A STUDY ON THE ETIOLOGY AND EPIDEMIOLOGY OF BLACK ROOT ROT OF STRAWBERRIES IN THE WESTERN CAPE

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Summary

Historically root diseases have been a production-limiting problem for the strawberry industry worldwide. In the Western Cape Province of South Africa the most serious root disease is black root rot, which causes losses of up to 30%, annually. The aims of this study were to investigate aspects of the etiology and epidemiology of this disease in the Western Cape, and to provide information that can be incorporated in an integrated disease management strategy. In Chapter 1 a summary of published information on this disease is presented. Disease symptoms include severe stunting of plants, which have black, rotted, reduced root systems. Even though this disease is of great economic importance, the etiology remains unresolved. However, soilborne fungal root pathogens, particularly *Pythium* and *Rhizoctonia* spp. have been implicated as major role players. Control of this disease, as well as the other root diseases affecting strawberries, has relied on soil fumigation with broad spectrum chemical fumigants, in particular methyl bromide. However, due to the ozone depleting action of methyl bromide it was decided at the signing of the Montreal Protocol to remove this chemical from the market. This action has caused great demand for alternative measures to control root diseases on many crops including strawberries. Development of integrated disease management strategies is dependent upon a more complete understanding of the etiology, biology and ecology of the disease complex.

In Chapter 2 fungal pathogens associated with diseased plants were isolated and Koch's postulates were carried out. The most frequently isolated fungal pathogens were *Pythium irregulare*, *Rhizoctonia* spp. and *Cylindrocarpon destructans*. Two morphotypes of *Rhizoctonia* were isolated *viz.* a brown and a white type. *Pythium irregulare* was isolated more frequently in July than in September, and was not isolated at all in November. *Rhizoctonia* spp. were present at all sampling dates but were more frequently isolated in November than at the other times. All the fungi that were tested were pathogenic and caused root lesions. *Cylindrocarpon destructans* and *Coniella fragariae* did not have a stunting effect on the plants. These results confirm a major role for *Pythium* and *Rhizoctonia* in the black root rot

complex and suggest that there is a complimentary seasonal variation in occurrence between these two pathogens.

The *Rhizoctonia* species and anastomosis groups of isolates obtained from diseased strawberries in the Western Cape Province were determined, and their pathogenicity and relative virulence assessed. Both binucleate and multinucleate types were recovered from diseased roots and identified as *R. fragariae* and *R. solani*, respectively. All isolates of *R. solani* were members of anastomosis group (AG) 6, whereas three AG types were identified among isolates of *R. fragariae*, *viz.* AG-A, AG-G and AG-I at a relative occurrence of 69%, 25%, 6% respectively. All *Rhizoctonia* isolates were pathogenic to strawberry, but *R. solani* (AG 6) was the most virulent causing severe stunting of plants. This is the first species confirmation and AG type identification of *Rhizoctonia* taxa causing root rot of strawberries in South Africa.

An assessment of the presence and quantity of black root rot pathogens associated with soils prior to fumigation and post fumigation with methyl bromide, was made in Chapter 4. Isolations were also made from nursery plants to determine whether any black root rot pathogens were in the plants before transplanting. Results demonstrated that after fumigation the soil was free of all pathogenic fungi associated with the disease. However, the main pathogens involved in black root rot, *viz. Rhizoctonia fragariae, R. solani* and *Pythium* spp. were isolated from nursery plants. The fact that the plants are already infected with these pathogens renders the prospects for control of this disease difficult. Further studies are urgently required to develop production practices that can be included in disease management programmes.

In vitro studies were carried out to determine the EC_{50} values of different fungicides for isolates of *Pythium irregulare*, *Rhizoctonia fragariae* AG-A, AG-G and AG-I and *R. solani* AG 6. Benomyl, fludioxonil and tolclofos-methyl were used in these tests. Field trials were also conducted using these fungicides. In Chapter 5 it is shown that in general application of fungicides improved the yield and did not affect the survival rate of strawberry plants. Fludioxonil showed potential for short-term use. Applications of fungicides that targeted specific fungal genera were not sufficient to control the disease. Seasonal fluctuation of *Pythium* and *Rhizoctonia* spp. became apparent with the occurrence of *Pythium* being relatively high early in the season but low late in the season. Conversely, the occurrence of *Rhizoctonia* was low at the beginning of the season but high late in the season. In the short-term there

is potential for fungicide applications as part of an integrated disease management strategy, but the economic feasibility of this practice needs to be assessed.

In this study the major pathogens causing black root rot were identified in the Western Cape Province of South Africa, and important information regarding the epidemiology of the disease was reported. These results can be incorporated in an integrated management strategy to reduce losses of strawberry production, attributable to black root rot.

Opsomming

Wortelsiektes is wêreldwyd 'n produksie-beperkende probleem vir die aarbeibedryf. . Swartwortelvrot, wat jaarliks verliese van tot 30% veroorsaak, is die belangrikste wortelsiekte in die Wes-Kaap Provinsie van Suid-Afrika. Die doelwitte van hierdie studie was om aspekte van die etiologie en epidemiologie van die siekte in die Wes-Kaap te ondersoek en inligting wat in geïntegreerde siektebestuurstrategië ingesluit kan word, te voorsien.

In Hoofstuk 1 word 'n opsomming van gepubliseerde inligting aangaande die siekte uiteengesit. Siektesimptome sluit ernstige verdwerging van plante met swart verotte en verkleinde wortelstelsels in. Alhoewel die siekte van groot ekonomiese belang is, is die etiologie grootliks onbekend. Grondgedraagde wortelpatogene swamme, spesifiek *Pythium* en *Rhizoctonia* spp., is egter as belangrike rolspelers geïdentifiseer. Tot dusver het die beheer van hierdie siekte sowel as ander wortelsiektes van aarbeie berus op grondberoking met breë spektrum chemiese berokingsmiddels, spesifiek metielbromied. As gevolg van die osoonafbrekende aksie van metielbromied is daar egter tydens die ondertekening van die Montreal Protocol besluit om dié middel van die mark te verwyder. Hierdie besluit het 'n groot aanvraag na alternatiewe beheermaatreëls vir wortelsiektes van verskeie gewasse, insluitende aarbeie, veroorsaak. Die ontwikkeling van geïntegreerd siektebestuurstrategieë is egter afhanklik van 'n meer volledige begrip van die etiologie, biologie en ekologie van die siektekompleks.

In Hoofstuk 2 is die patogene swamme wat met die siekte geassosieer word, geïsoleer, en is Koch se postulate uitgevoer. Die mees algemeen geïsoleerde patogene swamme was *Pythium irregulare*, *Rhizoctonia* spp. en *Cylindrocarpon destructans.* Twee morfotipes van *Rhizoctonia* is geïsoleer, nl. 'n bruin tipe en 'n wit tipe. *Pythium irregulare* is meer dikwels in Julie as in September geïsoleer, maar glad nie in November nie. *Rhizoctonia* het tydens alle monstertye voorgekom, maar is meer dikwels in November geïsoleer. Al die swamme wat getoets is, was patogenies en het letsels op die wortels veroorsaak. *Cylindrocarpon destructans* en *Coniella fragariae* het nie'n verdwergingseffek op plante gehad nie. Hierdie resultate bevestig die

dominante rol van *Pythium* en *Rhizoctonia* in die swartwortelvrot kompleks en dui op 'n komplementêre seisoenale variasie in die voorkoms van hierdie twee patogene.

Die *Rhizoctonia* spesies en anastomose groepe (AG) van die isolate geisoleer vanaf siek aarbeiplante in die Wes-Kaap Provinsie is bepaal, en die patogenisiteit en relatiewe virulensie is beraam. Sowel tweekernige as multikernige tipes is vanaf siek wortels geïsoleer en respektiewelik as *R. fragariae* en *R. solani* geïdentifiseer. Alle isolate van *R. solani* was lede van anastomose groep 6, terwyl drie AG tipes, nl. AG-A, AG-G en AG-I onder die *R. fragariae* isolate geïdentifiseer is met relatiewe voorkomste van 69%, 25%, 6% respektiewelik. Alle *Rhizoctonia* isolate was patogenies op aarbeie, maar *R. solani* (AG 6) was die mees virulente en het ernstige verdwerging van plante veroorsaak. Hierdie is die eerste bevestiging van spesies en identifisering van AG tipes van *Rhizoctonia* taksa wat wortelvrot van aarbeie in Suid Afrika veroorsaak.

In Hoofstuk 4 is 'n beraming van die voorkoms en hoeveelheid swartwortelvrot patogene geassosieer met grond voor, en na beroking met metielbromied, gemaak. Isolasies is ook vanaf kwekeryplante gemaak om te bepaal of enige swartwortelvrot patogene voor oorplanting in die plante teenwoordig was. Die resultate het getoon dat grond na beroking vry was van alle patogeniese swamme geassosieër met die siekte. Die hoof patogene betrokke in die swartwortelvrot kompleks, nl. *Rhizoctonia fragariae, R. solani* en *Pythium* spp. was egter in die kwekery plante teenwoordig. Die feit dat plante reeds met hierdie patogene geïnfekteer is, maak die vooruitsigte vir die beheer van hierdie siekte moeilik. Verdere studies word dringend benodig vir die ontwikkeling van produksiepraktyke wat by siektebestuursprogramme ingesluit kan word.

In vitro studies om die EC_{50} waardes van die isolate van *Pythium irregulare*, *Rhizoctonia fragariae* AG-A, AG-G en AG-I en *R. solani* AG 6 vir die fungisiedes benomyl, fludioxonil en tolclofos-metiel te bepaal, is uitgevoer. Hierdie fungisiedes is ook in veldproewe getoets. In Hoofstuk 5 is getoon dat aanwending van fungisiedes die opbrengs verbeter het en nie die oorlewing van aarbeiplante beïnvloed het nie. Fludioxonil het potensiaal vir korttermyn gebruik getoon. Die aanwending van fungisiedes wat spesifieke swamgenera teiken, was nie voldoende om die siekte te beheer nie. Seisoenale fluktuasies van *Pythium* en *Rhizoctonia* spp. het duidelik geword met die relatief hoë voorkoms van *Pythium* vroeg in die seisoen, maar lae voorkoms laat in die seisoen, terwyl die voorkoms van *Rhizoctonia* laag was aan die

begin van die seisoen, maar hoog later in die seisoen. In die korttermyn is daar potensiaal vir fungisiedtoedienings as deel van 'n geïntegreerde siektebestuurstrategie, maar die ekonomiese haalbaarheid van hierdie praktyk moet bepaal word.

In hierdie studie is die hoof patogene wat swartwortelvrot van aarbeie in die Wes-Kaap Provinsie van Suid-Afrika veroorsaak geïdentifiseer, en belangrike inligting rakende die epidemiologie van die siekte is aangeteken. Hierdie resultate kan in 'n geïntegreerde bestuurstrategie geïnkorporeer word om verliese van aarbeiproduksie, toeskryfbaar aan swartwortelvrot te, verminder.

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1: The Black Root Rot Disease Complex: A Review

Abstract

Historically root diseases have been a production-limiting problem for most strawberry producing countries for example Australia, the Netherlands, Japan, the United Kingdom and the USA. In the Western Cape Province of South Africa the most serious and costly root disease is black root rot disease complex, causing annual losses of up to 30%. The disease is recognised by the severe stunting of the plants and black, rotted and reduced root systems. Even though this disease is very important internationally little is known about its etiology. Control of this disease, as well as the other root diseases affecting strawberries, has relied on soil fumigation with chemical fumigants and in particular methyl bromide. However, due to the ozone depleting action of this chemical it is currently being removed from the commercial market. This has caused a great demand for alternative measures to control root diseases on many crops including strawberries. In Australia and the USA intensive research has already been done on short- as well as long-term alternatives to methyl bromide. However, in South Africa there has not been any research done into alternatives to methyl bromide. Soil drenches with fungicides can be used as a short-term solution, but only a few are registered for use on strawberries in South Africa. The present study reviews information on the main issues associated with the black root rot complex of strawberries.

Introduction

Black root rot is a serious disease of strawberries that reduces annual production by about 30% in the Western Cape Province (Botha *et al.* 2001). This disease has also been reported in Australia (Porter *et al.* 1999), the Netherlands (Klinkenberg 1955), Japan (Watanabe *et al.* 1977), the United Kingdom (UK) (Wardlaw 1927) and the USA (Heald 1920; Coons 1924; Wilhelm and Paulus 1980; Yuen *et al.* 1991; Duniway 1998). In the Western Cape Province production area it is currently recognised as the most important yield-limiting root disease of strawberries. Although the strawberry industry in South Africa is small by comparison with other

countries it is none the less an important component of local agriculture and tourism. Furthermore, the Western Cape Province supplies about half of the national production of strawberries. The area under strawberry cultivation in this region is approximately 100–150 ha comprised mostly of small-scale farms, some of which are subsistence farmers. Thus, in these cases strawberry production is the only source of income for families, thereby adding an important local socio-economic aspect to this crop. Recognition of the relationship between root disease and yield losses in strawberries has a long and tortuous history. The use of methyl bromide as a soil fumigant had a major impact on strawberry production, but with the imminent removal of this product from the market, root diseases of strawberries have resurfaced, thereby providing new challenges to the industry.

The effect of methyl bromide on black root rot disease

Historically, root diseases have been a limiting factor to the productivity of strawberry fields worldwide. Until the 1920's the estimated yield of strawberries in California was about 2 t/ha (Wilhelm and Paulus 1980). New strawberry cultivars were then introduced and yields increased dramatically up to an average of 5–6 t/ha, but producers often suffered losses as a consequence of root diseases. This situation was more or less maintained until the 1960's when methyl bromide was introduced as a soil fumigant. In the USA, strawberry productivity escalated four to five fold up to 25 t/ha (Wilhelm and Paulus 1980). The use of methyl bromide not only gave control of soilborne pathogens, nematodes and weeds, but plants also showed increased vigour, productivity and fruit weight. Fumigation with methyl bromide has almost always resulted in uniform control of pests, pathogens and weeds with a nutrient benefit to crops, thus enabling maximum cropping opportunities with high yielding varieties that have a short turn-around time (Porter *et al.* 1999). With the diminished effect of root diseases on yields, the industry (worldwide) was lulled into a false sense of security that root disease problems had disappeared or were not important in this crop. Thus, the emphasis on research and breeding programmes was on fruit properties and yields, rather than on breeding for resistance to diseases and on investigating methods of production unreliant on methyl bromide (Yeun *et al.* 1991). Recently, however, methyl bromide has been identified as a high-risk chemical that depletes atmospheric ozone. It is also harmful to beneficial soil biota, the environment and the user

(Ristaino and Thomas 1997; Porter *et al.* 1999). In 1992 it was decided that, due to the risks involved in the use of this fumigant, methyl bromide would be removed from the market. This process was initiated with the signing of the Montreal Protocol. In accordance with this protocol the subscribing members pledged to decrease their use of methyl bromide until it is finally phased out. South Africa is a signatory to the protocol and it is expected that this chemical will no longer be available for commercial use in South Africa by 2005 (Ellis 2000). In the Western Cape Province about 60% of the area used in strawberry cultivation is fumigated, thus a large proportion of the industry faces an eminent crisis since their production is dependent upon this soil fumigant.

In the absence of methyl bromide black root rot is one of the main root diseases limiting strawberry production worldwide. The etiology of this disease has not been clearly defined and varies amongst different sites. In general a complex of fungal pathogens has been implicated in the disease (Maas 1984). Black root rot can therefore also be described as a syndrome since it is characterized by a number of symptoms.

