on crop rotation with bulbous crops. Host ranges from pathogenic *Pythium* isolates varied from one to all five of the tested bulb crops.

The results also revealed that iris, crocus, hyacinth, tulip, and lily differed in their susceptibility for pathogenic *Pythium* isolates, although tulip and lily are closely related. Therefore, crop rotation with iris, crocus, hyacinth, and tulip or lily could still be effective in reducing populations of *Pythium*.

In this thesis, a number of agronomic approaches have been proposed in order to control *Pythium* root rot in ornamental bulb culture. Because the suggested alternatives depend on living organisms, their efficacy may vary under a wide range of environmental, cultural, and biotic conditions. The best results may be expected when different strategies are combined. The generated knowledge has contributed to a better understanding of *Pythium* root rot in flower bulb culture, and actuated a gradual change in the grower's mentality concerning the possibilities for reduction of the use of chemical control. Growers have adjusted the implementation of several soil management practices within the crop rotation as a result of the reported findings.

Concluding remarks on root infection in bulb crops

Successful infection depends on the probability that host and pathogen encounter, followed by interaction with the root surface and the influence of root physiology. The chance that roots and *Pythium* meet is determined by density and dispersal of both. Infection occurred mainly at root tips and root development (i.e. density and susceptibility of root tips in time and place) is distinctly crop specific. *Pythium* dispersal through growth in soil, is negatively correlated with microbial biomass and activity, and suppression could be induced by different microbial communities. *Pythium* growth rate is stimulated by soil fumigation and flooding, and reduced by compost amendment. Thus, these soil treatments affect the probability of infection. They have more influence on eventual disease severity than initial *Pythium* inoculum density. On the root surface, crop specific interactions occurred between the host, *Pythium* and the rhizosphere microflora. These interactions were also influenced by soil treatments, and may include competition for nutrients, other forms of antagonism, or induced resistance by the microflora.

Concluding remarks on practical implications

All bulb fields seem to be infested with one or more pathogenic *Pythium* spp. with various host ranges. Crop rotation with bulb crops could be effective in reducing populations of *Pythium*. The efficacy, however, may vary between fields.

Soil fumigation and flooding reduce disease suppression against *Pythium*. Therefore, application of these measures should be avoided. Other cultural practices, like subsoil ploughing, may have similar effects. If necessary, these measures should be applied two years or more before cultivating a susceptible crop.

Several interactions between host plant, *Pythium* and soil microflora are crop specific. Therefore, disease control measures should be tested for individual crops. Amendment of matured VFG-compost can reduce disease severity in iris, but stimulates disease severity in crocus. Compost amendment could best be implemented within the crop rotation after measures like soil fumigation or flooding, prior to iris but not prior to crocus. In general, regular compost application on sandy soil may improve physical, chemical, and biological properties of the soil, stimulating the soil microflora and general suppressiveness.

Control measures against *Pythium* in iris should be effective towards spring and summer, whereas for crocus they should especially be effective within two months after planting. Based on disease severity, the benefit of planting late appeared considerable for crocus and seemed relatively small for iris. Whether delayed planting will be profitable or not, depends on the susceptibility of the crop to *Pythium*, but also on sensitivity to several physiological effects of late planting on bulb yield and quality. For example, many commercially important cultivars of *Crocus* are very susceptible to *Pythium*, but also sensitive to calcification, sprout breakage and growth retardation as a result of late planting. Thus, it is not likely that planting late will be profitable for this group of cultivars.

Future Research

Mechanisms of disease suppression by the soil microflora may change in time due to environmental changes and crop development. Periodical monitoring of microbial parameters may elucidate some of the observed phenomena, such as the high microbial biomass and activity after compost amendment resulting in relatively poor disease suppression. Also, the mechanism(s) responsible for the differential plant responses on compost amendment remain(s) unclear. This needs to be studied in more detail. In the field, effects of compost amendment on disease suppression in general may interact or be overruled by other aspects within commercial farming systems. In order to evaluate effects of compost amendment under these circumstances interdisciplinary, system-level research is needed rather than disciplinary research.

Many biological agents against *Pythium* spp. are well documented and tested under controlled circumstances. However, the efficacy of these agents may be crop specific and influenced by environmental factors, and needs to be evaluated under standard conditions for bulb cultivation.

