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Studies of the life cycle of *Diplocarpon rosae* Wolf on roses and the effectiveness of fungicides on pathogenesis

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Dedicated to

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Abstract

The blackspot disease of roses caused by *Diplocarpon rosae* Wolf teleomorph (anamorph *Marssonina rosae*) is a widespread and important disease on outdoor grown roses. The development of *D. rosae* in rose leaves is not clearly understood and no detailed well-documented photographs of the fungus development in the host are available. The control of this pathogen heavily relies on fungicides. The objective of this study was to provide a detailed growth pattern the *D. rosae* inside the rose leaves and to test the effectiveness of fungicides, the strobilurins and azoles, on the various fungal structures formed by this fungus.

The aggressiveness of various Kenyan and Germany isolates was investigated. A German isolate was used to study the life cycle of *D. rosae*. The life cycle of the fungus was studied using different staining techniques for the light microscopy and the conventional preparation for electron microscopy: A conidium germinated to form a germ tube, from which an appressorium was sometimes formed. A brown ring that was presumably melanized was formed at the point of penetration of the host cuticle. Upon penetration an infection vesicle was formed in the subcuticular region. Primary hyphae spread from the infection vesicle into the subcuticular and intercellular regions in the host. The subcuticular, intercellular and intramural hyphae formed haustoria of varying shapes in the epidermal cells. The intercellular hyphae also formed haustoria in palisade mesophyll cells. Intracellular hyphae were formed just before the formation of the reproduction structures. Brown heavily ornamented structures formed in the overwintering leaves, opened in spring of the following year to release small thin-walled structures. No ascospores were formed in the subepidermal apothecia-like structures. The strobilurins completely inhibited germination of the conidia of *D. rosae* but the azoles did not. The azoles were able to destroy the fungal structures in the host even when they were applied after the fungus was fully established in the host but the effectiveness of the strobilurins was limited. Protective and curative tests of the effectiveness of the fungicides were carried out with 100 ppm of the active ingredients.

The study showed that *D. rosae* is a hemibiotroph: The biotrophic phase is marked by the formation haustoria and the necrotrophic phase by the formation of the intracellular hyphae. The weather conditions in Germany may not be suitable for the development of the ascospores in the subepidermal apothecia. The strobilurins were only effective when applied protectively. The azoles were effective when applied pre and post inoculation.

Zusammenfassung

Der Sternrußtau (Schwarzfleckigkeit), Hauptfruchtform *Diplocarpon rosae* Wolf, (Nebenfruchtform *Marssonina rosae*), ist eine häufige und weit verbreitete Krankheit an Freilandrosen. Bisher war weder die Entwicklung des Pilzes im Blattgewebe geklärt, noch waren in der Literatur fotografische Aufnahmen zur endophytischen Pilzentwicklung zu finden. Die Bekämpfung des Pilzes wird überwiegend mit Fungiziden durchgeführt. Die Untersuchungen vorliegender Arbeit umfassen sowohl mikroskopische Studien zur exakten Ausbreitung des Erregers im Rosenblatt als auch die Wirkung unterschiedlicher Fungizide, insbesondere Strobilurine und Azole, auf pilzliche Strukturen.

Zunächst wurde die Aggressivität verschiedener Isolate aus Kenia und Deutschland getestet. An einem deutschen Isolat konnte der Entwicklungszyklus von *D. rosae* mit verschiedenen Färbetechniken lichtmikroskopisch sowie nach konventioneller Präparation elektronenmikroskopisch studiert werden: Nach der Konidienkeimung entsteht eine Keimhyphe, von der aus meist ein Appressorium gebildet wird. Auf der Wirtskutikula zeigt sich dann an der Stelle der späteren Penetration ein brauner Ring, der vermutlich melanisiert ist. Nach der Penetration bildet sich in der subkutikulären Region ein Infektionsvesikel. Primäre Hyphen durchdringen von diesem Infektionsvesikel ausgehend subkutikuläre und interzelluläre Wirtsgewebezonen. Subkutikuläre, interzelluläre und intramurale Hyphen bilden in den epidermalen Zellen Haustorien unterschiedlicher Formen. Interzelluläre Hyphen produzieren ebenfalls Haustorien im Palisadenparenchym. Intrazelluläre Hyphen entstehen erst kurz vor der Bildung der Reproduktionsorgane. Braune, stark ornamentierte Strukturen werden vom Pilz auf den überwinternden Blättern gebildet. Diese öffnen sich im Frühjahr des Folgejahres und entlassen dünnwandige Organe. In diesen subepidermalen Apothezien-ähnlichen Strukturen entstehen aber keine Ascosporen. Strobilurine hemmten die Konidienkeimung vollständig, während Azole einen weniger starken Einfluss auf die Keimung hatten. Dagegen zerstörten die Azole alle Pilzstrukturen im Inneren des Gewebes, auch wenn sie sich bereits gut etabliert hatten. Diese Reaktion konnte bei Einsatz der Strobilurine nicht so deutlich beobachtet werden. Alle Fungizidtests zur protektiven und kurativen Wirkung wurden mit jeweils 100 ppm reinem Wirkstoff durchgeführt.

Die Untersuchungen zeigten, dass *D. rosae* eine hemibiotrophe Lebensweise besitzt: die biotrophe Phase besteht aus der Haustorienbildung und die nekrotrophe Phase aus der Bildung interzellulärer Hyphen. Die deutschen Witterungsbedingungen eignen sich offenbar nicht für eine subepidermale Apothezienbildung. Strobilurine zeigten nur nach protektiver Applikation eine Wirkung. Azole wirkten sowohl bei prä- als auch bei post-inokulativer Behandlung.

Table of contents…….....…….Page

Abbreviations

dpi	days post inoculation
PDA	potato dextrose agar
MEA	malt extract agar
BMA	biomalt agar
WA	water agar
NBT	nitroblue tetrazolium
ERL	Epoxy resin low (Spurr's resin)
a.i.	active ingredient
ppm	parts per million
G1	Germany isolate 1
K1	Kenya isolate 1
TEM	transmission electron microscope
EHM	extrahaustorial membrane
EHX	extrahaustorial matrix
SD	standard deviation

1 Introduction

The rose is a widely studied plant and much has been written about it. Without delving much in to the history suffice it to say that the wild roses are believed to have their origins in Europe, America, the orient and China, and in the Middle East (Beales 1997, Joyaux 2003). Rose cultivation dates way back to around 3000 B.C. in the ancient Chinese gardens (Beales 1997). Three important organizations are concerned with the formulation of classes and the nomenclature of roses: The American Rose society through their publications "Modern Roses", The World Federation of Roses Societies, and the British Association Representing Breeders (Baeles 1997). Beales (1997) has also given what he calls a simplified classification of roses based on their flower characteristics. The details of rose classification and the associated controversies are not the subject here, but it is important to mention that roses belong to the family Rosaceae, and Genus *Rosa*.