Symptoms

Plants affected by black root rot are characterised by stunted growth, they wilt under heat stress and have brittle, blackened root systems (Strong and Strong 1931, Wilhelm and Paulus 1980) (Figs. 1 and 2). They also have fewer crowns of reduced diameter than unaffected plants and will produce less fruit of reduced quality. The leaves of the affected plants are typically smaller and fewer runners are produced (Elmer and LaMondia 1999). Diseased plants show a loss in productivity and vigour due to the rotted roots. Black root rot of strawberry has often been associated with fields that have been in production for a long time (Ellis 2000). The first symptoms are seen in only a few plants, mostly in areas were drainage is insufficient and where the soil has become compacted due to cultivation. When the stunted plants are removed from the soil, black root rot symptoms are seen showing blackened brittle, dead roots (Wing *et al.* 1995). Affected plants are devoid of feeder roots and many of the larger roots have broken off at rotted portions where the cortical tissue has collapsed, which gives broken off roots a "rats-tail" appearance (Fig. 3 and 4). Symptoms are usually most prominent in the last few weeks before harvest. Some of the plants die during the

production season because of root rot, but those that survive will be stunted and will produce a reduced crop of small strawberries. Replanting strawberries in fields previously cultivated with black root rot affected strawberries, is not successful and the disease incidence will increase significantly (Ellis 2000).

Etiology

According to Strong and Strong (1931), black root rot of strawberries had been reported by various authors in the early 1900's, and was referred to as a disease condition of unknown etiology. In the 1920's a number of investigations into the cause of the disease were carried out in different strawberry growing areas across the globe and various fungi and bacteria were implicated in the etiology. In the USA, Heald (1920) and Coons (1924) ascribed the disease to *Rhizoctonia*. In Scotland, Wardlaw (1927) implicated a species of *Pythium* and an unidentified sterile fungus in the cause of the disease. Plakidas (1930) also found a *Pythium* sp. to be highly pathogenic to strawberries in the USA. Although Berkeley (1924) had implicated bacteria as causal agents, he was unable to confirm pathogenicity. In contrast to Berkeley (1924), Strong and Strong (1931) were of the opinion that the disease was caused by *Coniothyrium fuckelii* Sacc. and *Hainesia lythri* (Desm.) Höhn.

The possible role of nematodes in this complex was only realised in the late 1950's, when the root knot- or meadow-nematode (*Pratylenchus penetrans* Cobb.) was thought to be the causal agent of black root rot (Raski 1956). Root feeding by this nematode caused stunting of plants and blackening of roots. Experiments conducted by Chen and Rich (1962) revealed that *P. penetrans* only predisposed plants to fungal infection by creating wounds. The idea that nematodes were the sole causal agents of black root rot was, thereafter discarded (Chen and Rich 1962).

***Pythium* species associated with black root rot of strawberries.** Since the 1960's many fungi, including species of *Pythium*, have been implicated as the cause of the disease. In the USA *Pythium sylvaticum* Campbell & Hendrix was most commonly isolated, occurring in 76% of the fields surveyed, but *P. irregulare* Buisman and *P. perniciosum* Serbinow were also frequently isolated (Nemec and Sanders 1970). Pathogenicity tests confirmed that *P. sylvaticum* was pathogenic and Nemec (1970) subsequently proposed that it was the primary pathogen in the Illinois area. Other

Pythium species that were isolated from the strawberry plants in the Illinois area included *P. dissotocum* Drechsler, *P. ultimum* Trow, *P. hypogynum* Middleton, *P. rostratum* Butler and *P. acanthicum* Drechsler. Denman (1994) made isolations from black root rot affected plants at the end of the 1993 season in the Western Cape, South Africa. A low level of *P. irregulare* was isolated from affected plants. In some studies on black root rot of strawberries, researchers in different areas of the world placed more emphasis on the role of *Rhizoctonia* spp. and *Pratylenchus penetrans* than on *Pythium* (Ribeiro and Black 1971; Maas 1984; Elmer and La Mondia 1999).

Factors affecting disease development by *Pythium* spp. Nemec (1970) demonstrated that soil moisture is critical for successful infection of strawberries by mycelia and germinating sporangia of *Pythium* spp. An abundance of soil water promoted germination of sporangia and production of zoospores as well as root infection. However, some researchers reported that *Pythium* was not able to penetrate the host directly but needed a wound or other opening to obtain access to host tissue (Adegbola and Hagendorn 1969). Excessive irrigation favoured disease development by *P. sylvaticum* in California (Nemec 1970) but, zoospore formation was not observed. *P. sylvaticum* was noted for its ability to colonize runner plant roots early in the growing season, which suggests that this pathogen either has a preference for young host tissue, specific nutritional requirements in the rhizosphere or requires an optimum temperature for infection to take place. It has been suggested that *Pythium* only plays a role in areas where there is enough free water in the soil and optimum temperature conditions (Nemec and Sanders 1970).

***Pratylenchus penetrans* associated with black root rot of strawberries**. In the past it was suggested that the root lesion nematode, *P. penetrans*, was the primary cause of black root rot disease (Klinkenberg 1955; Raski 1956). Townshend (1962) confirmed the pathogenicity of *P. penetrans* on strawberries in 1962. It was believed that nematodes provided soilborne fungi with an infection point through the wound they made when feeding (Chen and Rich 1962). Plants growing in nematode infested soil showed root rot symptoms similar to those with black root rot (Goheen and Smith 1956). It was thought that when the nematodes were not present the fungi associated with black root rot could not infect, and no disease would develop (Chen and Rich, 1962). However, Raski (1956) conducted experiments in soil from a black root rot

infested strawberry field. This trial showed that *P. penetrans* could not cause black root rot without the presence of the other pathogens implicated. However, nematodes do contribute to black root rot, if they are present in soil along with the other pathogens involved in the disease complex. Surveys were carried out using eight commercial strawberry cultivars that were planted in soil collected from strawberry rhizospheres at various locations in New Hampshire. It was evident from this survey that there was a definite association between *P. penetrans* and soilborne fungal pathogens forming part of the black root rot complex (Chen and Rich 1962).

***Rhizoctonia* associated with black root rot of strawberries.** The other organism that is most commonly associated with the black root rot complex is *Rhizoctonia*. This filamentous soilborne fungus survives long periods in soil by living on plant debris (Cotterill 1993) or by forming sclerotia (Banniza *et al.* 1999). The genus *Rhizoctonia* consists of many host specific and non-specific, as well as pathogenic and non-pathogenic species. Since De Candole first described the genus *Rhizoctonia* in 1815, more than a hundred species have been placed in the genus (Ogoshi 1987). *Rhizoctonia* is mostly found as an imperfect basidiomycete fungus. Different species of *Rhizoctonia* have teleomorphs in different genera. For example *Rhizoctonia solani* J.G. Kühn has a *Thanatephorus* Donk teleomorph, while *Rhizoctonia fragariae* S.S. Husain & W.E. McKeen has a *Ceratobasidium* D.P. Rogers teleomorph. In general mycelia of *Rhizoctonia* species exhibit extensive right angle branching near the distal septum (Ogoshi 1987). Clamp connections are not present, nor are conidia. A rhizomorph is not formed, and sclerotia are not differentiated into a rind and a medulla (Ogoshi 1987).

Using these morphological characteristics it was found recently that only forty-nine of the one hundred species described are true *Rhizoctonia* spp., but also, some species of *Sclerotium* Tode and other genera are in fact *Rhizoctonia* spp. (Ogoshi 1987). It has been recognized that there are great differences amongst the various isolates identified as *R. solani*. Differences in pathogenicity, sclerotial morphology, cultural appearance on media and other physiological characteristics are evident. These differences have been utilised to form a number of intraspecific groups or ISG's which assist in the identification of *R. solani*. However, the initial step in species identification of *Rhizoctonia* is to determine the nuclear status of the isolate. The genus *Rhizoctonia* contains isolates that either have multinucleate cells

(and this is one of the main criteria for isolates to be classified as *Rhizoctonia solani*) or binucleate cells.

To determine the nuclear status of isolates, rapid and effective nuclear staining techniques are necessary. Many different nuclear staining methods are available. However, some of the methods are complicated and time consuming. For example the HCL-Giemsa staining method was used frequently because of its reliability, and the distinctiveness of the stained nuclei, which made them easy to count (Herr 1979). However the process of fixation and acid hydrolysis is very time consuming and tedious. Trypan blue was used as an alternative to the HCL-Giemsa, but the trypan blue did not stain all the nuclei of all the isolates, neither did aniline blue. The acridine orange method proved to be excellent for quantifying nuclei in consecutive cells on specific strands of hyphae (Yamamoto and Uchida 1982). The nuclei were not difficult to locate and the method has been proven reliable although one of the disadvantages of this method is the need for a fluorescence microscope. The safranin O-KOH method is equally rapid and can be used with bright field microscopy. The safranin apparently stains the nucleolus, rather than the nucleus (Yamamoto and Uchida 1982). Both these methods have been proven useful in staining *Rhizoctonia* nuclei and are simple to use, rapid and give reliable results.

Once the nuclei have been counted, the anastomosing group must be determined. Anastomosis is the process during which compatible hyphae from two different isolates fuse and exchange genetic material. The anastomosing group that an isolate belongs to is of importance because the morphology and virulence of different AG types within a species varies greatly (Muyolo *et al.* 1993). Eleven anastomosis groups have been identified and confirmed in *R. solani* based on hyphal anastomosis, cultural morphology, virulence, disease type and DNA base-sequence homology. All these factors (anastomosis group, pathogenicity, cultural morphology etc.) are important facets of identification as demonstrated by the AG-1 group of *R. solani* which has recently been sub-divided into three groups AG-1 IA, AG-1 IB and AG-1 IC (Muyolo *et al.* 1993). .

Parmeter *et al.* (1967) found isolates of *Rhizoctonia* in strawberries that were similar to *R. solani* but had a *Ceratobasidium* teleomorph and not a *Thanatephorus* teleomorph. These *R. solani*-like isolates had predominantly binucleate hypal cells (Martin 1988). Isolates of *Rhizoctonia* from California also had binucleate hyphal

cells and were identified as *R. fragariae*. This led Parmeter *et al.* (1967) to confirm the identity of their isolates as *R. fragariae.*

Many other binucleate *Rhizoctonia* spp. have been identified in a range of hosts. Burpee *et al.* (1980) divided the binucleate group of *Rhizoctonia* species into seven different anastomosing groups (CAG-groups) and *R. fragariae* was placed in one of these groups, CAG 2. However Ogoshi (1983) separated the binucleate *Rhizoctonia* spp. into at least 15 anastomosing groups (AG-groups) and three of these groups, *viz.* AGA, AGG, or AGI were associated with *R. fragariae*.

Factors affecting disease development by *Rhizoctonia* spp. In a study carried out by LaMondia and Martin (1989) in Connecticut the three *R. fragariae* AG types most commonly associated with black root rot were AG-G, AG-A and AG-I. Variable levels of virulence of the isolates were also observed for these isolates at different temperatures. AG-A and AG-G were more virulent at 24°C and AG-I was more virulent at 10°C. Black root rot symptoms developed at the entire range of temperatures (10–24°C). *Rhizoctonia solani* can cause disease under a wide range of environmental conditions. In general *R. solani* is active under warm temperatures of between 24 and 28°C (Martin 1987). However, some anastomosing groups have cooler optimal temperature conditions, for example AG 4 that causes disease on some turf grasses has an optimal temperature of 20°C. Soil disturbance has been shown to have a negative affect on *Rhizoctonia* disease development, and the use of modified seed drills or sowing points that disturb the soil below the seeding depth during seeding can decrease *Rhizoctonia* root rot of wheat (Roget 1995). Deep ploughing also buries sclerotia, and the lower levels of oxygen deep in the soil cause the demise of sclerotia.

Environmental factors affecting the development of black root rot

In 1984 it was suggested that black root rot was not caused by one specific agent, but by a number of different factors. Although soilborne fungi and root-lesion nematodes were involved, other environmental effects such as freezing injury and water logging were also implicated (Maas 1984). Environmental factors largely predisposed plants to infection (Wing *et al.* 1995) and agronomic practices often aggravated the situation.

Sandy soils with good drainage promoted the development of healthy roots, whereas soils with a high clay content and bad drainage supported poor root health and more severe disease development (Wing *et al.* 1995). Chemical components of soils did not have a major effect on the development of black root rot, but cultural practices did. Any factor that decreased the vigour of the roots increased the chance of black root rot developing.

Cultural practices that included, for example, high planting beds that caused shorter water saturation periods and allowed better oxygen concentration, led to plants that had better root health. It was also shown that if fields had been planted with strawberries within the previous five years, or had been used for strawberry cultivation for a number of years continuously, or if the current plantings were old, poor root health and black root rot symptoms were prevalent.

The repeated use of the herbicide terbacil (Sinbar, E.I. du Pont de Nemours and Co., Wilmington, Delaware) stressed plants, making them more susceptible to disease (Wing *et al.* 1995). Prevailing weather conditions throughout the season also affected plant health and the development of black root rot. In the Western Cape Province a wet winter followed by a hot, dry and windy spring and summer promotes the development of severe disease (S. Denman personal communication).

Management of black root rot

Control of black root rot has relied mostly on the use of soil fumigants such as methyl bromide (Yuen *et al.* 1991; Elmer and LaMondia 1999; Porter *et al.* 1999; Botha *et al.* 2001). Due to the imminent retraction of methyl bromide from use, an urgent need for alternative control measures has developed. There are a few different approaches to the solving of this problem (Denman and Botha 1999). The one approach is to replace methyl bromide with different chemical fumigants such as chloropicrin and metham sodium. In Australia research has been carried out on the use of these alternative fumigants, and the combination of these fumigants with lower levels of methyl bromide, as a short-term solution to the problem (Porter *et al.* 1999). Long-term solutions have not yet been developed, but the only plausible solution for the sustainable control of soilborne pathogens is in the form of an integrated control system (ICS) (Porter *et al.* 1999). This approach includes the use of biofumigants, biological control, crop rotation, nutrient management and resistant cultivars.

In California the search for an alternative to methyl bromide has been carried out in two areas. Firstly in the use of other chemical fumigants, and secondly in the use of non-chemical methods. With regard to the former, good results have been achieved by using 1,3 – Dichloropropene with chloropicrin and also with chloropicrin alone (Yeun *et al* 1991). Vapam® (methyl isothiocyanate) has also shown promising results (Duniway 1998). Unfortunately the use of chemical fumigants is not a long-term solution to the problem. Chloropicrin, commonly known as tear gas, is user- and environmentally-unfriendly and will probably not remain a viable option for much longer.

Practices such as crop rotation, ammonium sulphate [$(NH_4)SO_4$] amendments and soil solarisation (Elmer and La Mondia 1999) have been investigated and appear promising. It was found that a single rotation with Saia oats (*Avena strigosa* Schreb.) and the use of $(NH_4)SO_4$ resulted in larger strawberries, a higher early yield and less damage from black root rot (Elmer and La Mondia 1999). The addition of organic amendments such as biofumigants or organic composts has also been investigated, but the results have been variable and the structure and quality of the soil greatly influence the efficacy of these amendments. Yields from organically treated soil have not been as good as those from chemically treated soils (Duniway 1998).

In South Africa there has not been any research done into alternatives to methyl bromide. Fungicides can be used as a short-term solution, but only a few are registered for use on strawberries in South Africa (Botha *et al.* 2001). Many problems are associated with the use of fungicides. If used too often, pathogens can become resistant to fungicides, rendering them ineffective. Fungicide treatments are not always economical and are not environmentally considerate. Another problem with using fungicides to control black root rot is the spectrum of pathogens that are involved in the disease complex. Fungicides target specific groups of pathogens but in this complex a variety of phylogenetically divergent pathogens are involved. Thus one fungicide alone will not be effective for controlling black root rot. A combination of different fungicides will have to be used, and the right combinations have to be found.