In general, molecular tools will considerably facilitate ecological research on disease suppression. DNA-based microbial community profiles were poor predictors of *Pythium* suppression. However, community profiles based upon rRNA, or the use of other methods that focus on the active populations in a sample, may be more useful to gain insight in the nature of suppression and may reveal potential

parameters. In recent years, molecular research is aimed at the development of micro-array's for measurement of numerous physical, chemical and biological characteristics of soil, including the presence of potential pathogens and parameters for disease suppression. The current knowledge about suitable parameters for suppression of *Pythium* in bulb cultivation is not adequate for this purpose. However, combined with bio-assays, the micro-array technique may reveal new relations between soil characteristics and disease suppression.

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Samenvatting

Het merendeel van de voorjaarsbloeiende bolgewassen wordt in Nederland geteeld op zandgrond, in een vruchtwisseling met uitsluitend bolgewassen. Wortelrot veroorzaakt door *Pythium* spp. is een van de belangrijkste ziekten op deze grondsoort. Aantasting kan leiden tot 40% financiële opbrengstderving uit leverbare bollen. In dit proefschrift wordt het onderzoek beschreven naar de ontwikkeling van alternatieve bestrijdingsmethoden om het gebruik en de afhankelijkheid van chemische middelen tegen *Pythium* wortelrot te verminderen. Hierbij is gekeken naar effecten van diverse teeltmaatregelen op de aantasting en op de ziektewering door de bodemmicroflora, en naar het vóórkomen en de pathogeniteit van *Pythium*-soorten in bollenpercelen. Iris en krokus zijn gebruikt als toetsgewas.

Tot 1990 was er weinig bekend over het moment van infectie en de daaropvolgende ziekteontwikkeling in bolgewassen. Uit veldwaarnemingen bleek dat de plantdatum een effect zou kunnen hebben op de schade door *Pythium*, hetgeen mogelijk was gerelateerd aan de bodemtemperatuur. Om na te gaan of de infectie en de daaropvolgende ontwikkeling van wortelrot in iris en krokus wordt beïnvloed door de plantdatum en de bodemtemperatuur, zijn beide bolgewassen op twee data in het najaar geplant in met *Pythium* spp. besmette grond (Hoofdstuk 2). De ziekteontwikkeling in krokus was opvallend anders dan in iris. In krokus was er een sterke toename van de aantasting vlak na het planten, terwijl dit in iris vooral in het voorjaar en de zomer optrad. In beide gewassen verliep de ziekteontwikkeling elk jaar hetzelfde, ongeacht de *Pythium* soort waarmee de grond was besmet. De verschillen in de ziekteontwikkeling tussen iris en krokus leken afhankelijk van het gewas en niet van het pathogeen. Het verloop van de ziekteontwikkeling kon voor beide gewassen worden verklaard op basis van de wortelontwikkeling en de aard van aantasting. In iris werden het hele groeiseizoen nieuwe wortels gevormd en wortelpunten van zowel hoofd- als zijwortels waren altijd vatbaar. De aantasting en nam toe gedurende het groeiseizoen en correleerde met de toename van het aantal wortelpunten. In krokus ontwikkelden alle wortels zich gelijktijdig en waren uitsluitend gedurende een korte periode na planten vatbaar voor *Pythium*. Wortelpunten die niet werden aangetast bleken na twee maanden ongevoelig te zijn geworden. Bij geen van beide gewassen is er een verband gevonden tussen de ziekteontwikkeling en de bodemtemperatuur tijdens het groeiseizoen. Laat planten resulteerde in een vertraagde wortelontwikkeling en in uitstel van de aantasting en ziekteontwikkeling in beide gewassen. In krokus resulteerde de combinatie van een beperkte vatbare periode met uitstel van infectie in minder aantasting. In dit opzicht leek laat planten een aanzienlijk voordeel op te leveren voor krokus. Voor iris was het voordeel slechts beperkt. Of laat planten voor de teler rendabel is hangt af van de vatbaarheid van het

gewas voor *Pythium*, maar ook van de gevoeligheid voor diverse fysiologische effecten op de bolopbrengst en bolkwaliteit als gevolg van laat planten.