The rose has established itself as the most popular garden plant in the world as well as the most important commercial cut flower grown under glass (Horst 1983). Cut flowers contributed to 2,787 million of trade turnover in Europe in the year 2000 (Eurostat 2000). A fifth of the cut flowers were from none European countries and roses made 47 % of the imports from none European countries, which was worth 282 million Euros in the year 2000 (Eurostat 2000). According to the year 2000 statistical analysis the main destination for all cut flower imports into Europe was Netherlands but the highest consumer of cut flowers in Europe was Germany. The highest ranked exporters of cut flowers into the Europen Union (EU) were Kenya (25 %), Colombia (17 %) and Israel (16 %) to mention but a few (Eurostat 2000).

Despite the popularity of the rose, its cultivation is associated with various diseases. The most important rose diseases caused by fungal pathogens are: powdery mildew caused by *Sphaerotheca pannosa*, the blackspot disease by *Diplocarpon rosae*, and rust by *Phragmidium* species. The most important bacterial disease is the bacterial wilt caused by *Agrobacterium tumefaciens*.

The blackspot of roses is a foliar disease recognized by black spots on the upper side of the leaf. The symptoms are brown to black circular spots with an irregular margin on the upper surface of the leaf, which are followed by yellowing and premature defoliation. The disease is caused by a fungal pathogen with the perfect stage *Diplocarpon rosae* wolf (Wolf 1912) and the imperfect stage that is called *Marssonina rosae* (Lib.) Lind (Baker 1948). Other synonyms to the imperfect stage are *Asteroma rosae*, *Actinonema rosae*, and *Marsonina rosae* (Baker 1948, Horst 1983) among many others.

In temperate regions the first infections in spring are caused by the conidia or ascospores found in the overwintered plant material. The fungus overwinters on dormant stems, thorns, fallen leaves, and buds (Lyle 1943, Cook 1981). The conidia are spread by wind, rain splash and probably by animal vectors including insects and arachnids (Palmer et al 1978). Records of the apothecia containing eight ascospores are rare and the importance of the genetic variation generated by sexual recombination is not clear (Walker 1995). The formation of apothecia has been reported in North America (Wolf 1912, Aronescu 1934), Great Britain (Knight and Wheeler 1977, Cook 1981) and in Russia (Dudin 1972).

The disease was first described in Europe in Sweden in 1815, then in Belgium in 1827 and later in other European countries (Baker 1948). The first report in North American were in 1831, South America in 1880, Australia 1892, Africa in 1920, and in China in 1910 as summarized by Baker (1948). Today the disease has a worldwide distribution, even occurring on oceanic islands like the Philippines and Hawaii (Horst 1983, Drewes-Alvarez 2003).

Blackspot of roses is an important, devastating and widespread disease. In out door growing of roses black spot disease is generally present, often as a major problem, and frequently in epidemic proportions (Horst 1983). It is a minor problem in greenhouse roses because humidity is regulated very carefully but is the most important disease of out door roses (Horst 1995). The rose plant is used in gardens and landscaping for its aesthetic value, but the blackspot infections make the roses unsightly due to the black spots on the leaves, yellowing and premature defoliation. The pathogen causes defoliation and weakening of the plants (Drewes-Alvarez 2003). The premature defoliation leads to reduced vigour (Smith et al 1988) and even death in very susceptible varieties (Black et al 1994). The damage caused by the *D. rosae* is greater than the leaf spots because of the added effect of the premature defoliation. Disease damage cannot be assessed only in terms of size of lesion but always includes the defoliation aspect.

Symptom expression in the blackspot disease has been reported to differ with pathogenic races, varieties, and environmental conditions. Rose cultivars differ in their susceptibility to *D. rosae* and pathogenic races of this pathogen have been reported by several authors (Debener et al 1998, Malek et al 1996, Bolton and Svejda 1979, Jenkins 1955, Knight

and Wheeler 1978b). In a study using a set of differentials of rose cultivars, Malek et al (1996) identified at least five physiological races of *D. rosae*.

The widely cultivated tea hybrids and floribunda roses are susceptible to the blackspot pathogen (Reddy et al 1992, Walker et al 1995). Some of the wild species of roses have resistance but they lack the desired aesthetic traits (Reddy et al 1992). High resistance to blackspot has been found in genotypes of *R. banksiae* Ait, *R. carolina* L., *R. laevigata* Michx., *R. multiflora* Thunb. Ex Murray, *R. rugosa* Thunb., *R. roxburghii* Trait., *R. virginiana* Herrm. and *R. wichurana* Crèp (Drewes-Alvarez 2003). The resistance mechanism in roses is thought to operate through mechanical resistance of the cuticle (Castledine et al 1981), diffusible substances (Walker et al 1995, Knight and Wheeler 1978) or to be a post penetration defence response (Wiggers et al 1997). Debener (2003) reported that in a recent screening of tetraploids and diploids carrying resistance from *R. multiflora* with single conidial isolates the presence of two dominant genes Rdr1 and Rdr2 was revealed, but this does not in any way rule out horizontal resistance.

The resistance found in the wild roses needs to be transferred into the modern cultivars. Attempts to transfer resistance from the wild species to the modern roses by introgressive breeding led to a resistant F1 hybrids but the resistant was lost in the first backcross maybe due to lack of pairing partner at meiosis (Walker et al 1995). The integration of resistance genes by crosses with wild species is complicated due to varying ploidy levels in rose species and possible linkage of resistance genes with undesirable traits (Dohm et al 1996). In addition to that, breeding of rose cultivars that are resistant to blackspot is very time consuming because of the generative reproduction and complex genetic

constitution of roses (Malek et al 1996). Research for the development of resistance against the disease are still under way (Debener et al 1996, Debener 1996), but the control of black spot still requires intensive use of systemic fungicides (Walker et al 1995, Reddy et al 1992).

Once established on plants, blackspot is difficult to control despite a combination of practices that include sanitation measures and fungicide applications (Behe et al 1993). Good cultural practices include: Removing disease leaves from the ground and pruning canes with infected leaves to reduce the overwintering potential of the pathogen; avoiding dense planting to allow good air circulation through the leaf canopy (Horst 1983); avoiding overhead irrigation since it favours infection; avoiding excessive watering during dark and humid weather; avoiding keeping the rose leaves wet for long hours, as this provides the water needed for the conidia to germinate.

In general, a good understanding of a pathogen's life cycle is a prerequisite to its effective control. Despite several research contributions to the understanding of the development of *D. rosae* in roses, there are still discrepancies in the observations made on its life cycle. The existing histological studies of *D. rosae* (Aronescu 1934, Frick 1944 and Palmer et al 1978) were limited by the scope of the then available techniques and equipment. Aronescu (1934) and Frick (1944) made drawings of their microscopic observations, while Palmer et al (1978) commented on the inability to make better photographs due to poor sectioning apparatus. There are no detailed ultra structure studies of the infection process of *D. rosae* on roses. Therefore, there is need to have a detailed and more accurate documentation of the infection process of *D. rosae* using

modern technology and there is need to shed more light into the discrepancies in the life cycle of this pathogen.

Given that the management of *D. rosae* heavily relies on chemical control, there is need to keep on testing effectiveness of new fungicides active ingredients in controlling this pathogen. Due to the increase in the general environmental protection awareness world wide, the need to replace the fungicides in the market with more environmentally friendly ones has never been more timely. This can be achieved through testing of new more environmental friendly fungicidal active ingredients with the hope of offering better disease management.