Rhizoctonia is the one fungus that has been most consistently associated with the black root rot disease complex in most cases and areas. Although not much information is available on the effects of fungicides on strawberry black root rot, reports of fungicides against some of the pathogens involved in black root rot, but

occurring on other crops, are available. Pre-plant treatments of carboxin and iprodione have been recommended against *R. solani* and gave excellent results on *Brassica* species (Kataria *et al.* 1993). Other fungicides that have been used and recommended are PCNB (Pentachloronitrobenzene) and metalaxyl. PCNB controlled *Rhizoctonia*-induced rotting effectively on strawberries and metalaxyl *Pythium*-induced diseases (Castillo and Peterson 1990). It was shown that colonisation of alfalfa roots by *Pythium* or *Rhizoctonia* was also reduced by the use of these fungicides (Hancock 1993). Fosetyl-Al controlled *Pythium*-induced diseases and can be used as an alternative to metalaxyl (Castillo and Peterson 1990). Metalaxyl and fosetyl-Al are also used on strawberries for the control of certain fruit rots, such as leathery rot caused by *Phytophthora cactorum* Lebert & Cohn (Madden *et al.* 1991). In South Africa not many fungicides have been tested for use on strawberries and only captab and copper oxide are registered for use on this crop for Botrytis fruit rot and leaf spot.

Alternative control recommendations for the control of black root rot include avoiding abiotic stress, such as drought or injury and heavy, wet soils, and crop rotation in a two-year interval with grains. Other cultural practices can also deliver a certain level of control. Erosion control by means of cover crops is of importance in areas where strawberries are planted year round. The losses of soil due to runoff water can be considerable and of great importance. The use of barley as a cover crop has shown to be very effective to reduce the pathogen levels in the soil (Vliet *et al.* 1996).

Crop rotation. Crop rotation has proven effective in many root and stem rotting diseases caused by *Pythium* spp. as well as *Rhizoctonia solani* and binucleate *Rhizoctonia* species including (*R. cerealis*) in crops such as cucumber, snap-beans, carrots, wheat and barley (Vilich 1993; Davis and Nunez 1999; Sumner et al. 1999). Crop rotation as a method of controlling of black root rot was investigated by Elmer and LaMondia (1999). Plots with a history of black root rot were seeded with sorgho-sudangrass (*Sorghum bicolor* (L.) Moench. × *S. sudanense* Stapf.) or Gary (*Avena sativa* L.) or Saia oats. Gary and Saia oats as well as Triple S sorgho-sudangrass were shown to be poor hosts for *Rhizoctonia fragariae* and *P. penetrans*, and suppressed disease (Elmer and LaMondia 1999). Other crops such as canola (*Brassica napus* L.) and buckwheat (*Fagopyrum esculentum* Moench.) did not have any effect on the population densities of *R. fragariae* and *P. penetrans*, or even increased the numbers

of these pathogens in the soil (Elmer and LaMondia 1999). Plots sown with Gary oats showed reduced levels of infestation with *R. fragariae* when compared with the controls. Rotation with grains, however, is not acceptable to the local strawberry farmers. Strawberries are grown on small areas that are not large enough to produce grains economically. Other crops need to be evaluated for use in a rotation plan with strawberries. The crop used has to be chosen and evaluated very carefully, because certain crops can increase the population densities of pathogens, and thus increase the disease intensity.

Resistant cultivars. Wing et al. (1995) evaluated resistance of twenty commercial strawberry cultivars to black root rot. Under the experimental conditions used in this trial none of the cultivars showed any resistance to this disease complex. . Martin (2000) evaluated the growth and yield of 14 cultivars on non-fumigated, soil that had a history of black root rot. All cultivars except "Laguna" showed significantly reduced growth when compared to the control that was planted on methyl bromide + chloropicrin fumigated soil. Seven cultivars (Aromas, Capitola, Parisbad, Douglas, Gaviota, Languna and Parker) had yields that were more than 70% of that of the control plants (Martin 2001). This was only a preliminary trial however, this is a crucial area of research that urgently needs to be addressed. It is essential to know and understand the etiology of black root rot in order to establish an effective resistance testing programme.

Biological control. Biological control through the addition of microorganisms to the rhizosphere, such as cyanobacteria, can be an effective means of control of certain soilborne diseases of strawberries (Kulik 1995). Many bacterial species are natural inhabitants of soil and some can fix nitrogen, thus enhancing soil fertility as well as controlling plant disease (Kulik 1995). The uses of other fungi as control for pathogenic fungi have also been evaluated. *Rhizoctonia* AG-K induced resistance to *R. solani* AG 4 on soybeans and had the potential to be used as biocontrol agent. Mycorrhizal fungi (*Glomus* spp.) were shown to increase the production of strawberry runners by up to 76% in the first year (Niemi and Vestberg 1992).

The most effective, economical and environmentally safe control strategy for black root rot of strawberries is an Integrated Disease Management Program. The

principles on which these programs rely are as follows: optimisation of pest control in an ecologically and economically sound manner; emphasis on co-ordinate use of multiple tactics to enhance stable crop production; and maintenance of pest damage below injurious levels while minimising hazards to humans, animals, plants, and the environment (Cooley et al. 1996). The system includes chemical control, but it limits the use of chemicals by utilising other control measures that are available for use.

The aims of the work reported in this thesis include identification of some of the elements that cause and affect black root rot of strawberries in the Western Cape Province. Furthermore, we wanted to provide information that could be included in an integrated disease management program that is suitable for strawberry farmers locally.

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Fig. 1. Patchiness in strawberry fields indicates loss to black root rot.

Fig. 2. The severely stunted strawberry plants in the background suggests that they are infected with black root rot compared with the healthy plants in the foreground.

Fig. 3. Typical black root rot symptoms on strawberry plants. Root lesions and broken off roots that give a "rats-tail" appearance are characteristic of the disease.

Fig. 4. Collapsed root tissue and ring-like lesions along the root length are also typical symptoms associated with black root rot of strawberries.

2: An investigation of aspects of the etiology and epidemiology of black root rot of strawberries in the Western Cape Province

Abstract

In the present study strawberry plants showing typical symptoms of black root rot were sampled in July, September and November (1998 – 2000) from various areas in the Western Cape Province. Isolations were made from the diseased material and fungi commonly associated with the black root rot complex were subsequently used for pathogenicity tests. The most frequently isolated fungal pathogens were *Pythium irregulare*, *Rhizoctonia* spp. and *Cylindrocarpon destructans*. Two morphotypes of *Rhizoctonia* were isolated *viz.* a brown and a white type. *Pythium irregulare* was isolated at higher frequencies in July than in September, and was not isolated at all during November. *Rhizoctonia* occurred on all sampling dates but a higher occurrence was evident during November than at the other times. All the fungi that were tested were pathogenic and caused lesions on the roots. *Pythium irregulare* had a slight stunting effect on the strawberry plants, but the brown morphotype of *Rhizoctonia* caused severe stunting. There was marked variation in virulence of the different *Rhizoctonia* isolates tested. *Cylindrocarpon destructans* and *Coniella fragariae* did not have a stunting effect on the plants. These results confirm a major role for *Pythium* and *Rhizoctonia* in the black root rot complex and suggest that there is a complimentary seasonal variation in occurrence between these two pathogens.

Introduction

Black root rot is a very serious disease of strawberries worldwide and has been reported in Australia (Porter *et al.* 1999), the Netherlands (Klinkenberg 1955), Japan (Watanabe 1977), the United Kingdom (UK) (Wardlaw 1927) and the United States of America (USA) (Heald 1920; Coons 1924; Wilhelm and Paulus 1980; Yuen *et al.* 1991; Duniway 1998). In 1931, Strong and Strong (1931) described this disease as a condition of unknown etiology, and much effort has been put into establishing the etiology of this disease since the 1920's. Previously, black root rot was attributed to

single pathogens. In the USA, Heald (1920), Coons (1924), Ribeiro and Black (1971), Martin (1988) and Martin (2000) ascribed the disease to *Rhizoctonia*. In Scotland, Wardlaw (1927) implicated a species of *Pythium* and an unidentified sterile fungus as the cause of the disease. Plakidas (1930) also found a *Pythium* sp. to be highly pathogenic to strawberries in the USA. Although Berkeley and Jackson (1924) suspected bacteria to be the causal agents, they were unable to confirm this. Strong and Strong (1931) were of the opinion that the disease was caused by *Coniothyrium fuckelii* Sacc. and *Hainesia lythri* (Desm.) Höhn. In spite of claims made by these researchers, none of the pathogens they implicated could produce typical black root rot symptoms in inoculation trails. The possible role of nematodes in this complex was only realised in the late 1950's, when the root knot- or meadow-nematode (*Pratylenchus penetrans* Cobb.) was thought to be the causal agent of black root rot (Raski 1956). Root feeding by this nematode caused stunting of plants and blackening of roots. However, experiments conducted by Chen and Rich (1962) revealed that *P. penetrans* only predisposed plants to fungal infection by creating wounds that were invaded by other soilborne pathogens. The idea that nematodes were the sole causal agents of black root rot was thereafter discarded (Chen and Rich 1962).

In 1984 it was suggested that black root rot was not caused by one specific agent, but by a number of different factors. Although soilborne fungi and root-lesion nematodes were involved, environmental effects such as freezing injury and water logging were also implicated (Maas 1984). Environmental factors largely predisposed plants to infection (Wing *et al.* 1995) and agronomic practices often aggravated the situation. Chemical components of soils did not have a major effect on the development of black root rot, but cultural practices did. Strawberries growing in fields that had been cultivated with strawberries in the preceding five years developed severe black root rot. Furthermore, poor root health and black root rot symptoms were also prevalent if the plants in the current plantings were old. It was later discovered that the repeated use of the herbicide terbacil (Sinbar, E.I. du Pont de Nemours and Co., Wilmington, Delaware) stressed plants, making them more susceptible to black root rot (Wing *et al.* 1995). Prevailing weather conditions throughout the season also affected the development of the disease. Wing *et al.* (1995) maintained that the disease symptoms were most apparent shortly before and during the fruiting stage and that wet, cool conditions seemed to favour disease

development. In the Western Cape Province a wet winter followed by a hot, dry and windy spring and summer promoted the development of severe black root rot (S. Denman personal communication).

In South Africa (Western Cape Province) Denman (1994) first reported this condition, implicating both *Pythium* as well as *Rhizoctonia* species as major pathogens. It is now recognised that black root rot is one of the largest production-limiting factors in the local industry, causing losses of approximately 30% annually. The aims of this study were to evaluate the relative virulence of fungal pathogens commonly associated with black root rot of strawberries and to monitor their seasonal occurrence.

Materials and Methods

Isolation of pathogens. Plants with typical symptoms of black root rot were sampled from various areas in the Paarl and Stellenbosch regions in July, September and November from November 1998–2000. Plants that were stunted, wilted, chlorotic or necrotic were selected and dug out of the fields using a spade. Material was placed in polythene bags in a cooler box and brought back to the laboratory for isolation. In total 630 plants were collected.

Plants were prepared for isolations immediately following arrival. The foliage was cut off and the remainder of the material washed to remove soil particles. The plants were then surface disinfested for 1 min in a 1% sodium hypochlorite (NaOCl) solution prepared from commercial bleach, followed by 30 sec rinse in 50% ethanol and two rinses of 1 min each in sterile water. The plant material was then air dried in the laminar flow cabinet. Isolations were made by dissecting pieces of tissue approximately 5 mm long from the margin between healthy and diseased tissues of the roots and plating them onto a range of culture media. The crowns were split longitudinally and tissue was excised from the centre of the crowns and plated on the culture media. The media used included PARPH (*Phytophthora* selective medium) (Solel and Pinkas 1984) PARP (*Pythium* selective medium adapted from PARPH by omitting hymexazol), V-8 juice agar (Galindo and Gallegly 1960), water agar (Biolab, Midrand, South Africa) (WA), 17% corn meal agar (Difco) (CMA) and fresh homemade potato dextrose agar (200 g potatoes, 20 g dextrose, 12 g bacteriological

agar in 1 L water) (PDA). As soon as fungi started growing out of the plant material hyphal tips were transferred to divided plates containing carnation leaf agar (CLA) (Fisher *et al.* 1982) in one half of the dish and PDA in the other half. Plates were incubated under near-ultraviolet and cool white light with a 12 h photoperiod at 25°C for three weeks. Isolates were identified by microscopic examination. Single conidial or hyphal tip isolates were obtained of common pathogens associated with black root rot were stored for further use. The occurrence of common pathogens was expressed as a percentage based on the number of isolates per species out of the total number of fungi isolated per sampling date.

Pathogenicity tests. Pathogenicity tests were carried out using randomly selected isolates of the following fungi: *Coniella fragariae* (Oudem.) Sutton, *Cylindrocarpon destructans* (Zinssm.) Scholten, *Pythium irregulare* Buisman and *Rhizoctonia* DC (two morphotypes *viz.* a brown type and a white type) (Table 1). Three isolates were included per pathogen except for *Rhizoctonia* (white colony), of which seven isolates were used. All isolates are maintained in the Department of Plant Pathology culture collection (STE-U), University of Stellenbosch (Table 1).

Pathogen-free plants propagated from tissue culture plantlets were used. The plantlets were transplanted into sterilised composted pine bark medium and glass jars were inverted over them in the hardening off process. Potted plants were placed on metal mesh racks in the glasshouse at temperatures of between 25–35°C for four weeks. After the hardening off period the glass jars were removed and the plants were kept in the greenhouse for a further four weeks.

Sand-bran inoculum was prepared using the method described by Lamprecht *et al.* (1988). Washed river sand (400 g) and wheat bran (20 g) was mixed together in 500 mL Schott bottles and 60 mL sterile water was added. The mixture was autoclaved at 120°C and a pressure of 1.5 kgf/cm^2 for 15 min and then left to cool. This process was repeated for three successive days after which four 5 mm plugs of a single isolate were used to inoculate each bottle. Fungi were left to colonise the sand-bran medium for seven days at 25°C with a 12 h day / night interval, after three days the bottles were shaken to facilitate even colonisation. Inoculum (25 g in 475 g potting mix) was mixed into sterilised potting medium and the eight-week-old cv Tiobelle plants were planted into the pots. Plants were kept in a growth chamber at

25°C with a 12 h day/night interval for five weeks (Martin 2000). The trial was set out in a complete randomised design. An experimental unit consisted of nine inoculated plants per pathogen. Three plants per isolate and three isolates per pathogen.

Five weeks after inoculation plants were lifted from the soil and carefully washed to remove the potting medium from the roots. The plants were then surface disinfested for 1 min in a 1% sodium hypochlorite (NaOCl) solution prepared from commercial bleach, followed by 30 sec rinse in 50% ethanol and two rinses of 1 min each in sterile water. The plant material was then air dried in the laminar flow cabinet. Isolations were made by dissecting pieces of tissue approximately 5 mm long from the margin between healthy and diseased tissues of the roots and placing them onto fresh homemade potato dextrose agar (PDA). After isolation the plants were placed into paper bags, dried in a drying oven at 60°C for a week, and weighed.

Statistical treatment of data. Dry mass data were subjected to analysis of variance (ANOVA). Fisher's Least Significance Difference method (LSD) was used to test for significant differences between fungi at the 5% and 10% significance levels (Lyman-Ott 1993). The Shapiro-Wilk test (Shapiro and Wilk 1965) was performed to test for normality in the residuals. The data were analysed using SAS version 8.2 (SAS 1999).