Teeltmaatregelen zoals grondbewerking beïnvloeden de plantengroei en ziekteontwikkeling in de landbouw (Kennedy & Smith, 1995; Van Bruggen, 1995). In de bloembollenteelt op zandgrond worden chemische grondontsmetting en inundatie toegepast ter bestrijding van diverse ziekten en onkruiden. *Pythium* spp. kunnen overleven of worden geherintroduceerd na deze behandelingen, terwijl vele andere micro-organismen sterk in aantal teruglopen of verdwijnen (Bollen, 1979; Mitchell & Alexander, 1962). *Pythium* zou hierdoor meer kans kunnen krijgen om aantasting te veroorzaken Grondontsmetting en inundatie hebben een verschillend werkingsmechanisme en verschillende soorten van het bodemleven sterven als gevolg van deze maatregelen. Om te bepalen of deze cultuurmaatregelen een averechts effect kunnen hebben op het optreden van *Pythium* wortelrot, zijn de effecten van inundatie en ontsmetting met cis-dichloorpropeen of methylisothiocyanaat (metam-Na) getest op de ziektewering tegen *Pythium* spp. in pot- en veldexperimenten (Hoofdstuk 3). Zowel inundatie als grondontsmetting leidden tot een verminderde ziektewering en een verhoogde aantasting en opbrengstderving in iris en krokus. Het is aangetoond dat de ziektewering biologisch van aard was en dat de mate van aantasting meer werd beïnvloed door het effect van de grondbehandelingen op de bodemmicroflora dan door de besmettingsdruk van *Pythium*. Na de inundatiebehandeling herstelde de ziektewering zich binnen een periode van twee jaar. Na grondontsmetting was de ziektewering binnen deze periode slechts gedeeltelijk hersteld en blijvende effecten kunnen niet worden uitgesloten. Een zorgvuldige planning van deze maatregelen binnen het bouwplan kan van groot belang zijn. Een minimale periode van twee jaar tussen de behandeling en de teelt van een vatbaar gewas kan de gelegenheid bieden aan andere micro-organismen om de grond te koloniseren en zodoende de kansen voor *Pythium* verkleinen.

Onderzocht is of toevoeging van compost het herstel van de bodemmicroflora en de ziektewering na grondontsmetting en inundatie zou kunnen versnellen door de (her-) introductie van specifieke antagonisten of een overmaat aan concurrerende micro-organismen door (Hoofdstuk 4). Toepassing van compost kan de plantgezondheid direct en indirect beïnvloeden door veranderingen in de fysische, chemische en biologische eigenschappen van de grond (Windels, 1997). Het is bekend dat bodempathogenen, waaronder *Pythium* spp., kunnen worden onderdrukt door compost (Hadar & Mandelbaum, 1986; Kuter *et al*., 1988; Lumsden *et al*., 1983; Mandelbaum *et al*., 1988; Tuitert *et al*., 1998). In de bollenteelt op zandgrond is de toepassing van nutriëntarme organische stof, zoals compost, essentieel om het organisch stofgehalte op peil te houden. Compost van groente-, fruit- en tuinafval (GFT-compost) is in Nederland alom beschikbaar. Toepassing van deze compost in

het veld is echter beperkt tot zes ton droge stof per hectare per jaar, of twaalf ton droge stof per hectare per twee jaar (ca. 1% w/v), vanwege het gehalte aan zware metalen. In kas- en veldproeven is onderzocht of verrijking van de grond met kleine hoeveelheden (0.5%-5% w/v) gerijpte GFT-compost effect had op de onderdrukking van *Pythium* wortelrot in iris en krokus. Beide toetsgewassen, iris en krokus, reageerden opnieuw heel verschillend. In potexperimenten met iris resulteerde toevoeging van compost aan geïnundeerde en ontsmette grond tot minder aantasting, terwijl toevoeging van compost aan onbehandelde grond geen effect had op de aantasting. Het effect van de compost op de ziektewering werd niet beïnvloed door de rijpingstemperatuur van de compost (10°C of 20°C) voorafgaand aan de toediening, of door de temperatuur tijdens de teelt (9°C of 18°C). In potexperimenten met krokus leidde de compostbehandeling tot stimulering van de aantasting in onbehandelde, geïnundeerde en ontsmette grond. In veldexperimenten met krokus resulteerde toevoeging van compost aan onbehandelde en ontsmette grond ook in meer aantasting en minder opbrengst in de met *Pythium* besmette veldjes. Er is geconcludeerd dat het gewas de doorslaggevende factor is tussen ziekteonderdrukking of ziektestimulering als gevolg van verrijking met GFT-compost. Het mechanisme dat hiervoor verantwoordelijk is, is tot nu toe onduidelijk. Factoren die van invloed zouden kunnen zijn, zijn bijvoorbeeld aan- of afwezigheid van geïnduceerde resistentie door de micro-organismen uit de compost of verschillen tussen iris en krokus in wortelontwikkeling, exudatie en micro-organismen in de rhizosfeer. Het onderzoek in dit proefschrift heeft zich geconcentreerd op de mechanismen van ziektewering, zonder verder in te gaan op de verschillen in respons tussen de gewassen.