The conidia of the blackspot pathogen germinate to form germ tubes on the host surface but further development of the fungus takes place below the host cuticle and even within the host cells. The control of this type of pathogen requires the use of contact as well as systemic fungicides. Couch (1995) distinguishes fungicides by their topical or physical mode of action: contact fungicides stay outside of plants, and penetrants penetrate the plant in some manner. Localized penetrants diffuse into leaf surfaces. Acropetal penetrants are transported by xylem towards the leaf tips after they penetrate (upward movement) but do not move in the phloem towards the root tips. Systemic penetrants are fungicides that are transported by both xylem and phloem. Penetrant fungicides in general offer the possibility of prolonging disease control, as the fungicide protects plant surfaces, but they also may inhibit pathogens in the early stages of infection inside the plant.

One group of fungicides that have been widely used for the control of many leaf spot pathogens including *D. rosae* are the demethylation inhibitors or DMI fungicides. This is a homogenous group of fungicides showing a common mode of action within the fungal sterol biosynthesis pathway i.e. the inhibition of demethylation at position 14 of lanosterol or 24-methylene dihydrolanosterol, which are precursors of sterols in fungi (Kuck et al 1996). Azoles such as imidazole and triazoles derivatives are the most important DMI fungicides. DMIs have a broad spectrum of fungicidal activity with most fungi from the Ascomycetes, Basidiomycetes and Fungi Imperfecti. As reported by Kuck et al (1996), a multitude of leaf spot pathogens are effectively controlled by several DMIs. According to the report of Newmann and Jacob (1996), all DMIs are able to penetrate the plant cuticle and /or seed coat to some extent and they are then translocated in either the apoplast (transport in coherent network of free space, cell walls and non-living cells) or symplast (transport in the coherent network of protoplasts connected by plasmodesmata). DMIs have minimal symplastic/basipetal movement i.e. movement in the living part of the plant which is the phloem and cytoplasm, but their translocation is predominantly apoplastic and protection is confined to such cases where sufficient quantities of active ingredients have been applied to the basal parts of the leaf or shoot (Kuck et al 1996). The DMIs of interest in this study are two triazoles, namely myclobutanil and tebuconazole.

A new group of fungicides with much promise in the control of a wide range of fungal pathogens are the strobilurins. Strobilurin fungicides were developed following the discovery of naturally occurring β-methoxyacrylates, the simplest being strobilurin A and oudemansin A produced naturally in certain species of mushroom fungi, including

Strobilurus tenacellus and *Oudesmansiella mucida* (Ypema and Gold 1999). These fungicides are now more properly referred to as Q_oI. Industrial chemists improved these natural fungicides by making chemical modifications that resulted in compounds that were less subject to break down on the leaf surface by sunlight. More strobilurins continue to be released in the market. Some of the fungicides active ingredients found in this group include trifloxystrobin, pyraclostrobin, kresoxim-methyl and azoxystrobin (Bartlett et al 2001).

As reported by Sauter et al (1995), all Q_oI fungicides share a common biochemical mode of action: they all interfere with energy production in the fungal cell. They block the electron transfer at the site of quinol oxidation (the Qo site) in the cytochrome bc_1 complex, thus preventing ATP formation. They are site-specific fungicides.

Ebeling (2003) reporting on one of these active ingredients, namely trifloxystrobin, said that it has a very favourable profile concerning toxicology, residue behaviour, environmental fate and ecotoxicology. It has low acute toxicity, no likelihood of causing acute hazards, no genotoxic or carcinogenic potential and also no signs of neurotoxicity. The active ingredient rapidly degrades in the environment and no risk to the consumer is expected by the way of residue intake through food.

With important exceptions, the Q_oI control an unusually wide array of fungal diseases, including diseases caused by water molds, downy mildews, powdery mildews, leaf spotting and blight fungi, fruit rotters, and rusts. They are used on a wide variety of crops, including cereals, field crops, fruits, tree nuts, vegetables, turf grasses and ornamentals (Stark-Urnau et al 1997, Gold and Leinhos 1995). However, the effectiveness of these

new fungicide active ingredients on the fungal structures formed by *D. rosae* has not been studied in detail.

This study was undertaken with the main aim of carrying out a detailed study of the life cycle of *Diplocarpon rosae* in roses leaves in order to clarify the discrepancies found in literature. The objectives also included an investigation into the state and the plant parts in which the fungus overwinters in the weather conditions in Germany. The study targeted at assessing the aggressiveness of *D. rosae* isolates from Kenya and Germany by visual observation of the isolates that led to the earlier development of disease symptoms, and through biochemical assays of relative accumulation of reactive oxygen species (ROS) in leaves of plants inoculated with different isolates. The objectives encompassed a disease control aspect in which the effectiveness of the novel fungicide active ingredients, the strobilurins, on the fungal structures within the host tissues at different developmental stages was compared with the effectiveness of the fungicide active ingredients already in the market.

2 Materials and methods

2.1 Research organisms

2.1.1 Pathogen

The pathogen used was *Diplocarpon rosae* Wolf, which is the teleomorph state and the anamorph is called *Marssonina rosae* (Lib.) Lind. Two isolates from Germany collected in 2001 and 2002 from Duesseldorf and Bonn respectively, and 20 isolates from Kenya collected in 2001 from Kiambu, Nairobi, Naivasha, Nyeri and Thika districts were used.

2.1.2 Plants

The following cultivars of roses were used in this work: a floribunda rose cv. Frensham of parentage [Floribunda seedling X crimson Glory] obtained from Belle Epoque Rozenwekerij breeders, Netherlands; two hybrids teas cv. Claudia and cv. Rosita that were obtained from Koster breeders in Grefrath, Germany.

2.2 Plant cultivation

Rose cv. Frensham bushes were cut back to 3-5 cm from the budding point and the roots wetted before potting. The pots were at least 17 cm in diameter and 25 cm deep as recommended by the breeders. The initial planting soil was composed of "Klasmann special mixture": field soil: compost soil in the ratio of 2:2:1 and 5 g "plantosan" long-term fertilizer (20:10:15). The hybrids teas were planted in pots of 10.5 cm and in the same soil mixture but only 2 g of the "plantosan" fertilizer per plant was added. The potted plants were maintained in the green house with a minimum temperature of 20 ^{o}C ± 5 and 16 h light all the year round. Heaters that came on when the temperature fell below 21 °C and air circulation that automatically started by the opening of the windows when

the temperature was above 25 °C maintained the greenhouse temperature. The day length of 16 h was maintained by using Philips sodium vapour lamps SGR 140 with a lighting strength of 300 $\mu E/m^2$. The plants were fertilized with 2.5-3 g/l "Flory 2 spezial" (16:9:22) at the rate of 200 ml per plant every 2 weeks for the cv. Frensham and 20 ml per plant for the two hybrid tea cultivars.

2.3 Pathogen isolation and inoculum harvesting

Diseased leaves with fully developed black spot symptoms that had been sampled from different locations were incubated in moist chambers at room temperature (20 $^{o}C \pm 5$) for 3 days. Leaves from each location were incubated separately. The leaves were then washed with sterile water and the concentration of conidia in the suspension adjusted to 1 x 10^5 conidia/ml. Inoculum from each location was considered as an isolate from that particular region.

2.4 Inoculation of plants

Inoculations were carried out on both intact plants growing in pots and detached leaves.