Results

Isolation of pathogens. Many fungi were isolated from the strawberry plants but the fungal pathogens commonly associated with black root rot in the Western Cape Province included *Coniella fragariae*, *Cylindrocarpon destructans*, *Pythium irregulare* and *Rhizoctonia* sp(p) (Table 2). *Coniella fragariae, P. irregulare* and *Rhizoctonia* sp(p) were consistently isolated from diseased plants (Table 2). In 1998 the isolations yielded predominantly *Coniella* (42.7%) but the occurrence of *Coniella* decreased in 1999 (3.45%) and 2000 (2.79%).

Pythium irregulare was not isolated at the end of a season (November). There was a consistently high incidence of *Pythium* during July but this decreased as the

season progressed and by the end of the season *Pythium* could no longer be isolated (Table 2, Fig. 1).

Two morphologically different groups of *Rhizoctonia* were isolated, one that formed brown colonies and the other white colonies. The *Rhizoctonia* sp(p) were consistently isolated throughout the season. The general pattern of occurrence for the two morphotypes was similar. In July and September a relatively low occurrence was recorded, but this increased to a high occurrence in November (Table 2, Fig. 1).

In November 1998 a very high occurrence of *C. destructans* was recorded (14.58%), but it was not isolated again until November 2000. On the latter occasion it only comprised 0.02% of the fungi isolated (Table 2). All pathogens were re-isolated from infected plants.

Pathogenicity tests. All isolates were pathogenic to strawberry cv Tiobelle plants, which was evident by the necrotic lesions that developed on the roots (Figs. 2 and 3), as well as the reduced root volume and dry mass relative to the controls (Figs. 4 and 5) and all the fungi tested were re-isolated from infected plants. Using dry mass as an indicator of virulence, differences among the species tested were observed. The control plants had a higher dry mass (3.07 g) than the inoculated plants (Table 3). However, the dry masses of plants inoculated with either *Coniella fragariae* or *Cylindrocarpon destructans* were not significantly ($P = 0.05$) lower than the control. Plants inoculated with either morphotype of *Rhizoctonia* had significantly ($P = 0.05$) lower dry masses than plants inoculated with any of the other pathogens. The brown morphotype of *Rhizoctonia* was more virulent than the white morphotype and caused plants to have a significantly ($P = 0.05$) lower dry mass (0.96 g) (Table 3). The plants treated with *Pythium irregulare* had significantly lower dry masses than the control.

Discussion

The isolation data confirmed that a number of the fungal pathogens commonly associated with black root rot in other countries (Maas 1998) were also isolated from roots of symptomatic strawberry plants from the Western Cape Province. The two fungal pathogens that were consistently isolated throughout this study were *P. irregulare* and *Rhizoctonia* sp(p).

Pythium irregulare was frequently isolated from diseased roots in July and less frequently in September in the Western Cape Province. In Illinois (USA) *P. irregulare* was also associated with this disease by Nemec and Sanders (1970). However, the involvement of *Pythium* spp. in the disease has been considered less important in some studies (Wilhelm and Paulus 1980, Elmer and LaMondia 1999).

Rhizoctonia sp(p) on the other hand are often considered major role players in the black root rot complex (Maas 1998; Martin 2000). In the present study two morphotypes of *Rhizoctonia* were clearly evident. These morphotypes may represent different species and further studies are required to establish this (see Chapter 3). Within the white morphotype of *Rhizoctonia* there were big differences in the dry mass of plants inoculated with the different isolates. These differences might, however, be attributed to different AG types.

The general patterns evident in the occurrence of both the *Pythium* and *Rhizoctonia* spp. suggests that there is a root niche predominance of different species that is dictated by seasonal conditions. *P. irregulare* was not present in diseased plants when the most severe symptoms of black root rot were evident (at the end of the season - November) but was isolated in high numbers at the beginning of the season (winter - July). Conversely, *Rhizoctonia* spp. were more prevalent at the end of the growing season (summer), although they were present throughout the season. Thus, it appears that *Pythium* is the dominant fungal pathogen isolated in winter and *Rhizoctonia* in summer. When strawberry plants are small and the young roots soft and thin they are very susceptible to *Pythium* infection (Plakidas 1930; Maas 1998). In the Western Cape Province strawberries are planted in the autumn (April) when temperatures are cool and rainfall occurs These environmental conditions and the presence of young roots on transplanted plants are excellent for *Pythium* infection. Root development is thus curtailed due to the infection by this pathogen in winter in this area. However when the fruiting season starts in spring (September), temperatures rise, the rainy season ends and the incidence of *Pythium* decreases. By the end of the season, conditions are dry and hot and *Pythium* is no longer isolated from the plants. Incidence patterns of *Pythium*, and *Rhizoctonia* on strawberry roots therefore display opposite trends. This is the first report of the difference in seasonal incidence patterns of *Pythium* and *Rhizoctonia* on strawberry roots with black root rot symptoms. It provides information on the epidemiolgy of this multifaceted disease complex, as well as providing the first step towards an integrated pest management

system by making it possible to apply specific fungicides at the optimum time in the season. Although further research is necessary, this information will be of great importance for developing and implementing an effective black root rot management program.

Although both *Coniella* and *Cylindrocarpon* have been associated with disease of strawberry plants, in the work reported here, they did not have a significant stunting effect. *Coniella fragariae* has been associated only with fruit rot on strawberries (Maas 1984), which would explain the minimal pathogenic effect on the roots and dry mass of the plants. Therefore, it can be concluded that *Coniella* is not part of the complex of primary pathogens causing black root rot.

Although the etiology of black root rot of strawberries has not been completely resolved, this work has confirmed a role for *Pythium* and *Rhizoctonia* in the disease complex in the Western Cape Province. Seasonal succession of the main role players in the disease has also been demonstrated and this should be considered in studies investigating disease management strategies.

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Table 1. Fungal pathogens used in pathogenicity trials

Species[a]	STE-U number
Coniella fragariae	4600
	4601
	4602
Cylindrocarpon destructans	4603
	4604
	4605
Pythium irregulare	4606
	4607
	4608
Rhizoctonia (brown colony)	4609
	4610
	4611
Rhizoctonia (white colony)	4612
	4613
	4614
	4615
	4616
	4617
	4618

[a] All isolates are maintained in the Department of Plant Pathology culture collection, University of Stellenbosch.

Table 2. Seasonal occurrence in the Western Cape Province of fungal pathogens commonly associated with black root rot of strawberries

	Occurrence (%)[a]						
	1998	1999			2000		
Species	Nov	July	Sept	Nov	July	Sept	Nov
Coniella fragariae	42.7	22.2	16.0	3.5	2.0	3.9	2.8
Cylindrocarpon destructans	14.6	0.0	0.0	0.0	0.0	0.0	0.02
Pythium irregulare	0.0	27.8	4.0	0.0	6.1	2.0	0.0
Rhizoctonia (br)[b]	8.4	2.9	5.6	7.1	2.3	1.8	3.8
Rhizoctonia (wh)	8.2	8.2	6.4	13.5	3.9	2.1	8.1

[a] Occurrence is expressed as a percentage based on the number of isolates of a species out of the total number of isolates of fungi isolated at a time.

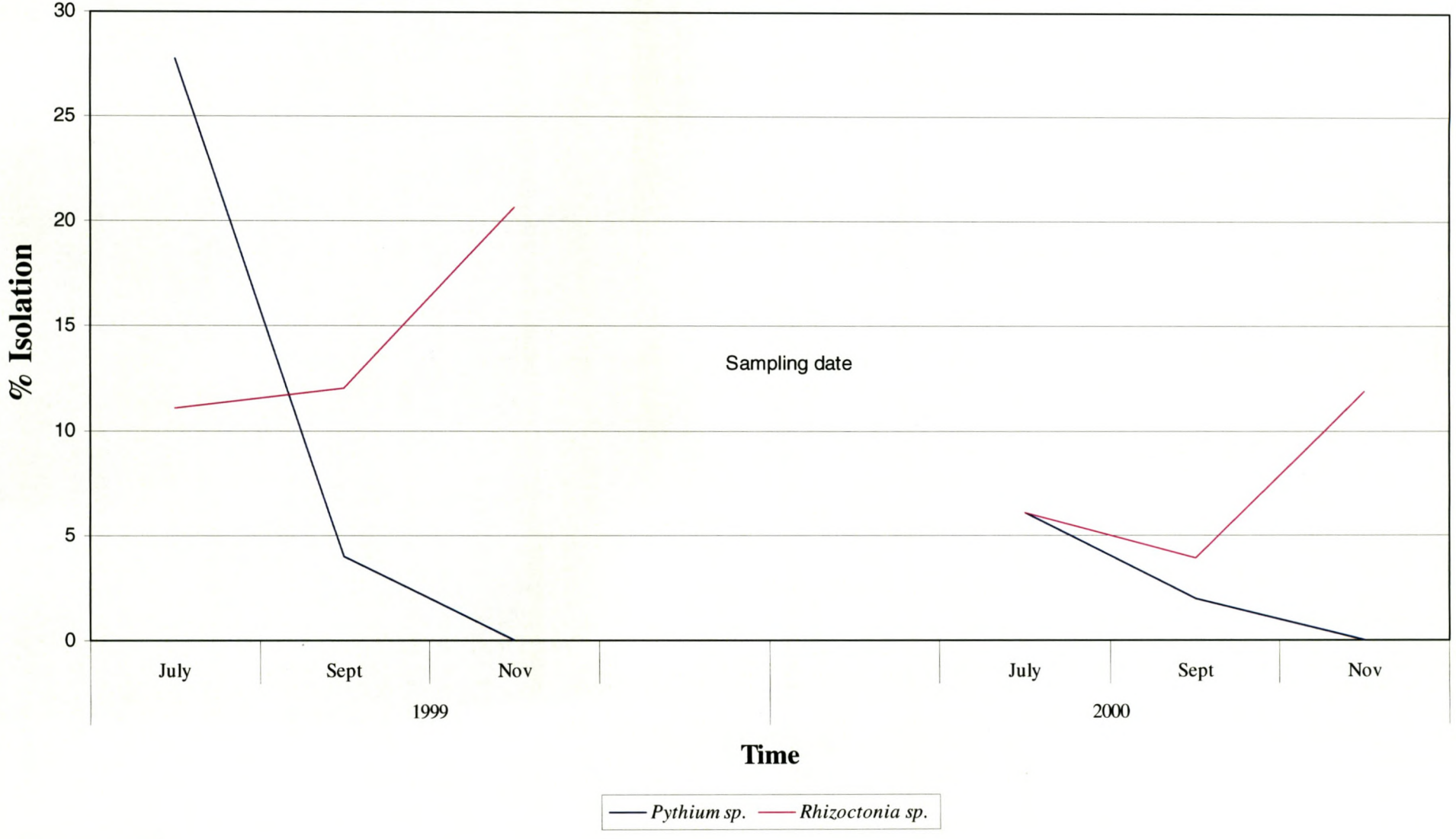
[b] *Rhizoctonia* (br) = brown morphotype, *Rhizoctonia* (wh) = white morphotype.

Table 3. Dry mass of strawberry plants inoculated with fungal pathogens

Fungus	Dry mass (g)[a]
Coniella fragariae	2.55 **ab**
Cylindrocarpon destructans	2.55 **ab**
Pythium irregulare	2.39 **b**
Rhizoctonia (brown morphotype)	0.96 **d**
Rhizoctonia (white morphotype)	2.29 **c**
Control	3.07 **a**

[a]Means with the same letter are not significantly different at $P = 0.05$.

Fig. 1. Seasonal interaction between *Pythium* and *Rhizoctonia*



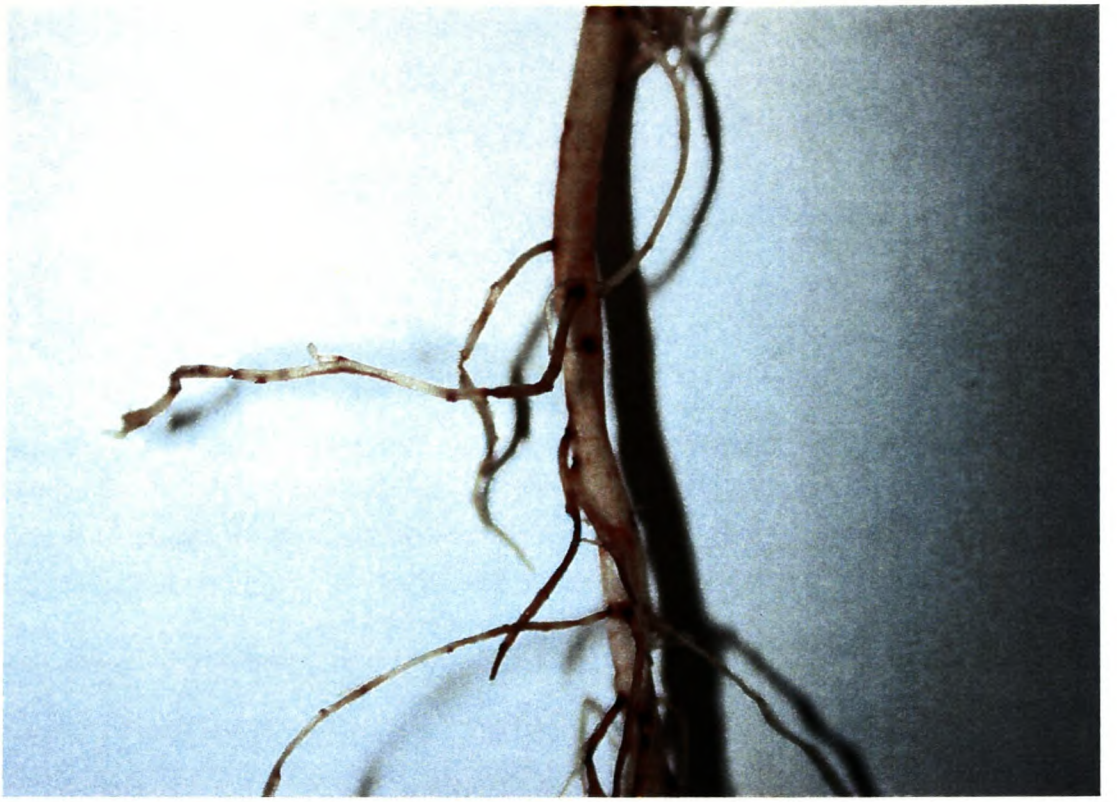


Fig. 2. Lesions on fine lateral root caused by *Pythium irregulare*

Fig. 3. Root lesions with a rats-tailed appearance formed by *Rhizoctonia* spp.

Fig. 4. Root system infected with *Rhizoctonia* (white colony) below picture

Fig. 5. Root system infected with *Rhizoctonia* (brown colony)

3: Characterisation and pathogenicity of *Rhizoctonia* isolates associated with black root rot of strawberries in the Western Cape Province

Abstract

Black root rot is an important disease of strawberry caused by a complex of fungi including species of *Rhizoctonia.* In this study the *Rhizoctonia* species and anastomosis groups isolated from diseased strawberries in the Western Cape Province were determined, and their pathogenicity and relative virulence assessed. Both binucleate and multinucleate types were recovered from diseased roots and identified as *R. fragariae* and *R. solani*, respectively. Anastomosis grouping of the isolates was carried out on a sub-sample using the conventional method of hyphal fusion, and molecular techniques were employed to confirm results of the former. RFLP analysis of the 28S RNA gene and DNA sequence analysis of the internal transcribed spacer (ITS) regions were used to further characterize relationships among the isolates of *Rhizoctonia.* The molecular results correlated with those obtained from the conventional methods. All isolates of *R. solani* were members of anastomosis group 6, whereas three AG types were identified among isolates of *R. fragariae, viz.* AG-A, AG-G and AG-I at a relative occurrence of 69%, 25%, 6%, respectively. Pathogenicity trials were conducted on eight-week-old cv. Tiobelle plants. All *Rhizoctonia* isolates were pathogenic to strawberry, but *R. solani* (AG 6) was the most virulent causing severe stunting of plants. *R. fragariae* AG-A and AG-G were not as virulent as *R. solani* but also caused stunting. *R. fragariae* AG-I was the least virulent, and did not cause stunting of the plants. However, isolates incited small, pale, spreading lesions on infected roots. This is the first species confirmation and AG type identification of *Rhizoctonia* taxa causing root rot of strawberries in South Africa and in the Southern Hemisphere.