Om inzicht te krijgen in de aard van de ziektewering tegen *Pythium* en de effecten van grondbehandelingen zoals grondontsmetting, inundatie en de toepassing van compost, is het effect bepaald van deze behandelingen op een aantal microbiële parameters en de microbiële soortensamenstelling. Onderzocht is of er een verband bestaat tussen de ziektewering tegen *Pythium* en de activiteit van de bodemmicroflora (Hoofdstuk 5) en/of de aanwezigheid van specifieke groepen (Hoofdstuk 6). Verschillende niveaus van ziektewering zijn gerealiseerd door het uitvoeren van diverse grondbehandelingen: onbehandelde grond, gesteriliseerde grond met 1% compost, ontsmette grond en geïnundeerde grond (beide met hetzelfde niveau van aantasting), en gesteriliseerde grond (de volgorde van de behandelingen is gerangschikt van laag naar hoog percentage aantasting in iris veroorzaakt door *Pythium macrosporum*). Parameters zoals microbiële biomassa, dehydrogenase activiteit, glucoseopname en ademhaling, waren negatief gecorreleerd met de groeisnelheid van *Pythium* door grond. Dit geeft aan dat een hoge microbiële biomassa en activiteit de groei van *Pythium* door grond onderdrukt. Maar, met geen enkele combinatie van microbiële parameters kon de ziektewering

(aantasting in iris) worden verklaard. De waarde van de microbiële parameters als maat voor de ziektewering bleek sterk afhankelijk van de gegevensset (grondbehandelingen) die gebruikt werd in de statistische analyse. Met name de compostbehandeling, met relatief hoge waarden, had een zeer grote invloed op de correlatie tussen de microbiële parameters en de ziektewering. Concurrentie om koolstof was niet het belangrijkste mechanisme voor de ziektewering, aangezien er geen correlatie is gevonden tussen de glucoseopname en aantasting. Andere aspecten van de pathogeen-ontwikkeling moeten dus van invloed zijn, zoals interacties op het worteloppervlak. Daar komt nog bij dat de verschillende grondbehandelingen via verschillende mechanismen de ziektewering kunnen beïnvloeden. Veranderingen in de soortensamenstelling en de diversiteit van de microflora als gevolg van de grondbehandelingen zouden deze mechanismen verder kunnen ophelderen.

Om te analyseren of de samenstelling van de microbiële gemeenschap is gerelateerd aan de ziektewering tegen *Pythium* zijn profielen gemaakt van bacterie- en schimmelgemeenschappen in de grond met behulp van denaturatie gradient gel electroforese (DGGE) van PCR-geamplificeerde ribosomale RNA genen (Hoofdstuk 6). Vier PCR-DGGE strategieën (twee gericht op bacteriën en twee gericht op schimmels) zijn gebruikt om de microbiële gemeenschappen in onbehandelde, ontsmette, geïnundeerde, en met compost verrijkte, gesteriliseerde grond te vergelijken. De dominante groepen zijn geïdentificeerd door de DGGE-banden te sequencen. Deze gemeenschapsprofielen, gebaseerd op microbieel DNA, waren slechte indicatoren voor de ziektewering tegen *Pythium*. De dominante microbiële populaties waren consistent aanwezig in de profielen, zelfs na rigoureuze behandelingen zoals ontsmetting en inundatie. Het meest uitgesproken effect op de dominante populaties in de grond is gevonden bij de compostbehandeling in gesteriliseerde grond. Het herstel van de ziektewering tegen *Pythium* in de met compost verrijkte grond was geassocieerd met een geheel andere microbiële gemeenschap dan de ziektewering in onbehandelde grond. Dus, microbiële gemeenschappen van verschillende samenstelling kunnen resulteren in vergelijkbare niveaus van ziektewering tegen *Pythium*.