Intact plants: The intact plant inoculations were carried out on plants with a minimum of six fully opened leaves. Potted plants were placed overnight in an incubation chamber with almost 100 % relative humidity and a temperature of 20 °C. The plants were then sprayed with a conidial suspension (1 x10^5 conidia/ml) and placed back in the incubation chamber for another 2 d. The plants were then taken out of the incubation chamber and put back in the green house. The untreated control plants were sprayed with sterile distilled water.

Detached leaves: For the detached leaf technique, the youngest fully opened (penultimate) leaves, -which is usually about the fifth youngest leaf and not from a flowering stem -were used. Leaflets were detached from the plants and washed in running tap water for 10 min and then surface sterilized in 3 % sodium hypochloride for 3 min. The leaflets were then rinsed in three changes of sterile distilled water and placed on sterile moist filter papers in 90 mm petri dishes with the adaxial side up as described by Palmer et al (1966). Alternatively 1cm diameter leaf discs were cut out from the sterilized leaflets on either side of the midrib using a sterile core borer and placed on the filter papers. The leaflets or the discs were inoculated with 5 µl droplets of the conidial suspension (5 x 10^4 conidia/ml) and incubated at 20 °C. The use of discs was avoided because they dried up earlier than the intact leaflets and they were also more often contaminated.

A lower inoculum concentration was used on the detached leaves so that the germinating conidia at infection site could be well spread out from each other in a manner that observations of each germinating conidia could be made without obstruction from the others. In the control the leaflets were inoculated with 5 µl droplets of sterile distilled water.

2.5 Disease assessment

The conidial suspension of the different isolates was used to inoculate two hybrid tea roses/cultivar/isolate. The hybrid teas were used in disease assessment with the aim of isolate characterization because of their ability to produce new leaves within a short time. The growth pattern of the different isolates on the plants was compared with respect to

germination rate, and disease severity. The germination percentage for each isolate was calculated and a disease scale of 0 - 100 % was used to determine the disease severity. The disease scale was divided into five classes of disease severity as follows:

0 % - no symptoms,

1-20 % - spots less than 5 mm, no yellowing and no defoliation,

20- 40 % - spots less than 5 mm, yellowing and/or defoliation,

40-60 % - spots 5-10 mm, no yellowing and no defoliation,

60-80 % - spots of 5-10 mm, yellowing and/or defoliation,

80-100 % - spots of a diameter greater than 10 mm, yellowing and/or defoliation

The results were used to select isolates showing significant differences in germination rate and disease severity, which were then used as representative isolates in subsequent experiments.

The same disease scale was used to compare the growth rate of the representative isolates on both intact plants and detached leaves, and at different incubation temperatures, 10 °C, 15 °C, 20 °C and 25 °C. The number of days required from inoculation to symptom expression on the detached leaves and intact plants were recorded. On the detached leaves ten droplets of inoculum were put on each leaflet and the number of black spots that developed per leaflet were recorded.

2.6 Maintenance of the pathogen and multiplication of the inoculum

The diseased leaves with fully developed black spot symptoms were incubated in a moist chamber for 3 d and then stored at –20 ^{o}C for long-term storage. To make a conidial suspension the leaves were allowed to defrost, then washed in sterile distilled water and

the conidia concentration adjusted to 1 x 10^5 conidia/ml before being used to inoculate the healthy plants. To multiply the inoculum detached leaves were inoculated and leaves with fully developed symptoms were stored at –20 °C.

2.7 Evaluation of the fungal growth *in vitro*

Three different types of artificial growth media namely, Potato dextrose agar (PDA), Biomalt agar (BMA) and Malt extract agar (MEA) were used in their full and half strengths. In addition to these was 1 % water agar (WA) also used.

2.7.1 Preparation of growth media

Following are the contents of the different growth media:

Water agar: 10 g agar, 1000 ml H_2O.

PDA: 39 g industrially prepared PDA in 1000 ml H_2O or pulp from 200 g potato cubes, 15 g dextrose and 20 g agar, in 1000 ml H_2O. Half strength PDA was composed of 19.5 g industrially manufactured PDA and 7.5 g agar, in 1000 ml H_2O, or pulp from 100 g potato cubes, 7.5 g dextrose, 20 g agar, in 1000 ml H_2O.

Biomalt agar: 20 g biomalt and 20 g agar in 1000 ml H_2O. Half strength biomalt agar was composed of 10 g biomalt and 20 g agar in1000 ml H_2O.

Malt extract agar: 30 g malt extract, 3 g peptone from soja bean meal and 15 g agar in 1000 ml H_2O. Half malt extract agar was composed of 15 g malt extract, 1.5 g peptone from soja bean meal and 15 g agar, in 1000 ml H_2O.

To a litre of each of the autoclaved and cooled (below 80 °C) artificial media were 50 mg of each of the following antibiotics added: streptomycin sulphate, penicillin G. and

chlortetracycline hydrochloride. 10-15 ml of artificial media were poured into each petri dish.

2.7.2 Incubation on growth media

Four points at the bottom of each of the petri dishes were marked with a permanent marker and the growth medium above these marked points was seeded with 10 µl of a conidial suspension containing 1x 10^4 conidia/ml. The seeded petri dishes were then incubated at 20 °C. Germination of the conidia and further development until a new crop of conidia was formed were closely followed. The evaluations were carried out by cutting out a block of inoculated artificial media and mounting it on a microscopic slide in 10 µl of diethanol. The block was then observed under UV-light in the fluorescence mode as described in section 2.8.3.2.

2.8 Microscopic investigations

To investigate the growth pattern of *D. rosae* on *Rosa* species, different microscopic and histochemical techniques were used. Microscopic evaluations were done using a light microscope as well as a transmission electron microscope.

2.8.1 Light Microscopy

For all light microscopy, a Leitz DMR photomicroscope from Leica was used. The Leitz-microscope was equipped with Nomarski-interference-contrast and with UV-excitation for epifluorescence. The filter combinations that were used are given in table 1. Images of the observed specimens were photographed with a fitted digital camera and could be observed on a connected screen. The images were saved using the program “Discus” (Technisches Büro Hilgers, Koenigswinter, Germany).

Table 1: Filter combinations for the incident light fluorescence microscope

exciter filter(nm)	chromatic beam splitter (nm)	barrier filter (nm)
BP 340-380	FT 400	LP 430
BP 355-425	FT 455	LP 460

2.8.2 Transmission electron microscopy

A Zeiss EM 109 transmission electron microscope was used for all electron microscopy evaluations.

2.8.3 Specimen preparation techniques

2.8.3.1 Fresh specimen

Samples taken at 4-h intervals for the first 72 h post inoculation (hpi) were used in the observation of post-germination and pre-penetration fungal structures found on the surface of the leaf and on artificial growth media. Leaf pieces of 1 cm^2 were cut out from either side of the midrib and mounted on a microscopic glass slide in 10 µ l of 0.05 % diethanol with the adaxial side to the glass slide. The specimens were evaluated without discolouration of leaf tissue or fixation of fungal tissue. 1 cm^2 blocks of artificial growth media were also treated the same way.