Introduction

Black root rot is a serious yield limiting disease of strawberries that reduces production by about 30% annually in the Western Cape Province and it is currently recognised as the most important root disease of strawberries in this area (Botha *et al.* 2001). This disease has also been identified in Australia (Porter *et al.* 1999), the Netherlands (Klinkenberg 1955), Japan (Watanabe 1977), the United Kingdom (Wardlaw 1926) and the USA (Heald 1920; Coons 1924; Wilhelm and Paulus 1980; Yuen *et al.* 1991; Duniway 1998). Despite its significance the etiology of black root rot has not yet been fully resolved, and appears to vary according to the site in which it occurs. However, in general it is accepted that a complex of fungal pathogens is the primary cause of the disease (Maas 1998; Denman 1994; Ellis 2000; Botha *et al.* 2001).

Rhizoctonia spp. have consistently been implicated in the black root rot complex (Heald 1920; Coons 1924; Herr 1979; La Mondia and Martin 1989; Denman 1994). Two species, *R. fragariae* S.S. Husain & W.E. McKeen and *R. solani* J.G. Kühn are considered the major pathogens contributing to disease development (Ribeiro and Black 1971; La Mondia and Martin 1989; Martin 1988; Martin 2000). In a study carried out in Connecticut, USA only 2.7% of the isolates were identified as *R. solani* (AG 5), the rest were *R. fragariae*, including AG-G (52.7%), AG-A (27.3%), and AG-I (17.3%) (Martin 1988). Similar results were obtained in California where *R. fragariae* dominated the population of *Rhizoctonia* recovered from strawberries (Martin 2000). However, the ratio of the different AG types differed from those in Connecticut with AG-A dominating (68%) followed by AG-I (21.3%) and AG-G (10.7%). Although *Rhizoctonia* spp. were isolated from strawberry plants with symptoms of black root rot in the Western Cape Province of South Africa, isolates were not identified to species level and anastomosis groups were never determined (Denman 1994).

Recently molecular techniques have enabled researchers to identify AG types of *Rhizoctonia* spp. more efficiently. The internal transcribed spacer or ITS regions of the nuclear encoded ribosomal RNA genes (rDNA) have been shown to differ sufficiently to distinguish among the different anastomosis groups (Cubeta *et al.* 1991; Mazzola 1997; Gonzalez *et al.* 2001).

Despite the fact that different species and AG types of *Rhizoctonia* have been associated with the black root rot complex of strawberries, not much has been recorded about their potential role in this disease complex. Ribeiro and Black (1971) suggested that *R. fragariae* existed as an endophytic mycorrhiza in strawberries, and that environmental conditions might affect the pathogenicity of this fungus. Martin (1988) demonstrated that the relative virulence of anastomosis groups of *R. fragariae* differed in a temperature-dependent manner. AG-I was the most virulent at 15°C causing severe disease, but at higher temperatures AG-A and AG-G were more virulent than AG-I.

The aims of the work carried out in this study were therefore to identify the *Rhizoctonia* spp. associated with black root rot of strawberries in the Western Cape Province, and to determine their AG types. In addition, studies were conducted to determine the pathogenicity of each species, and relative virulence of the AG types in strawberries.

Materials and Methods

Isolation of *Rhizoctonia* spp. During 1999–2000, 630 diseased plants were collected from strawberry farms in the Western Cape Province. Plants were taken to the laboratory in cooler boxes and prepared for isolations immediately. Leaves were removed and the remainder of the material was washed. Plants were surface disinfested for 1 min in a 1% sodium hypochlorite (NaOCl) solution (prepared from commercial bleach), and 30 sec in 50% ethanol, then rinsed twice (1 min each) in sterile water and air dried in a laminar flow cabinet. Isolations were made by dissecting three pieces of tissue approximately 5 mm long from the margin between healthy and diseased tissues of the roots and placing them onto a range of culture media. The crowns were split longitudinally and tissue was excised from the centre of the crowns and two pieces plated on culture media. Media used included 2% V-8 juice agar (Galindo and Gallegly 1960) (V-8 agar), PARPH (*Phytophthora* selective medium) (Solel and Pinkas 1984), PARP (a medium selective for *Pythium* adapted from PARPH by omitting the hymexazol), water agar (WA) (Biolab, Midrand, South Africa), corn meal agar (Difco) (CMA) and fresh homemade potato dextrose agar

(PDA) (200g potatoes, 20 g dextrose, 12 g agar in 1 L water). Hyphal tips of fungi emerging from plant material were subcultured and transferred to divided plates containing carnation leaf agar (CLA) (Fisher *et al.* 1982) in one half of the dish and PDA in the other half. Plates were incubated under near-ultraviolet and cool white light with a 12 h photoperiod at 25°C for three weeks. Microscopic examination of cultures was carried out and isolates identified as *Rhizoctonia* spp. were subjected to the staining procedures outlined by Yamamoto and Uchida (1982). AG typing and molecular confirmation of identifications were carried out according to the methods described by Mazzola (1997).

Nuclear staining of *Rhizoctonia* spp. Nuclear staining was performed using a fluorocrome, acridine orange stain and viewing the stained specimen through a fluorescence microscope (Yamamoto and Uchida 1982). Isolates were cultured on clarified 2% vegetable juice agar without $CaCO_3$ (Yamamoto and Uchida 1982). Four sterilized microscope glass cover slips (Marienfeld cover glasses number 1, 22 x 22 mm) were placed on the medium next to the inoculum disc and plates were incubated for 48 h at 25°C with a 12 h photoperiod under cool white light.

The stain was prepared as follows: Veronal acetate stock was made with 0.971 g sodium acetate, 1.471 g soluble sodium diethylbartiturate and 50 mL distilled water. Veronal acetate solution was then prepared by mixing 50 mL 0.01M hydrochloric acid, 74 mL distilled water and 100 mL veronal acetate stock, the pH was adjusted to 4.5 using NaOH or HCl. Veronal acetate buffer solution pH 4.5 (100 mL) was mixed with 2.5 mg of 0.0025% acridine orange.

After 48 h the cover slips that were overgrown with mycelium growth were mounted, mycelium side downwards, into the drop of stain on a microscope slide and examined using a Zeiss Axioskop microscope equipped with a epifluorecence condenser and a high-pressure mercury lamp. The Zeiss 02, 06 and 18 filters were used. The nuclei stained bright green and were clearly visible and easy to count when freshly stained. The nuclei of twenty cells per isolate were counted to confirm the nuclear status of each isolate.

AG typing using conventional methods. A sub-sample of isolates representing 10% of the isolates examined for each species was selected randomly and used for the

anastomosis tests. In establishing AG types the unknown isolates and tester strains (Table 1) were paired by plating one unknown isolate opposite a known AG type on 2% WA in Petri dishes. Analysis of hyphal fusion was conducted by examining the zone of hyphal interaction at 100 x magnification using a light microscope.

AG typing using molecular techniques. The anastomosis results were confirmed using restriction analysis of the 28S rRNA gene of the isolates, according to the procedure outlined below.

DNA extraction. DNA extraction was carried out according to the method described by Mazzola *et al.* (1996). Erlenmyer flasks (250 mL) containing 50 mL YM^{+} broth (3g yeast extract, 3g malt extract, 5g peptone and 20g glucose per litre water) were inoculated and placed on a rotary shaker at 200 rpm until a large mat of mycelium covered the surface of the medium. The mycelium was harvested onto a sterile filter paper disk that was placed in a Buchner funnel. The growth medium was gently suctioned from the mycelium, which was then washed with sterile H_2O. Excess H_2O was removed by blotting the filter paper between two sterile paper towels. Approximately 200 mg mycelium was placed into a 1.5 mL eppendorf tube and was lyophilized overnight without closing the caps of the tubes.

Dried mycelium was ground using a pipette tip and resuspended in 600 µL extraction buffer (200 mM Tris-Cl, pH 8.0, 250 mM NaCl, 25 mM EDTA, pH 8.0 and 0.5% non-sterile SDS), and shaken briefly in a vortex machine. After resuspension the mixture was microfuged for 5 min at maximum speed and the upper aqueous layer removed to a new tube. The DNA was extracted once with 70% volume phenol and 30% volume chloroform. After centrifugation the extraction was repeated, the upper layer transferred to a new tube and mixed with ~55% volume isopropanol to precipitate the DNA.

After precipitation the solution was microfuged for 1 min at maximum speed to pellet the DNA, and the supernatant was carefully removed and discarded. The DNA was washed once with 70% ETOH, microfuged again for 3 min at maximum speed to reform the DNA pellet and the supernatant was again discarded. The DNA was resuspended in 100 µL TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and placed in a heat block at 55°C for 1 h to dry. The DNA was diluted 4:1000 in TE buffer (10 mM Tris-Cl and 0.1 mM EDTA) for use in PCR.

PCR amplification. PCR amplification of DNA was conducted using the two primers LR7 (5'-TACTACCACCAAGATCT-3') and LROR (5'-ACCCGCTCAACTTAAGC-3') (Cubeta *et al.* 1991). A final volume of 25 µL per reaction was used which included 5 µL dilute DNA sample, 2 units *Taq* polymerase, 1X manufacturers reaction buffer with 1.5 µM $MgCl_2$, and 200 µM of each deoxynucleoside triphosphate. The amplification reactions were conducted in a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Norwalk, CT) using the following reaction conditions, 94°C for 1 min, 49°C for 2 min and 74°C for 3 min, which were repeated for 30 cycles (Cubeta *et al.* 1991).

Restriction enzyme digestion. Restriction enzyme digestion was used to distinguish between the different AG types. Digestion reactions were set up using isolates of known AG type as well as the unknown isolates. PCR products (2 µL) were mixed with the one of the following restriction enzymes *Hpa*II, *Hha*I or *Taq*I (10 units; Invitrogen Life Technologies, Carlsbad, CA, USA), sterile distilled water (15 µL) and enzyme reaction buffer (1X) in a total reaction volume of 20 mL. The mixtures were incubated overnight at room temperature (~25°C). Reaction products were then separated on a 1.5% agarose gel in Tris-borate-EDTA buffer. The gel was stained using ethidium bromide and DNA fragments were viewed using an ultraviolet transilluminator.

Pot trials. Pathogenicity tests were carried out in pot trials using eight-week-old strawberry cv Tiobelle plants. To ensure that plants were pathogen free, hardened off tissue culture plantlets were used. Plantlets were transplanted into sterilised composted pine bark medium and hardened off with glass jars inverted over them for four weeks in the glasshouse at 25–35°C. After the hardening off period glass jars were removed, and plants were kept for an additional four weeks before pathogenicity trials were carried out.

Sand-bran inoculum was prepared using the method described by (Lamprecht *et al.* 1988). Washed river sand (400 g) and wheat bran (20 g) were mixed together in 500 mL Schott bottles and 60 mL sterile water was added. The mixture was autoclaved at 120°C and 1.5 kgf/cm^2 for 15 min, and then left to cool. This process was repeated for three successive days after which each bottle was inoculated with

four 5 mm plugs of a single *Rhizoctonia* isolate and incubated at 25°C for a week before use.

Pilot trial. A pilot trial was carried out to determine the level of inoculum that would enable differentiation of pathogenicity and relative virulence of the various *Rhizoctonia* isolates. A single isolate of *R. solani* and one of *R. fragariae* were selected randomly and sand-bran inoculum was prepared as described previously. One plant was used for each inoculum concentration (1, 5 and 10% mass of inoculum: mass of planting medium) and uninoculated, sterile sand-bran was used in the controls. Inoculum was mixed into sterilised potting medium and eight-week-old cv Tiobelle plants were planted into the pots. Plants were kept in a growth chamber at 25°C with a 12 h day / night interval for five weeks as described by Martin (2000). The experiment was laid out as a complete randomised design. At the end of the trial plants were removed from the soil and carefully washed. Five roots were selected at random from each plant and the percentage of root necrosis was calculated by measuring the total length of each root and summing the lengths of the lesions formed on each root, and expressing the necrotic area as a percentage of the root length (Martin 1988). Isolations from necrotic areas that developed on roots were made on PDA as described previously.

Pathogenicity trial. The pathogenicity trials were conducted based on the results of the pilot trial and an inoculum level of 5% was selected. The planting medium of the control plants was amended with 5% sterile sand-bran. Using results of the anastomosis characterisation, seven isolates of *R. fragariae* (STE-U 4612, 4613, 4614, 4615 type AG-A, STE-U 4616, 4617 type AG-G and STE-U 4618 type AG-I) and three isolates of *R. solani* (STE-U 4609, 4610 and 4611) were selected for the pathogenicity trial. Three plants were inoculated per isolate. Tests were carried out as described previously and percentage root necrosis was assessed as stated above. In addition to recording percentage root rot, the above ground parts were dried at 60°C for 72 h and dry mass was recorded.

Statistical treatment of data. All data from the pilot trail and the pathogenicity tests were subjected to analysis of variance (ANOVA). Fisher's Least Significance

Difference method (LSD) was used to test for significant differences at the 5% confidence level (Lyman-Ott 1993). The data were analysed using SAS version 8.2 (SAS 1999).

Results

Isolation of *Rhizoctonia* spp. *Rhizoctonia* spp. were isolated from 44% of the diseased plants surveyed. Different morphological types were evident on visual inspection. The isolates were divided into two groups, those with very dark brown mycelium and those with white to light brown mycelium. Overall 59.3% of the *Rhizoctonia* species isolated were light coloured and 40.7% were dark brown.

Nuclear staining of *Rhizoctonia* spp. Nuclear staining revealed that all the dark brown isolates were multinucleate and were thus called *R. solani*. The light coloured isolates all had binucleate cells and were called *R. fragariae*. Thus 59.3% of the isolates obtained were *R. fragariae* and 40.7% of the isolates were *R. solani* (Table 2).

AG typing using conventional methods. All the multinucleate *R. solani* isolates were successfully anastomosed with AG 6, and were therefore designated AG 6. Anastomosis tests revealed that there were three AG groups of *R. fragariae*, *viz.* AG-A, AG-G and AG-I (Table 2). AG-I was represented at a very low level (6%), AG-A was the most frequently isolated type (69%) while 25% of the *R. fragariae* isolates were AG-G.

The restriction analysis of the 28S rRNA gene of the isolates confirmed the AG types assigned to the isolates using conventional methods. The banding pattern formed when RNA is digested with a restriction enzyme is shown in Fig. 1. The two *R. solani* isolates in lanes 14 and 15 have the exact same banding pattern as the AG 6 isolate in lane 13 and was therefore identified as AG 6. This process was repeated with all the unknown isolates of both *R. solani* and *R. fragariae* to support the AG types that were determined by analysis of hyphal anastomosis.

Pilot trial. Both *R. solani* and *R. fragariae* were pathogenic to strawberry causing root lesions and the pathogen was reisolated from lesions on the roots of plants grown

in artificially infested soils. There were no significant differences (P = 0.05) in percent root necrosis of plants grown in soils infested with 1% or 5% of *Rhizoctonia* inoculum (Table 3). However, at an inoculum concentration of 10%, percent root necrosis was significantly higher relative to all other treatments and much of the root system had disintegrated. The pattern of disease severity at the different inoculum levels was similar for both *Rhizoctonia* species. A degree of root discolouration was evident on some of the control plants, but no pathogenic fungi were isolated from these lesions.