Vruchtwisseling is over het algemeen een methode om de besmettingsdruk van bodempathogenen te verlagen (Hendrix & Campbell, 1973). Het succes van deze methode wordt mede bepaald door factoren zoals het vóórkomen en de waardplantenreeks van de ziekteverwekker. Om te bepalen of detectie en identificatie van *Pythium* spp. kan helpen bij de gewaskeuze in de vruchtwisseling is een inventarisatie gemaakt van *Pythium* soorten in bollenpercelen in vijf belangrijke regio's voor de bollenteelt in het westen van Nederland (Hoofdstuk 7). *Pythium* isolaten zijn geïdentificeerd met behulp van PCR-RFLP analyse. Uit vijfendertig percelen zijn 163 *Pythium* isolaten geïdentificeerd tot zestien verschillende soorten.

De meerderheid van de bemonsterde percelen bevatte een mengsel van soorten en elk perceel bevatte één of meer potentieel pathogene soorten. De meest algemeen voorkomende soorten waren *P. sylvaticum*, *P. ultimum*, *P. intermedium* en *P. heterothallicum*. Een selectie van de geïdentificeerde isolaten is getest op pathogeniteit voor vijf vatbare bolgewassen (iris, krokus, hyacint, tulp en lelie). Bij alle geteste *Pythium* soorten waren er verschillen in waardplantenreeks en agressiviteit tussen isolaten van dezelfde soort. Binnen soorten varieerde de waardplantenreeks van individuele *Pythium* isolaten van géén tot alle vijf van de geteste gewassen. Gebaseerd op deze resultaten lijkt het bemonsteren en identificeren van *Pythium* spp. in bollenpercelen geen zinvolle bijdrage te kunnen leveren aan de gewaskeuze in een vruchtwisseling met bolgewassen. Uit de resultaten blijkt echter ook dat iris, krokus, hyacint, tulp en lelie verschillen in hun vatbaarheid voor *Pythium* isolaten, hoewel tulp en lelie nauw verwant bleken. Vruchtwisseling met iris, krokus, hyacint en tulp of lelie zou daarom nog steeds effectief kunnen zijn om de besmettingsdruk van *Pythium* te beperken.

In dit proefschrift zijn een aantal agronomische benaderingen voorgesteld om *Pythium* wortelrot in de bloembollenteelt te beheersen. Omdat de voorgestelde methoden afhankelijk zijn van levende organismen, kan de effectiviteit variëren onder invloed van vele omgevingsfactoren. Het beste resultaat mag worden verwacht wanneer verschillende strategieën worden gecombineerd. De verkregen kennis heeft bijgedragen aan een beter inzicht in *Pythium* wortelrot in de bloembollenteelt en het heeft een geleidelijke mentaliteitsverandering teweeggebracht bij telers met betrekking tot de mogelijkheden voor verminderd gebruik van chemische bestrijdingsmiddelen. In navolging van de resultaten uit het onderzoek hebben telers de implementatie van een aantal beheersmaatregelen binnen het bouwplan aangepast.

Curriculum vitae

Gera van Os werd geboren op 15 september 1965 in Amstelveen. Na het behalen van het Gymnasium β-diploma aan de Scholengemeenschap Casimir in Amstelveen, begon zij in 1983 met de studie Biologie aan de Universiteit van Amsterdam. In 1986 startte zij tevens met de studie Plantenziektenkunde aan de Landbouwuniversiteit Wageningen. In 1989 behaalde zij voor beide studies het doctoraalexamen met als afstudeervakken Fytopathologie en Virologie.
In 1990 trad zij in dienst als wetenschappelijk onderzoeker bij het Laboratorium voor Bloembollenonderzoek in Lisse (thans Praktijkonderzoek Plant en Omgeving, Sector Bollen & Bomen). Van 1990 tot 1999 heeft ze onderzoek gedaan aan de biologische beheersing van *Pythium* wortelrot in de bloembollenteelt. In 1995 bleek het onderzoek zich dusdanig te ontwikkelen dat werd besloten om een promotietraject in gang te zetten, waarvan de resultaten staan beschreven in dit proefschrift. Vanaf 1999 tot heden richt het onderzoek zich op andere bodemgebonden ziekteverwekkers, zoals *Rhizoctonia solani* en *Verticillium dahliae*.

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Nawoord

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