2.8.3.2 Whole specimen

Leaflet samples from plants or detached leaves (inoculated as described in section 2.4) were taken at 4-hour intervals for the first 72 hours post inoculation (hpi). After the 3rd day post inoculation (dpi) samples were collected every day. These samples were fixed in

chloral hydrate fixing solution (section 2.8.2.2.1) and stained (section 2.8.3) before the light microscopic observations. 1cm^2 pieces were cut out from either side of the midrib before mounting on the microscopic slide. The same method was also used to prepare fallen leaves, overwintered leaves and stem barks for microscopic observations.

2.8.3.2.1 Fixation with chloral hydrate

In order to observe and describe the development of the fungal structures inside the leaf tissue the chlorophyll was first removed and the samples then stained with the desired staining solutions. The fungal structures were fixed on the leaf surface and the leaflets discoloured in saturated chloral hydrate (250 g/100 ml H_2O) at 60 ^oC for at least 3 d (older tissue required longer). After the leaflets were fully cleared off chlorophyll, they were used in the light microscopic evaluations.

2.8.3.3 Sectioned specimen

To observe the fungal structures inside the host cells using the transmission electron microscope, vertical sections of the leaf tissue were necessary. They were prepared in the method below.

2.8.3.3.1 Embedding in ERL-resin

Samples collected from the inoculated leaflets at intervals of 4 h from 48 hpi to 72 hpi, and then every day up to the symptom appearance were cut into small pieces of 2 x 4 mm^2. These pieces were immediately placed in a fixing solution of 8 % formaldehyde: 8 % glutaraldehyde: 0.2 M cacodylic acid sodium salt trihydrate buffer (1:1:2 v/v/v) and 0.005 g calcium chloride (Karnovsky 1965) for a minimum of 2 h at room temperature. The eppendorf tubes containing the cut pieces in the fixing solution were placed in an air

tight glass jar connected to a water stream pump to enable the fixing solution to quickly penetrate and fix the structures in the leaf sections and also to remove excess air from the leaf pieces. Cutting the samples into small pieces enabled the fixing solutions to quickly penetrate into the cells before any cellular alterations had occurred, therefore the fixation preserved the cell contents in as much of their original state as possible. The samples were then rinsed about ten times in cacodylic acid sodium salt trihydrate buffer (pH 7.35), and then further fixed in 2 % Osmium tetraoxide (OsO_4, Next Chimica, S. Africa) fixing solution of 0.4 % KCr_2O_7: 3.4 % NaCl: 2 % OSO_4 (1:1:2 v/v/v) according to Dalton (1955), and modified according to Wohlfahrt-Bottermann (1957) for 1-2 h. Osmium tetraoxide increases the electron absorption and leads to a better photo-contrast. The chromium in the fixing solution helps in maintaining the tissue structure and in fixation of the lipids (Plattner 1981).

The samples were then rinsed up to eight times in cacodylic acid sodium salt trihydrate buffer (pH 7.35) for 20 min per rinse followed by dehydration in increasing concentrations of ethanol/aqua bi-distilled water as follows:

20 min in 15 %,	
20min in 30 %,	
20min in 50 %	2 changes
20 min in 70 %	2 changes
20 min in 90 %	2 changes
60 min in 96 %	4 changes
60min in 100 %	2 changes

Finally the samples were rinsed in 2 changes of propylene oxide of 10 min each. This was followed by infiltration of the samples in increasing concentrations of ERL-resin.

The contents of ERL-resin (Spurr, 1969) are as follows:

10.0 g ERL-4206
4.0 g D.E.R. 736
0.4 g S-1 (Agar Aids)

The samples were put in different ratios of ERL-resin: propylene oxide (v/v) in the following order:

16 h	in 1:3
8 h	in 1:1
16 h	in 3:1
16 h	in 100 % ERL-resin

The samples were then polymerised in 100 % ERL-resin in flat embedding trays (Agar-Aids) at 70 ^{o}C for 8-12 h.

2.8.3.3.2 Sectioning

Semi-thin sections

Semi thin sections of 500 nm thickness were cut using a 45^{o} glass knife and thereby being directly suspended in distilled water. The semi-thin sections were later suspended in distilled water on a glass slide, dried on an electric plate at 70 ^{o}C and stained in 0.05 % toluidine blue (w/v) in 0.01 M phosphate buffer (pH 7.4). The sections were rinsed in tap water to removed excess stain, followed by a rinse in demineralised water and in xylene for 5 min each. The sections were mounted and sealed in entellan rapid mounting media (Merck) and allowed to dry over night in a fume chamber before being viewed under the bright field of a compound light microscope.

Ultra-thin sections

When the observations under the light microscope confirmed the presence of the desired structures in the semi-thin sections, ultra-thin sections were cut out of the same block end as the semi-thin sections. The ultra-thin sections (70-75 nm thickness) were cut with a Reichert-Jung Ultramicrotome Ultracut E of 70-72 nm thickness using a diamond knife (Sitte 1982, 1985) and placed on copper or nickel grids.

2.8.3.3.3 Contrasting

The ultra-thin sections were then contrasted. The enhancement of the contrast before observations under the electron microscope was achieved through the use of electron dense material in form of heavy metal salts. The contrasting was done according to Geyer (1973). The grids were laid out in drops of saturated 2 % uranyl acetate for 8 min and then rinsed twice in aqua bi-distilled water, then put in drops of lead acetate solution (Reynolds 1963) for 2 min and rinsed in 2 changes of aqua bi-distilled water. The uranyl acetate and lead acetate were centrifuged before use. The lead acetate solution was prepared as follows:

-1.33 g $Pb(NO_3)_2$

-1.76 g $Na_3(C_6H_5O_7)$ x 2 H_2O in 30 ml aqua bi-distilled water

-thoroughly mixed and stored in darkness for 30 min

-the pH was adjusted to a value of 12 using 8 ml 1 N NaOH

-topped to 50 ml with aqua bi-distilled water.

The grids were allowed to air dry before being stored in a grid box at room temperature. The contrasting was carried out in a darkened petri dish lined with parafilm. The grids were then observed with a Zeiss EM 109 transmission electron microscope.

2.8.4 Staining techniques

2.8.4.1 Blancophor

The conidia germination and formation of pre-penetration structures were confirmed by staining the specimen with fluorescence stain blancophor. The same stain was used to stain fungal structures on artificial growth media. Blancophor binds to polysaccharides with β-glycoside bonds. This stain does not penetrate the plant cuticle, it binds the cell wall of the pathogen on the plant surface so that conidia, germ tubes and appressoria clearly fluorescent under UV-light.

The fresh specimen of inoculated leaflet samples or small blocks of artificial medium were mounted in 10 µl of 0.1 % blancophor solution in 10 % ethanol/H_2O_{bd} (v/v) before being observed. The specimen were covered with a cover slip and observed with the BP340-380/FT 400/ LP 430 filter combination at the Leitz-microscope with the incident lights fluorescence mode.

2.8.4.2 Diethanol (Uvitex 2B)

To determine the germination rate and describe the pre-penetration fungal structures found on the leaf surface, fresh leaf specimen as well as artificial media blocks were stained in 10 µl of 0.05 % diethanol (w/v). Diethanol binds to polysaccharides with β-glycosidic bonds. The stain does not penetrate the plant cuticle; therefore it stains the cell

wall of the pathogen on the plant surface, so that the conidia, germ tubes and appressorium on the plant surface fluorescence under UV-light.