Pathogenicity trial. *Rhizoctonia solani* (AG 6) and *R. fragariae* (AG-G, AG-A and AG-I) were pathogenic to strawberry roots causing root necrosis similar to that seen on plants with black root rot symptoms. Using plant dry mass as an indicator of pathogen virulence *R. solani* was more virulent than binucleate isolates causing severe stunting, wilting and collapse of young strawberry plants. The mean dry mass of plants inoculated with *R. solani* (AG 6) was significantly lower (P = 0.05) than that of any of the other inoculated plants or the controls (Table 4). The dry mass of plants inoculated with *R. fragariae* (AG-A), was significantly lower than the control plants or the AG-I inoculated plants but was not significantly different from the AG-G inoculated plants (Table 4).

However, if the percent root necrosis was considered, the picture changed. It was significant that no root necrosis developed in the control plants (Table 5). Percent visible root necrosis caused by *R. solani* was significantly less (P = 0.05) than that caused by *R. fragariae*, with the exception of *R. fragariae* (AG-I) (Table 5). The differences in visible root necrosis caused by AG-G and AG-A isolates were not significant, but AG-G caused significantly more root necrosis than *R. fragariae* AG-I.

Root lesions resulting from infection by *R. solani* were small and restricted. Many (<10 mm long) ring like lesions occurred along the length of infected roots. In contrast, irrespective of AG type, *R. fragariae* typically incited a single or a few large spreading lesions on roots of strawberry. The lesions caused by the AG-I isolates were very pale by comparison with those caused by the other AG types.

Discussion

This study represents the first attempt to comprehensively characterise the species of *Rhizoctonia* associated with strawberries in the Western Cape Province of South Africa. Two species have been identified, *R. fragariae* and *R. solani*, and multiple AG types of *R. fragariae* have been confirmed.

Identification of the *Rhizoctonia* spp. and anastomosis groups has made it possible to compare the Western Cape Province situation to that in the USA (Connecticut and California) where black root rot is also a problem. *Rhizoctonia solani* was isolated at a much higher incidence in the former than in the latter areas. Furthermore, *R. solani* AG 6 was present in the Western Cape Province in contrast to *R. solani* AG 5 which was present in both Connecticut and California (Martin 1988; Martin 2000). RFLP analysis of the 28S rRNA gene provided further evidence to support the identification of these *R. solani* isolates as members of AG 6. The molecular techniques used for characterisation could be readily carried out in the laboratory and results were relatively easily interpreted, whereas interpretation of the anastomosis reaction was not always easy.

The binucleate isolates (*R. fragariae*) in the Western Cape Province contained the same three anastomosis groups (AG-A, AG-G, AG-I) as those recorded from the USA. The relative frequencies at which these AG types occurred in the sub-sample may not be representative of the entire area. In the sub-sample AG-A was the most frequently isolated AG type, which is contrary to that reported from Connecticut where AG-G was the most frequently isolated strain. The ecological significance of the occurrence of the different AG types has yet to be established.

The pathogenicity tests elucidated differences in the severity and morphology of the lesions caused by the two species of *Rhizoctonia*. In the present study *R. solani* was more virulent than *R. fragariae* causing root necrosis, wilting and severe stunting of plants. Plants inoculated with *R. fragariae* AG-A and AG-G were only slightly stunted and root tip necrosis was clearly evident.

In spite of the fact that *R. solani* was more virulent than *R. fragariae*, it is interesting to note that the percentage root necrosis caused by the former was significantly lower than that caused by *R. fragariae* (AG-A and AG-G). The latter (AG-A and AG-G) usually caused a single, large lesion at the root tips, which

rendered only the basal portion of the root dysfunctional. On the other hand, much smaller lesions were caused by *R. solani,* and more than one lesion occurred over the length of the root. Frequently lesions caused by *R. solani* were formed just below the crown area of the plant causing the roots to break off or become non-functional near the crown. The absorption and transport capacity of the root system would therefore be greatly reduced which would prevent nutrients and water from reaching the above ground plant parts. This might explain why plants infected with *R. solani* in the pathogenicity tests had a very low dry mass even though the percentage root necrosis was less than that of plants infected with the *R. fragariae* isolates.

The AG-I isolate caused lesions that were comparable to those induced by *R. solani* as opposed to those caused by the other *R. fragariae* anastomosing groups. However, the lesions caused by AG-I were far less severe and did not occur as close to the crown area as those caused by *R. solani.* It has been shown that AG-I is more virulent under cooler conditions (10–15°C) (LaMondia and Martin 1989; Martin 2000). The tests carried out in the present study were conducted at 25°C thus the relative virulence observed for the South African AG-I isolates may indicate that environmental conditions will have a significant impact on the interaction between these fungi and the plant host. Such a finding has been reported for other species and anastomosis groups of *Rhizoctonia* (Burton *et al.* 1988; Smiley and Uddin 1993). It will be important to establish the effects of temperature on the virulence of the different species and anastomosis groups, so that this information can be incorporated into an integrated disease management programme.

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Table 1. Species, source and anastomosis type of *Rhizoctonia* tester isolates

Species	Isolate number	AG type
R. fragariae	BN4[a]	AG-A
	C-662	AG-A
	C-653	AG-G
	AV-2	AG-I
R. solani	R-43	AG1-IC
	F56L	AG 2-1
	RI-164	AG 2-2IV
	Sc124	AG 3
	AH-1	AG 4 HGI
	RH-165	AG 4 HGII
	RH-184	AG 5
	HAM1-1	AG 6 HGI
	1529	AG 7
	SqR1	AG 9
	91507	AG 10
	Roth 25	AG 11
	AI 1-4	AG-BI

[a] All isolates were supplied by M. Cubeta from the University of North Carolina, Vernon James Centre, Plymouth.

Table 2. Occurrence of species and AG types of *Rhizoctonia* spp. isolated from strawberries in the Western Cape Province

Species	Occurrence (%)[a]	AG type	Occurrence (%)[b]
R. fragariae	59.3	AG-A	43.7
		AG-G	50.0
		AG-I	6.3
R. solani	40.7	AG 6	100

[a] Percentage based on the number of isolates of each species out of the total number of *Rhizoctonia* isolates obtained.

[b] Percentage based on the number of isolates of each AG type out of the total number of isolates of each species of *Rhizoctonia*.

Table 3. Percentage root necrosis formed at different concentrations of *Rhizoctonia fragariae* and *R. solani* inoculum used in the pilot trial

	Percentage root necrosis[a] formed at different inoculum concentrations		
Species	1 (%)[b]	5 (%)	10 (%)
R. fragariae	17 **bc**[c]	27 **bc**	76 **a**
R. solani	22 **bc**	34 **b**	58 **a**
Control	21 **bc**	7 c	31 **bc**

[a] The percentage of root necrosis was calculated by measuring the total length of each root, summing the lengths of the lesions formed on each root, and expressing the necrotic area as a percentage of the root length. Values are the means of five measurements per inoculum concentration.

[b] The percentage inoculum is based on the mass of inoculum as a percentage of the mass of the planting medium.

[c] Means followed by the same letter do not differ significantly ($P = 0.05$).

Table 4. Dry mass of the above-ground parts of plants grown in soil artificially inoculated with *Rhizoctonia* spp.

Species	AG type	Dry mass (g)[a]
R. fragariae	AG-A	1.79 **b**[b]
	AG-G	1.97 **ab**
	AG-I	2.77 **a**
R. solani	AG 6	0.84 **c**
Control	-	2.70 **a**

[a] Values are the means of nine readings per AG type.

[b] Means followed by the same letter do not differ significantly ($P = 0.05$).

Table 5. Percentage root necrosis caused by different *Rhizoctonia* species and AG types in artificially inoculated soils

Species	AG type	Root necrosis (%)[a]
R. fragariae	AG-G	47.0 **a**[b]
	AG-A	35.5 **ab**
	AG-I	21.0 **bc**
R. solani	AG 6	16.3 **c**
Control	-	0.0 **d**

[a] The percentage of root necrosis was calculated by measuring the total length of each root, summing the lengths of the lesions formed on each root, and expressing the necrotic area as a percentage of the root length. Values are the means of 15 readings per AG type.

[b] Means followed by the same letter do not differ significantly ($P = 0.05$).

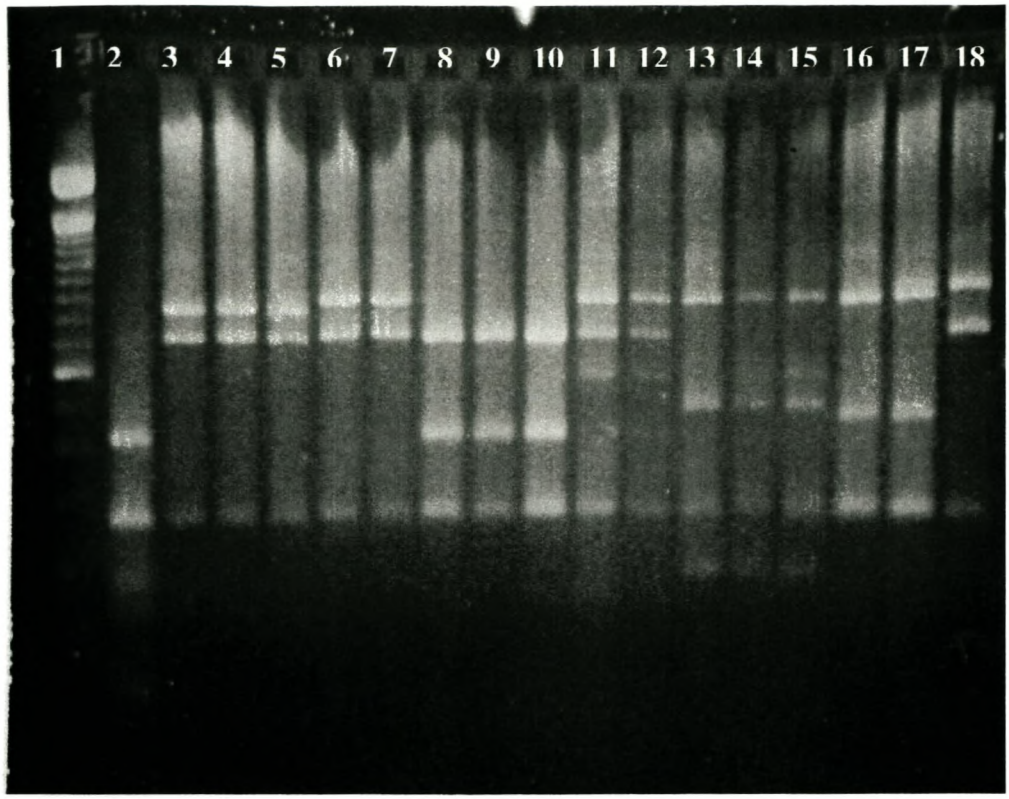

Fig. 1. Rhizoctonia isolates digested with a restriction enzyme HpaII.

Lane 1: 100-bp ladder, lane 2: AG 1-1, lane 3: AG 1-2, lanes 4 and 5: AG 2-2, lanes 6 and 7: AG 3, lanes 8–10: AG 4, lanes 11 and 12: AG 5, lane 13: AG 6, lanes 14 and 15: unknown isolates (STE-U 5246 and 4611), lanes 16–18: AG 8.

4: Soil and propagation material: Sources of inoculum of fungi associated with black root rot of strawberry

Abstract

In this study, an assessment of the presence and quantity of soil born fungal pathogens associated with soil from farmlands planted with strawberries was tested prior to - and post - fumigation with methyl bromide. Isolations were also made from nursery plants to determine whether any black root rot pathogens were in the plants before transplanting. Results demonstrated that after fumigation the soil is free of all pathogenic fungi associated with black root rot. However, the main pathogens involved in black root rot, *viz. Rhizoctonia fragariae, R. solani* and *Pythium* spp. were isolated from nursery plants. The fact that the plants are already infected with these pathogens renders the prospects for control of this disease difficult. Further studies are urgently required to develop production practices that would enhance the root environment and thereby reduce the detrimental effect of black root rot pathogens.

Introduction

Black root rot is the most important yield limiting root disease of strawberries in the Western Cape Province (Botha *et al.* 2001). Although the precise etiology remains unresolved (Heald 1920; Coons 1924; Plakidas 1930; Wilhelm and Paulus 1980; Yuen *et al.* 1991; Duniway 1998), there is general consensus that *Rhizoctonia* and *Pythium* spp. are major role players in the disease etiology (Maas 1984; Elmer and LaMondia 1999; Martin 2000; Botha *et al.* 2001). *Rhizoctonia* spp. survive long periods in soil on plant debris (Cotterill 1993), whereas *Pythium* spp. are both soil- and water-borne. *Pythium* spp. form oospores as survival structures in soils and plant debris, and in conditions of abundant moisture vast quantities of motile zoospores are formed from oospores and mycelia (van der Plaats-Niterink 1981).

An important aspect of integrated disease management is the reduction of inoculum available for disease. Soil is an obvious source of inoculum (Razik *et al.*

1989). In the 1960's the advent of methyl bromide as a soil fumigant in the strawberry industry resulted in a drastic reduction of losses caused by root disease (Wilhelm and Paulus 1980; Yuen *et al.* 1991). The use of methyl bromide as a soil fumigant resulted in soil being a reduced risk as a source of inoculum (Larson and Shaw 1995; Porter *et al.* 1999). However, methyl bromide has been identified as a high-risk chemical that depletes atmospheric ozone and is harmful to beneficial soil biota, the environment and the user (Ristaino and Thomas 1997; Porter *et al.* 1999). Since South Africa signed the Montreal Protocol in 1992, it is now expected that this chemical will no longer be available for commercial use in South Africa from 2005 onwards (Ellis 2000). Furthermore, in spite of pre-plant fumigation with methyl bromide and other fumigants, some farmers in the Western Cape Province still experienced serious losses attributable to black root rot. Thus, other methods to reduce soilborne inoculum will have to be sought.

In previous studies it was stressed that the control of black root rot was not only dependent on soil disinfestation, but also the use of pathogen-free propagation material (Razik *et al.* 1989). Since strawberry plants used for production are produced in open field nurseries, the chances of them being infected with black root rot pathogens, is high.

In this study, an assessment of the presence and quantity of soil born fungal pathogens associated with soil from farmlands planted with strawberries was tested prior to - and post - fumigation with methyl bromide. Isolations were also made from nursery plants to determine whether any black root rot pathogens were in the plants before they were planted for production purposes.

Materials and Methods

Soil. Immediately prior to fumigation, soil samples were taken from four different fields on a commercial farm in the Paarl district. In each field, soil was sampled to a depth of 25 cm using a soil auger on ten randomly chosen sites in the field. The ten samples from each field were pooled into plastic bags, and thus four bulk samples were brought back to the laboratory. Soil was transported in a cooler box and placed in a cold room at 5°C until used for soil dilution plates (within a week of sampling).