The fresh specimen of inoculated leaflet samples or small blocks of artificial medium on which the fungus were growing were mounted in 10 µl of 0.05 % diethanol (w/v) before being observed. The specimen was covered with a cover slip and observed with the BP340-380/FT 400/ LP 430 filter combination at the Leitz-microscope with the incident light fluorescence mode.

2.8.4.3 Fuchsin acid

The development of the pathogen in the plant tissue as well as the plant defence reaction to the pathogen invasion at different intervals after inoculation were described after observing leaflets fixed and discoloured with chloral hydrate and then stained with fuchsin acid. Fuchsin acid is a stain for light as well as fluorescence microscopy. It binds to carbohydrates. It stains the cell contents of the pathogen and damaged plant mesophyll cells. Fuchsin acid stains carbohydrates and fungal structures pink.

Samples that had been fixed and discoloured with chloral hydrate were stained for 12 h in 0.01 % fuchsin acid (Merck) in lacto phenol. The samples that were very darkly stained were left in chloral hydrate solution for sometime until they obtained the desired stain shade. Small pieces of 1 cm^2 were cut from the leaflets and mounted in lactophenol. The microscopic slide was then sealed with nail varnish. The leaf pieces were observed under the interference contrast.

2.8.4.4 Aniline blue

The fluorochrome aniline binds to various glucans of both plant and fungal origin (Nicholas et al 1994, Van Sengbusch et al 1983), and to plant polysaccharides (Smith and McCully, 1978). Its strong association with β-1,3-glucans such as callose is due to the loose packing of this polymer, thus providing greater accessibility of the fluorochrome to them.

A modification of the preparation method described by Hood and Shew (1996) was used. The leaflets that had been fixed and discoloured in saturated chloral hydrate were let to stand for 1-2 h in 1 M KOH. The leaflets were then rinsed twice in water and once in 0.067 M K_2HPO_4 solution at pH 9.0 (buffer). The pH adjustment is normally not needed. The leaflets were let to stay overnight in the buffer solution and were then stained for 10-20 min in 0.05 % (w/v) aniline blue (Serva) in 0.067 M K_2HPO_4. They were then mounted in the buffer or in 50 % glycerol in buffer at pH 9.0 with the abaxial surface to the glass. Stain solutions were stored in brown-glass bottles at room temperature for not more than several months.

2.8.4.5 Toluidine blue

Toluidine blue is a metachromatic stain that stains phenols, papillae, polysaccharides as well as other macromolecules, giving different colour tones. Since almost all plant and pathogen structures take up the stain, the metachromatic effects like the typical green – blue staining of phenols containing structures could only be differentiated on sectioned preparations.

Semi-thin sections prepared as already described were stained with 0.1 % (w/v) toluidine blue in 0.01 M phosphate buffer (pH 7) for 1 min, then rinsed once in tap water and twice in H_2O_{demin}. Due to the metachromatic properties of this stain the plant cells and fungal structures stained violet and could easily be differentiated under the light microscope. The sections were then mounted and sealed in Entellan (Sigma). The Microscopic slides were allowed to dry over night in a fume chamber before microscopic evaluations.

2.9 Estimation of reactive oxygen species (ROS) production in leaf tissue

The production of reactive oxygen species such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in leaf tissue was estimated.

2.9.1 Superoxide anion (O_2^-)

The quantity of the O_2^- in the inoculated leaflets was determined using the nitroblue tetrazolium (NBT) reduction test described by Doke (1983a). Fourteen leaf discs were taken from the infected plants at 4 h intervals from the 24^{th} hpi to 48^{th} hpi. The discs were placed in a test tube containing 3 ml of a reaction solution. The reaction solution was composed of 10 mM potassium phosphate buffer (pH 7.8), 0.05 % (w/v) NBT and 10 mM NaN_3. The discs were incubated in the suspension for 1 h at room temperature (20 $^oC \pm 5$) and the reaction was stopped by heating the test tubes containing the discs in a water bath at 85 °C for 15 min and cooling immediately in ice. The absorbance of the solution was measured at 580 nm. The NBT reduction by each set of discs was expressed as the mean absorbance of each of the three replicates taken at each interval. The means were compared statistically by Duncan's multiple range test.

2.9.2 Hydrogen peroxide (H_2O_2)

H_2O_2 production was assayed as described by von Tiedemann (1997). Ten fresh leaf discs were kept in the dark for 2 h in a solution containing 50 ml phosphate buffer (pH 7), 0.05 % (v/v) guiacol and 12.3 units/ml peroxidase (Sigma). After 2 h reaction time the absorbance of the reagent mixture was measured at 450 nm. Results were expressed as mean absorbance x 1000 without further conversions (von Tiedemann, 1997).

2.10 Fungicide tests

Four fungicides active ingredients (a.i.) with different modes of action were used for the fungicide tests (Table 2).

Table 2: The active ingredients of fungicides used in the study and their respective concentrations, common names and manufacturers

Active ingredient	Quantity	Common name	Manufacturer
Trifloxystrobin	500 g/kg	Flint	Bayer CropScience
Pyraclostrobin	250 g/l	Cabrio	BASF
Myclobutanil	60 g/kg	Biosynthase	Rohm and Haas
Tebuconazole	250 g/l	Folicur	Bayer CropScience

2.10.1 *In vitro* tests

Water agar (1 %) was prepared (see preparation of artificial growth media), autoclaved and then allowed to cool to 70 ^{o}C before a given amount of the active ingredients (a.i.) of the desired fungicides (Table 2) were added. The end concentration of the a.i. required was 10 ppm per litre of water agar. To maintain the end concentration of the a.i., water

agar was autoclaved with 50 ml water less. The amount of fungicide required to give 10 ppm in the end water agar solution was dissolved in 50 ml water and filtered into the autoclaved water agar using bacterial filters. The water agar was thoroughly mixed before being poured out into petri dishes. The water agar was inoculated with 10 ml conidial suspension containing 1 x 10^4 conidia/ml and the petri dishes were incubated in a moist chamber at 20 ^oC for 48 h. At 48 hpi a piece of water agar was cut out, mounted on a microscope slide in 0.05 % (w/v) diethanol and observed under the fluorescence light as already described. Germination rate was determined by observing the number of germinated conidia.

2.10.2 *In planta* tests

The protective and curative activities of the four fungicide active ingredients, trifloxystrobin, pyraclostrobin, myclobutanil, and tebuconazole were investigated on the test plants inoculated with the fungus *D. rosae*. The aim was to find out the effects of the active ingredients on disease development and on the pathogenesis related fungal structures. The concentration of the inoculum used was 1 x 10^5 conidia/ml.

2.10.2.1 Protective activity tests

Suspensions of 100 ppm a.i. of each of the four fungicides were prepared. To test the protective activity of the a.i., plants were first sprayed with 100 ppm suspensions of the fungicides. Leaves were detached from these fungicide treated plants after a determined period of time, placed on a moist filter papers in 90 mm petri dishes and inoculated with the conidial suspension. The petri dishes were then incubated at 20 °C. Some leaves from the fungicide treated plants were inoculated on the same day that the plants were treated

with the fungicides and others were inoculated a week after. Samples were collected from the 3rd day after inoculation to determine the effect of the fungicides on the conidia germination rate. In the untreated control, the leaflets were collected from plants sprayed with sterile water instead of the fungicide.