Before use, each soil sample was thoroughly mixed and stock solutions made up by adding 10 g soil to 100 mL of 0.1% sloppy, water agar (Biolab bacteriological agar) (WA) to give 0.1 g soil/mL suspension (10^{-1}concentration). The stock soil suspension was mixed thoroughly for 5 min on a magnetic stirrer at 500 rpm. Serial dilutions were made until a concentration 0.000001 g soil/mL (10^{-6}) was obtained. Three 1 mL aliquots of suspension of each concentration (10^{-1} to 10^{-6}) were plated onto several selective media; PARPH (*Phytophthora* selective medium) (Solel and Pinkas 1984), PARP (a medium selective for *Pythium* adapted from PARPH by omitting the hymexazol), *Rhizoctonia* selective medium (Sneh *et al.* 1991) and selective *Fusarium* agar (SFA) (Tio *et al.* 1977) as well as WA. The aliquots were spread evenly over the surface of the dish with sterile, L-shaped glass rods. The plates were air-dried for 30 min in a laminar flow cabinet and then incubated at 25°C for 7 d. Colony forming units (CFU's) were counted after 4 and 7 d and representative colonies (based on cultural morphology) were transferred to divided plates. The divided plates contained carnation leaf agar (Fisher *et al.* 1982) in one half of the dish and potato dextrose agar (PDA) in the other. The plates were incubated at 25°C under near-ultraviolet and cool white light with a 12 h light photoperiod. Fungi were identified after 3 weeks. The entire process was repeated three weeks after fumigation with methyl bromide (post-fumigation soil). Soil dilutions were carried out on pre- and post-methyl bromide fumigated soil in 1999 and 2000.

Nursery material. Nursery material was sampled from three different sources, *viz.* two commercial nurseries, one in Ceres (Tandfontein) and one in Sutherland, as well as a private nursery in Paarl (Loewenstein). Plants were randomly selected from batches delivered to farmers for planting. Ten plants per cultivar (Chandler, Selekta and Tiobelle) were collected from each nursery. The plants were taken to the laboratory, the foliage cut off and the remainder of the material washed to remove soil particles. Roots and crowns were surface disinfested for 1 min in a 1% sodium hypochlorite (NaOCl) solution prepared from commercial bleach, followed by 30 sec rinse in 50% ethanol and then two rinses of 1 min each in sterile water. After surface disinfestation the plant material was left in the laminar flow cabinet to air dry. Isolations were made by dissecting pieces of about 5 mm from the margin between healthy and diseased tissues of the roots and placing them onto a range of culture

media. The crowns were split open and tissue was excised from the centre of the crowns and plated on the culture media. The media used included V-8 juice agar (Galindo and Gallegly 1960) (V-8 agar), PARPH, WA, corn meal agar (Difco) (CMA) and fresh homemade PDA. Two root pieces and one crown piece were plated onto each of the media totaling 18 pieces of tissue from each plant. As soon as fungi started growing out of the plant material, hyphal tips were subcultured as described previously. Plates were incubated and identifications made as described above. Occurrence of fungi isolated from nursery material was calculated as a percent of the total number of fungi isolated on each sampling date.

Results

Soil. High numbers of fungi (particularly *Fusarium* and *Pythium*) were isolated from the pre-fumigated soil but no fungi were found in the post-fumigated soils (Table 1). *Fusarium oxysporum* Schlecht. emend. Snyd. & Hans. was the most frequently detected fungus in the soil, followed by *Pythium irregulare* Buisman. *Phytophthora* was not isolated from either the pre or post-fumigated soils. Low levels of *Rhizoctonia fragariae* S.S. Husain & W.E. McKeen AG-A and AG-G as well as *R. solani* J.G. Kühn AG 6 were obtained from pre-fumigated soil only in 1999 (Table 1).

Nursery material. The main pathogenic fungi implicated in black root rot (*R. fragariae, R. solani* and *Pythium* spp.) were isolated from the nursery material (Table 2). Other fungi were also isolated from nursery material, including the important fruit rot pathogens *Botrytis cineria* Pers. and *Colletotrichum acutatum* J.H. Simmonds ex J.H. Simmonds (Table 2).

Rhizoctonia fragariae (AG-G) was only detected in material from Loewenstein nursery (Table 2). *R. solani* (AG 6) on the other hand, was present in material from Sutherland and Tandfontein in both 1999 and 2000, but was not found in material from Loewenstein.

Pythium irregulare was only sampled from Loewenstein and Sutherland and there was a much higher incidence in the Loewenstein material than the Sutherland material. Other well-known and important strawberry fungal pathogens that are not

part of the black root rot complex such as *B. cinerea*, *Colletotrichum acutatum* and *Coniella fragariae* (Oudem.) Sutton were also consistently isolated from nursery material (Table 2). The occurrence of *Pestalotiopsis* sp(p) was exceptionally high in plants from Sutherland and *Trichoderma* sp(p) were relatively frequent in plants from Ceres (Tandfontein).

Discussion

Fumigation with methyl bromide was very effective at eliminating pathogenic fungi associated with black root rot from soil, and provided an environment almost devoid of microbes in which to grow strawberries. However, the primary fungal pathogens involved in the black root rot disease complex, *Pythium irregulare* and *Rhizoctonia fragariae* (Maas 1984; Elmer and LaMondia 1999; Botha *et al.* 2001) and *R. solani*, were isolated from nursery material. Clearly in the absence of a balanced soil microbiota, with an abundance of host material and favourable conditions for disease development, great losses can be experienced due to black root rot infection. The wet conditions that prevail soon after planting as well as the proliferation of young roots present early in the season are very favourable for infections caused by *Pythium* spp. *Pythium* is renowned for attacking juvenile roots (Nemec 1970) and if wet weather prevails, considerable damage would be caused to the young plants marking the beginning of damage attributable to the black root rot complex.

The fact that the plants are already infected with the pathogen makes the prospect of disease control difficult (Razik *et al.* 1989). Starting off with pathogen-free propagation material is one of the first steps in implementing an integrated pest management strategy for strawberries. Although plants are derived from sterile tissue cultured plantlets in the present system, they end up spending at least two years in open rooted nursery beds. During this two-year period they become infected with fungal pathogens, and when transplanted into soil fumigated with methyl bromide, the pathogens proliferate extensively (Razik *et al.* 1989). It is no simple task to propagate pathogen-free production material. The use of pathogen-free plants obtained from hydroponic systems is uneconomical at this stage. It is thus desirable to create a balanced soil and rhizosphere environment where microbial complexity prevents dominance of pathogens. Research should explore cultural practices that would create

and enhance this type of environment and the possibility of using bio-additives to boost microbial diversity should also be included.

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Table 1. Occurrence of soilborne fungal pathogens associated with black root rot in pre- and post-fumigated soil

	Occurrence (CFU /g soil)			
	Pre-fumigated soils		Post-fumigated soils	
Species	1999	2000	1999	2000
Fusarium oxysporum	770	820	0	0
Pythium irregulare	241	106	0	0
Rhizoctonia fragariae AG-A.	50	0	0	0
Rhizoctonia fragariae AG-G	35	0	0	0
Rhizoctonia solani AG 6	15	0	0	0

Table 2. Occurrence of fungi from nursery material from different farms in the Western Cape Province in 1999 and 2000

	Occurrence (%)[a]				
	Loewenstein	Sutherland		Tandfontien	
Species	2000	1999	2000	1999	2000
Botrytis cinerea	0.0	0.0	0.0	24.6	12.5
Colletotrichum acutatum	1.1	0.0	1.2	4.6	2.6
Coniella fragariae	8.9	7.1	0.0	0.3	0.0
Fusarium oxysporum	0.0	7.1	6.7	0.6	3.4
Penicillium spp.	3.3	3.6	1.3	10.8	2.8
Pestalotiopsis spp.	5.6	76.8	50.2	6.2	2.6
Pythium irregulare	5.3	0.01	0.0	0.0	0.0
Rhizoctonia fragariae AG-G	6.7	0.0	0.0	0.0	0.0
Rhizoctonia solani AG 6	0.0	5.4	4.9	0.3	1.2
Trichoderma spp.	1.1	0.0	2.3	27.7	35.8
Other fungi[b]	68.0	0.0	31.1	24.9	39.1

[a] Percentages calculated out of the total number of isolates obtained at each sampling date.

[b] Other fungi comprise a mixture of fungi from genera other than those listed.

5: The effects of fungicides on survival and yield of strawberries

Abstract

This study investigated the effects of fungicides on suppression of specific elements of black root rot of strawberries and elucidated ecological relationships between the major pathogens. *In vitro* studies were carried out to determine the EC_{50} values of isolates of *Pythium irregulare*, *Rhizoctonia fragariae* AG-A, AG-G and AG-I and *R. solani* AG 6 for the fungicides benomyl, fludioxonil and tolclofos-methyl. Additionally, field trails were established on a commercial farm in the Paarl area of the Western Cape Province. Three strawberry cultivars *viz.* Chandler, Selekta and Tiobelle were treated with fungicide drenches every fortnight from June to November. The four fungicides tested singly and in combination over two seasons (1999 and 2000) were benomyl, fludioxonil, metalaxyl and tolclofos-methyl. Yield and survival of plants were recorded and fungal isolations were made from roots and crowns during winter (June), spring (September) and at the end of the season (November). In the *in vitro* trials no inhibition of *P. irregulare* occurred and benomyl had the highest EC_{50} values for the *Rhizoctonia* spp. For each fungicide there were no significant differences in EC_{50} values among the *Rhizoctonia* spp. and AG types. Results of the field trials differed significantly in the two seasons. Treatment with fungicides generally improved yield but did not affect the survival of strawberry plants. However, tolclofos-methyl appeared to be phytotoxic and reduced yield and survival of plants. Treatment with fludioxonil showed potential for short-term use. Data obtained from fungal isolations clearly showed that decreasing infection of one of the pathogens involved would not be sufficient to control black root rot. Applications of metalaxyl lowered the occurrence of *Pythium* spp., but this resulted in a simultaneous increase in *Rhizoctonia* counts. This pattern was also reflected in the seasonal fluctuation of the two pathogens where the occurrence of *Pythium* was relatively high early in the season but low later in the season. Further work needs to be carried out on the application of fungicides as a short-term control for black root rot.

Introduction

Black root rot retards the establishment and productivity of new strawberry plants, especially in fields that have been cultivated with strawberries for periods longer than five years (Heald 1920; Coons 1924; Strong and Strong 1931; Ellis 2000). The etiology and epidemiology of this disease remain partly unresolved. However, it is accepted that a complex of fungal pathogens, of which *Pythium* and *Rhizoctonia* spp. are primary causal agents, is the main incitant of disease (Maas 1998; Ellis 2000; Botha *et al.* 2001).

Due to uncertain etiology of black root rot, control of the disease has been dependent upon the use of broad-spectrum soil fumigants, particularly methyl bromide and in some cases, chloropicrin (Maas 1998). The former fumigant has been identified as a high-risk chemical that depletes atmospheric ozone (Ristaino and Thomas 1997). It is also harmful to beneficial soil biota, the environment and the user (Porter *et al.* 1999). The Montreal Protocol was initiated in 1992 as a united international effort to ban or reduce use of ozone depleting products. Signatories of the Montreal Protocol pledged to stop usage of this product by 2005 – 2015 (Ellis 2000). With the imminent phasing out of methyl bromide, the strawberry industry in South Africa has identified alternative disease management tools as a critical research area.

Currently a number of different approaches to solving this problem are being investigated in major strawberry producing countries (Denman and Botha 1999). One approach is to replace methyl bromide with different chemical fumigants (Yuen *et al.* 1991; Duniway 1998; Porter *et al.* 1999). The use of alternative chemical fumigants, and the combination of these fumigants with lower levels of methyl bromide, is considered a short-term solution to the problem (Porter *et al.* 1999). However, Porter *et al.* (1999) emphasised the possibility of including biofumigation in an integrated disease management strategy. Many researchers (Yuen *et al.* 1991; Wing *et al.* 1995) have also pointed out the need for resistant cultivars to control black root rot of strawberry.

Mazzola (1998) stated that the development of selective chemical, cultural or biological additives to control disease is dependent upon a more complete understanding of the biology and ecology of the disease complex. He used a range of

semi-selective biocides that suppressed certain microbial populations as a means to elucidate apple replant disease soil microbiota and their interactions. He also evaluated the effect of these biocides on specific pathogens (Mazzola 1998). In South Africa no research has been carried out on alternatives to methyl bromide in the strawberry industry. One or two appropriately timed soil drenches with fungicides could be used in the short-term to aid integrated disease management programmes until other alternatives become available. As a step towards elucidating ecological relationships between the major pathogens associated with black root rot of strawberries in South Africa, trials were established in which the effects of fungicides on suppression of specific elements of black root rot could be assessed.

Materials and Methods

***In vitro* testing.** The effects of three fungicides on mycelial growth were assessed *in vitro* on *Pythium irregulare* Buisman, *R. fragariae* S.S. Husain & W.E. McKeen (AG-A, AG-G and AG-I) and *R. solani* J.G. Kühn (AG 6). Three isolates of *P. irregulare* (STE-U 4606, 4607 and 4608), two isolates of *R. fragariae* AG-A (STE-U 4612 and 4619), AG-G (STE-U 4617 and AB 524.3), a single isolate of AG-I (STE-U 4618) and two isolates of *R. solani* (STE-U 4611 and AB 268.3) were used. Three fungicides *viz.* benomyl, fludioxonil and tolclofos-methyl were tested by adding a series of dilute concentrations to molten 1.2% potato dextrose agar (PDA) (Biolab, Midrand South Africa) so that final concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 50 µg a.i./mL were obtained. For the controls, plates containing PDA without the addition of fungicides were used. Isolates were cultured on PDA under cool white light at 22°C for three days prior to inoculating the fungicide-amended medium. Plates containing approximately 20 mL of fungicide amended medium were seeded with 5 mm plugs by placing the plug in the middle of the Petri dish. Cultures were incubated under cool white light (12 h) at 22°C. Three replicate plates per isolate-fungicide-concentration combination were tested and the entire experiment was repeated once. After four days the mycelial growth was recorded by measuring the perpendicular diameters of the colonies. The mean colony diameters per fungicide-concentration were calculated for each isolate. Percentage inhibition was

then calculated by subtracting mean colony diameters from those of the controls. Percentage inhibition data were plotted against the fungicide concentrations and the most appropriate regression equation was fitted. The EC_{50} values (concentration at which 50% inhibition of mycelial growth was obtained) were calculated.

Field trails. Field trails were laid out on the farm Loewenstein in the Paarl district of the Western Cape Province. In 1999 five blocks (two each of cvs Tiobelle and Selekta and one of cv Chandler) were demarcated in commercially producing strawberry fields. In 2000 there were six blocks (two of each strawberry cultivar). Each block was composed of 17 rows and each row contained 35 plants. Plants in every alternate row were treated, the non-treated rows served to prevent possible cross-contamination of the treatments. Fungicides were applied singly or in combinations at fortnightly intervals a month after planting (June). Ten litres of fungicide were applied to each row as a soil drench, using a hand-held watering can. Seven fungicide treatments were applied per season at the manufacturers recommended dose (Nel *et al.* 1999). The control plants were drenched with water only. All plants were subjected to the watering, fertilisation and insecticide control measures used on the farm.

The following fungicides were used in the trial: metalaxyl (Ridomil, Novartis South Africa (Pty) Ltd, Isando RSA, 50 g a.i. /kg wettable powder) was used for its systemic action against the Oomycetes, which includes *Pythium,* tolclofos-methyl (Rizolex, Novartis South Africa (Pty) Ltd, Isando RSA, 500 g a.i./kg wettable powder) was used to target *Rhizoctonia* specifically, and a broad-spectrum systemic fungicide, benomyl (Benlate, E. I. du Pont de Nemours and Co. Wilmington Delaware USA, 500 g a.i./kg wettable powder) was also included in the trial to control the other pathogens implicated in this complex. The fungicide fludioxonil (Celesté, Novartis South Africa (Pty) Ltd, Isando RSA, 20 g a.i./L. flowable concentrate) was added in the 2000 experiment. The former three fungicides were used singly and in all possible combinations at the same ratios. Fludioxonil was not included in the combinations.