A time plan of the fungicide application, inoculation and sample collection intervals is schematically illustrated in figure 1**.**

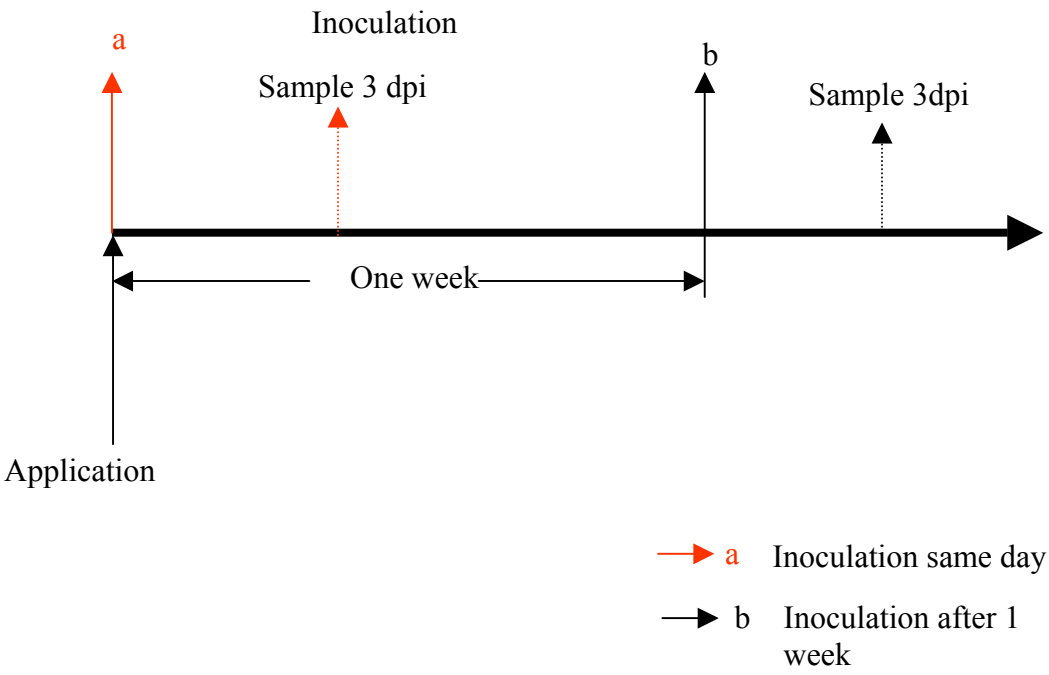

Figure 1: A schematic representation of the inoculation, fungicide application times and the respective sample collection intervals in experiments set up to determine the protective activity of the fungicide active ingredients**.**

2.10.2.2 Curative activity test

For the curative tests the plants were first inoculated and then treated with the fungicide after a predetermined time. Three experiments were set up; fungicides were applied 3 dpi

in one experiment, 7 dpi in another and 10 dpi in yet another set up. In the experimental set up where the plants were treated with the fungicides 3 dpi, the samples were collected from the plants on the 7^{th} and 10^{th} dpi. Where the plants were treated with the fungicides 7 dpi, the samples were collected on the 10^{th} dpi. In the set up where the plants were treated with the fungicides 10 dpi, the samples were collected 4 d after fungicide application i.e. 14 dpi. Untreated control samples for each experimental set up were collected at similar intervals from plants that had been inoculated with the same inoculum but sprayed with water instead of the fungicides. All the plants were observed for symptom development. The disease severity was assessed according to the disease scale given before. The disease scoring was done at 20 dpi to give the surviving inoculum time to reproduce.

A schematic representation of the inoculation, fungicide application and sample collection intervals is given in Figure 2.

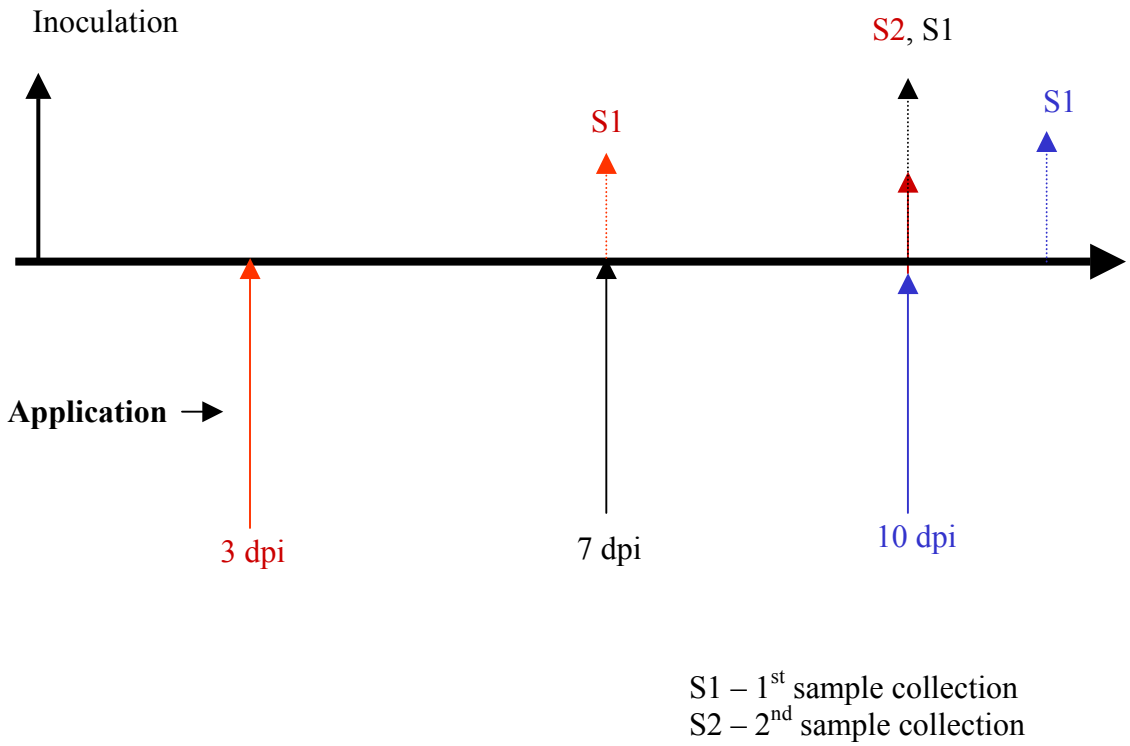

Figure 2: A schematic representation of the inoculation times, fungicide application times and the respective sample collection intervals in experiments to determine the curative activity of fungicide active ingredients**.**

2.11 Statistical analysis and software programmes.

Unless otherwise stated all statistical analysis were done using the MSTAT program. The mean comparison of more than two means was done using the Duncan's multiple range test and that of only two means using the LSD test. The photographs quality was improved using the computer program "Corel photo paint 9". The negatives of the electron microscope images were scanned using a 7400c hp scanner.