Yield was recorded at every picking from September to November in both production seasons (1999 and 2000). Throughout this period the strawberries were picked on a daily basis. The yield of each treated row was weighed and the mass recorded. The number of plants that survived in each treatment was counted at the

end of each experiment (November 1999 and 2000 respectively). Survival for each season was expressed as a percentage out of twenty plants.

Isolations were made from five plants selected at random per treatment, in winter (July), spring (September) and summer (November) of each year that the experiment was conducted (1999 and 2000). The plants were transported to the laboratory in a cooler box and prepared immediately for isolation. The leaves were removed and the remainder of the material washed to remove soil particles. Roots and crowns were surface disinfested for 1 min in a 1% NaOCl solution prepared from commercial bleach, followed by 30 sec rinse in 50% ethanol and then two rinses of 1 min each in sterile water. After surface disfestation the plant material was left in the laminar flow cabinet to air dry. Isolations were made by dissecting pieces approximately 5mm long from the margin between healthy and diseased tissues of the roots and placing them onto fresh homemade PDA (200 g potatoes, 20 g dextrose, 12 g bacteriological agar in 1 L water). The crowns were split open and tissue was excised from their centres and plated on the culture media. As soon as fungi started growing from of the plant material hyphal tips were sub-cultured by transferring them to divided plates containing carnation leaf agar (CLA) (Fisher *et al.* 1982) in one half of the dish and PDA in the other half. Plates were incubated under near-ultraviolet and cool white light with a 12 h photoperiod at 25°C for three weeks. Microscopic examination of cultures was carried out and isolates identified to generic level. Occurrence of fungi was recorded as the number of isolates obtained on each sampling date.

Statistical treatment of data. The EC_{50} values for the different species and AG types were compared for each fungicide using an analysis of variance (ANOVA). Where significant differences were found ($P < 0.05$) Fisher's LSD tests were applied to compare means at the 5% significance level. All the statistical analyses were carried out using SAS version 8.2 (1999).

Yield and survival data were also used in an ANOVA where the years, cultivars and fungicide treatments were compared. The Fisher's LSD tests were applied to compare means at the 5 and 10% significance levels.

Isolation data were subjected to a Chi-square (χ^2) test of independence to elucidate significant associations between species and time of sampling over the two

year test period. Where there were significant associations ($P < 0.05$) the frequencies within the tables were investigated. In the absence of any significant associations, χ^2 tests for uniform distribution were performed on the species and sampling times.

Results

***In vitro* testing.** The EC_{50} values for *Pythium irregulare* could not be calculated due to the fact that 50% inhibition was not achieved by any of the fungicides tested. All the isolates of the different *Rhizoctonia* species and AG types tested had significantly smaller EC_{50} values for fludioxonil and tolclofos-methyl than for benomyl (Table 1). However, the EC_{50} values of the different *Rhizoctonia* spp. and AG types were not significantly different for any given fungicide treatment ($P = 0.05$).

Field trials. When considering the yield data there were no significant ($P > 0.05$) year x cultivar x fungicide interactions (Table 2), and the only significant ($P = 0.0003$) main effect was the difference in yield obtained in the two years that the experiment was conducted (Table 2). In 1999 the mean yield (4093 g) was only 63% of that recorded in 2000 (6481 g) (Table 3).

In view of the significant year effect yield data for each year were also analysed separately. At the 95% confidence level ($P = 0.05$) there were no significant interactions or main effects in either year (1999 or 2000). However, at the 90% confidence level ($P = 0.1$) in 1999 the yield from cv Tiobelle was significantly ($P = 0.1$) less than that from cv. Selekta (Table 4). Furthermore, the yield from plants treated with benomyl plus metalaxyl was significantly greater than that of plants treated with tolclofos-methyl (Table 5). In 2000 at the 90% confidence level the yield from cv. Chandler was significantly less than that from Tiobelle or Selekta (Table 4). There were however, no statistically significant differences ($P = 0.1$) in yields amongst plants treated with the different fungicide drenches in 2000 (Table 5). In both years (1999 and 2000) low yields were obtained from plants treated with the combination of three fungicides.

With regard to plant survival, significant ($P = 0.0268$) interaction was found between the treatments and the years in which the experiments were conducted (Table

6). In the tolclofos-methyl treated plants survival was very low in 1999 (15%) but was much higher in 2000 (67%) (Table 7). Similarly, low survival was recorded in plants treated with metalaxyl plus tolclofos-methyl or with the combination of all three fungicides in 1999, but in 2000 the survival rate was much higher in these treatments (Table 7). Apart from these three treatments survival levels were similar in 1999 and 2000. Plants that received the combination of all three fungicides had a very low survival rate in both years (Table 7). The highest percentages of survival (about 70%) were recorded for control plants or those treated with benomyl, fludioxonil or a combination of benomyl plus metalaxyl. In general plants treated with tolclofos-methyl (singly or in combination with other fungicides) had a lower percentage survival than the controls. Strawberry plants receiving metalaxyl also had a slightly lower survival than the controls (Table 7).

Many different fungi and some bacteria were isolated from the strawberry plants receiving different fungicide treatments and sampled three times in each growing season. Since *Pythium* and *Rhizoctonia* spp. are considered the major causal agents of the black root rot complex (Maas 1998; Ellis 2000; Botha *et al.* 2001) only data pertaining to these fungi are presented here. In both years (1999 and 2000) the occurrence of *Pythium* spp. decreased from the first to the last sampling date (June through November). Conversely the occurrence of *Rhizoctonia* spp. increased as the season progressed (Table 8).

The Chi square test revealed that for the *Pythium* isolates there was not a significant year by treatment association. In general high counts of *Pythium* were recorded in the controls, benomyl, benomyl plus tolclofos-methyl and combination of the three fungicides (Table 9). Low counts were obtained in the metalaxyl, tolclofos-methyl and benomyl plus metalaxyl treatments. The data for the metalaxyl plus tolclofos-methyl treated plants were contradictory over the two years. In 1999 no *Pythium* was isolated from the latter treated plants but in 2000 a relatively large number of isolates was obtained. A similar pattern was recorded for plants treated with benomyl plus tolclofos-methyl.

By comparison the occurrence of *Rhizoctonia* spp. in the treated plants was variable. In general however, high numbers of isolates were consistently associated with the control or metalaxyl-treated plants (Table 9). The occurrence of *Rhizoctonia* isolates was also relatively high in plants treated with metalaxyl plus tolclofos-methyl.

Discussion

Results of this study have contributed to elucidating ecological relationships between the major pathogens associated with black root rot of strawberries in South Africa. This information can now be incorporated into an integrated disease management programme. Applications of metalaxyl lowered the occurrence of *Pythium* spp. as was expected since this fungicide specifically targets the Oomycota (Schwinn and Staub 1995). However, with lower *Pythium* counts there was an increase in *Rhizoctonia* counts. This pattern was also reflected in the seasonal fluctuation of the two pathogens where the occurrence of *Pythium* was relatively high early in the season but low later in the season. When *Pythium* counts were high (June), *Rhizoctonia* counts were relatively low and *vice versa*. Thus it can be concluded that by suppressing one of the major components of the black root rot complex for example *Pythium* spp., the niche that they occupied becomes vacant and another main role player (in this case *Rhizoctonia* spp.) colonises that open niche. This clearly shows that decreasing infection of one of the pathogens involved in the disease complex cannot control black root rot. However, based on the above information management practices such as avoiding excessive moisture in winter when the occurrence of *Pythium* is high can be implemented.

In the work presented here the use of fungicides in disease management has not been unequivocally demonstrated, and further studies are necessary before recommendations can be made. The differences in results from the field trials over the two seasons made interpretation of the effects of the fungicide treatments difficult. The lack of statistically significant differences in some of the parameters measured further complicated the interpretation of the results. However, it is important to recognise that although results were not always statistically significant the effects of the treatments could well make an economic difference to farmers. For example, in 1999 only plants treated with tolclofos-methyl yielded less than the controls but all the other plants yielded between 10–35% more fruit than the controls. Similarly in 2000 18% more yield was obtained relative to the control in the fludioxonil treated plants. These increases in yield translate into significant gains for farmers.

Survival of plants was similar in most of the treatments for both years of the trial. Most of the fungicides tested did not affect survival of the plants negatively

except for tolclofos-methyl, which seemed to have a phytotoxic effect on the plants (particularly in 1999) and many died before the fruiting season started. Lower yields were also obtained from plants treated with tolclofos-methyl, which is probably because a lot of plants died before fruit was harvested from them. The negative effect of tolclofos-methyl on plant survival has also been observed in the USA on apple seedlings (M. Mazzola, USDA-ARS, Fruit Tree Research Laboratory, 1104 N Western Ave, Wenatchee, WA 98801, USA, pers. comm.). Although there appears to have been negative effects of tolclofos-methyl on plant survival this fungicide was effective in reducing the number of *Pythium* and *Rhizoctonia* isolates. Consequently the method of application tested in this work does not appear suitable for strawberries but a lower concentration and different method of application may prove more effective for the strawberry industry.

Metalaxyl did not have any phytotoxic effects on the strawberry plants and the yield and survival of plants receiving this treatment were relatively high. Similarly yields and survival with benomyl treated plants were also high. The former fungicide reduced the *Pythium* count while the latter reduced the *Rhizoctonia* counts. A combination of the two fungicides reduced the counts of both pathogens, produced the highest yield in 1999 and did not affect survival. This treatment thus also appears promising, but both fungicides have a high resistance risk associated with them (Delp 1995; Schwinn and Staub 1995) and further investigations on only a few timely applications need to be carried out. Although fludioxonil was only applied in 2000, excellent results were obtained, and there is obvious potential for this fungicide as a part of an integrated disease management programme. The application of three fungicides at the same time had an adverse effect on the plants probably because the concentration of chemicals was too high or the combination of the chemicals had synergistic effects that negatively affected the plants. Thus, in the short-term there is potential for fungicide applications as part of an integrated disease management programme. However, further work needs to be carried out and the economic feasibility assessed before recommendations for reducing black root rot in the field can be made.

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Table 1. The *in vitro* EC_{50} values of various AG types of *Rhizoctonia fragariae* and *R. solani* for different fungicides

	EC_{50} values (µg a.i./mL)		
Pathogen	benomyl	fludioxonil	tolclofos-methyl
R. solani AG 6	0.40 **a**	0.01 **b**	0.04 **b**
R. fragariae AG-A	0.41 **a**	0.08 **b**	0.14 **b**
R. fragariae AG-G	0.48 **a**	0.10 **b**	0.14 **b**
R. fragariae AG-I	0.12 **a**	0.02 **b**	0.15 **b**

[a] Treatments followed by the same letter within rows do not differ significantly ($P = 0.05$).

Table 2. ANOVA of data on yields from strawberry plants drenched with fungicides

Source	DF	F Value	Pr > F
Year	1	15.61	0.0003
Cultivar	2	2.52	0.0921
Year*cultivar	2	1.98	0.1501
Fungicides	8	0.32	0.9531
Year*fungicide	7	0.17	0.9899
Cultivar*fungicide	16	0.12	1.0000
Year*cultivar*fungicide	14	0.18	0.9995

Table 3. Total yield of strawberry plants from the fungicide trial on Loevenstein Farm, Paarl in 1999 and 2000

Year	Mean yield[a] (g)
1999	4092.6 **a**[b]
2000	6481.1 **b**

[a] Total yields were comprised of strawberry fruit of cultivars Selekta, Tiobelle and Chandler harvested weekly between September and November, in the fungicide trials at Lovenstein Farm

[b] Means followed by the same letter do not differ significantly ($P = 0.05$).

Table 4. Mean yield from different cultivars of strawberries in 1999 and 2000

	Mean yield (g)	
Cultivar	1999	2000
Chandler	4169 **ab**[a]	4924 **b**
Selekta	4600 **a**	7072 **a**
Tiobelle	3548 **b**	7447 **a**

[a] Means followed by the same within columns letter do not differ significantly ($P = 0.1$).

Table 5. Mean yield of strawberry plants treated with various fungicides in 1999 and 2000

	Mean yield (g)[a]	
Treatment	1999	2000
Control	3565 **ab**[b]	6658 **a**
benomyl	4565 **ab**	6033 **a**
fluidioxonil	-	7854 **a**
metalaxyl	4745 **ab**	6625 **a**
tolclofos-methyl	3227 **b**	5988 **a**
benomyl + metalaxyl	4800 **a**	5959 **a**
benomyl + tolclofos-methyl	3998 **ab**	6767 **a**
metalaxyl + tolclofos-methyl	3940 **ab**	6742 **a**
benomyl + metalaxyl + tolclofos-methyl	3901 **ab**	5704 **a**

[a] Means were calculated by dividing the total yield of all replicates by the amount of replicates.

[b] Means followed by the same letter within columns do not differ significantly ($P = 0.1$).

Table 6. ANOVA on survival of strawberry cultivars drenched with fungicides in 1999 and 2000

Source	DF	F Value	Pr > F
Year	1	8.02	0.0070
Cultivar	2	2.63	0.0834
Year*cultivar	2	0.51	0.6026
Fungicides	8	2.90	0.0112
Year*fungicide	7	2.56	0.0268
Cultivar*fungicide	16	0.43	0.9650
Year*cultivar*fungicide	14	0.29	0.9930

Table 7. Mean survival of strawberry plants treated with various fungicides in 1999 and 2000

	Mean survival (%)[a]	
Treatment	1999	2000
Control	70 **a**[b]	71 **a**
benomyl	70 **a**	73 **a**
fludioxonil	-	71 **a**
metalaxyl	68 **a**	61 **a**
tolclofos-methyl	15 **c**	67 **a**
benomyl + metalaxyl	71 **a**	70 **a**
benomyl + tolclofos-methyl	62 **ab**	63 **a**
metalaxyl + tolclofos-methyl	50 **b**	63 **a**
benomyl + metalaxyl + tolclofos-methyl	25 **c**	59 **a**

[a] Percentage survival calculated out of 40 plants per treatment per year.

[b] Means followed by the same letter within columns do not differ significantly ($P = 0.05$).

Table 8. Occurrence of *Pythium* spp. and *Rhizoctonia* spp. in strawberry plants in 1999 and 2000

	Occurrence[a]			
	Pythium		*Rhizoctonia*	
Sampling date	1999	2000	1999	2000
June	14	36	14	28
September	5	10	17	27
November	7	0	30	72

[a] Occurrence is expressed as the number of isolates.

Table 9. Occurrence of *Pythium* and *Rhizoctonia* isolates at different times of the year from plants treated with various fungicides

	Occurrence[a] of *Pythium* spp.						Occurrence of *Rhizoctonia* spp.					
	1999			2000			1999			2000		
Treatment	1[b]	2	3	1	2	3	1	2	3	1	2	3
Control	5	1	0	3	1	0	2	3	6	3	2	17
benomyl	3	3	1	4	0	0	1	2	7	0	4	7
fludioxonil	-	-	-	6	0	0	-	-	-	3	7	8
metalaxyl	1	0	2	2	0	0	5	6	6	6	5	6
tolclofos-methyl	1	0	1	0	0	0	1	0	1	5	2	4
benomyl + metalaxyl	0	0	2	0	1	0	0	1	2	5	3	5
benomyl + tolclofos-methyl	1	0	0	6	3	0	0	1	4	4	2	10
metalaxyl + tolclofos-methyl	0	0	0	13	4	0	4	3	1	2	0	13
ben[c] + metalax + tcf-meth	3	1	1	2	1	0	1	1	3	0	2	2

[a] Occurrence is expressed as the number of isolates.

[b] 1 = June sampling date, 2 = September sampling date, 3 = November sampling date.

[c] ben = benomyl, metalax = metalaxyl, tcf-meth = tolclofos-methyl.