3 Results

3.1 Symptomatology of the black spot disease caused by *Diplocarpon rosae* on roses

3.1.1 Symptoms on inoculated plants

The first symptoms on the inoculated plants appeared as small brown spots from the 7th to the 10th dpi. Several very small brown spots were formed near each other to compose the fleck that one saw with the naked eye i.e. the spots observed at the beginning of symptom development were made up of several tiny brown points growing near each other (Figure 3b). On these spots the fungus grew under the cuticle to form what appeared like a network of railway tracks. On the surface of the leaves, acervuli, small blister-like structures that pushed the cuticle slightly upwards were formed (Figure 3c). The spots enlarged as more subcuticular mycelia and more acervuli were formed. The spots turned from brown to black with time and those next to each other merged to form bigger flecks. The spots increased in size to a maximum of 15 mm but where two or more spot merged the spot was bigger. Yellowing of the leaf tissues near the black spot occurred from the 14th dpi (Figure 3a). The leaf surface gradually turned yellow except for the black spot and the area around the spot that remained green forming “green islands” (Figure 3b). A closer examination of the black spots under a binocular microscope showed a mass of white conidia oozing out of the blister-like structures (Figure 3d). Infected leaves easily detached from the plant with the slight touch of a finger or slight wind movements. Where the diseased leaves did not turn yellow, they could still be more easily detached from the plants than the uninfected ones.

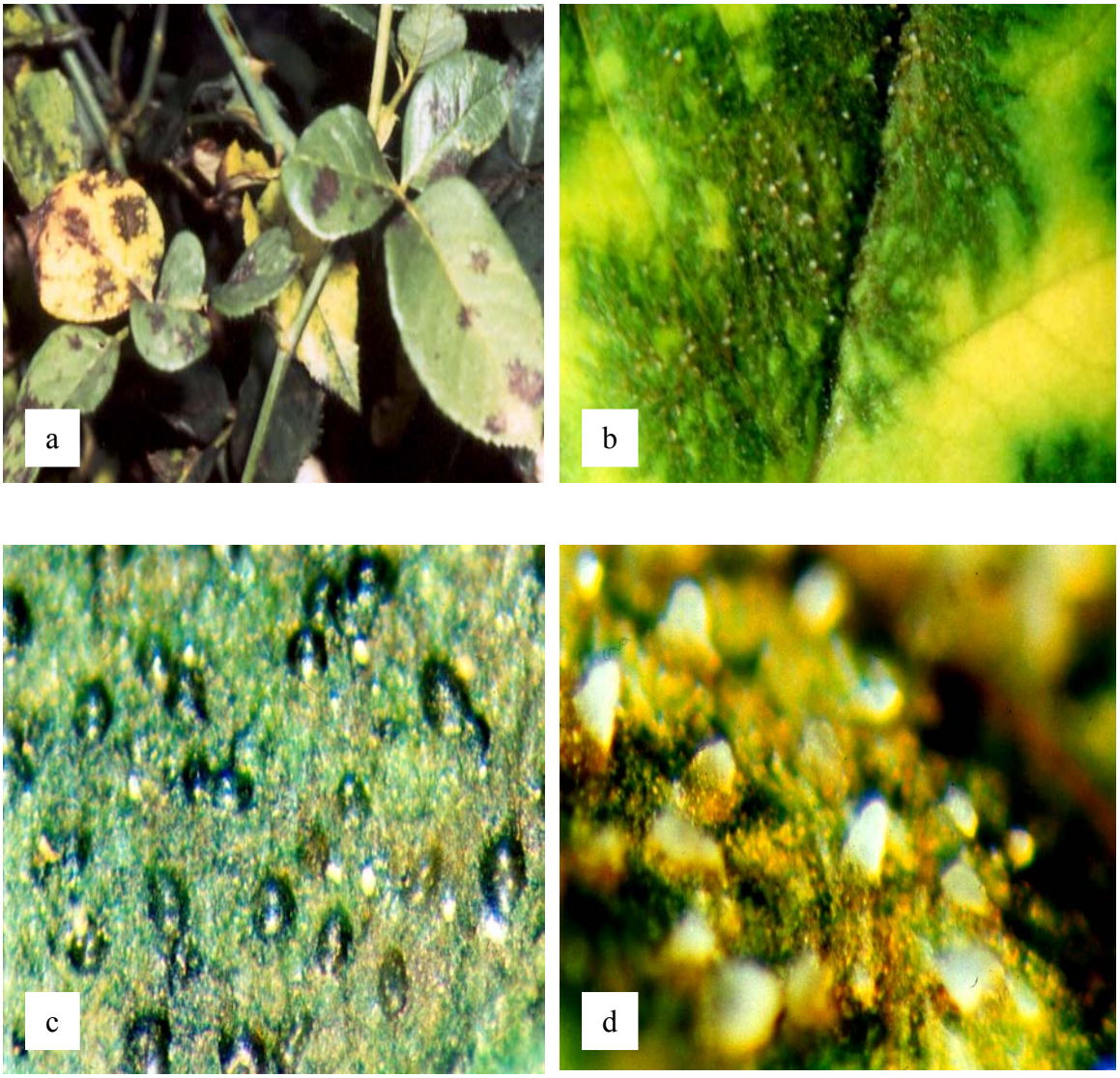

Figure 3: Photographs of rose leaves infected with *Diplocarpon rosae* showing, (a) the blackspot symptoms on leaves, note the complete yellowing of some of the leaves, (b) a close up of one of the black spots, the subcuticular hyphae appear like a network of roads or railway tracks, blister–like structures (acervuli) can be observed on the spots, the area around the black spot remains green but the rest of the leaf turns yellow to give the "green island" phenomen, (c) a close up of a sporulating spot showing the dome shaped unopened acervuli that have pushed the cuticle upwards, (d) a close up of a sporulating spot where a mass of white conidia oozes out of the acervuli.

3.1.2 Disease scoring

The disease scoring was done on the 20th dpi on the plants inoculated with the different isolates from Kenya and Germany. Although the symptom development process for the different isolates showed some slight variations, the end symptoms on the 20th dpi were the same. The variations that were observed in the symptom development in plants infected with the different isolates are discussed below.

According to the disease scale all the isolates from Kenya had a 100 % disease severity and those of Germany had also a 100 % disease severity. This was characterized by leaf spots on the upper side of the leaf with an average diameter greater than 10 mm. The diseased leaves turned yellow and fell from the plant prematurely. Since no noticeable differences were recognizable among the isolates from different regions, a representative isolate was chosen from each country. The isolate from Duesseldorf (G1) was chosen to represent the German isolates and an isolate from Thika (K1) was chosen to represent the Kenyan isolates.

3.2 Characterization of *D. rosae* isolates.

3.2.1 Germination rate

At 20 °C the isolate G1 had an average germination rate of 78.3 %. Out of all the germinated conidia 14.07 % formed more than one germ tube per conidium, and 7.36 % formed germ tubes from both cells of the conidia (Figure 4). The isolate K1 had an average germination rate of 57.9 %, and 20.83 % of the germinated conidia formed more than one germ tube per conidium. An average of 7.86 % of the germinated conidia germinated from both cells (MSTAT, Duncan's multiple Range tests). In general the very

long germ tube did penetrate the host but seem to just grow aimlessly over the host surface.

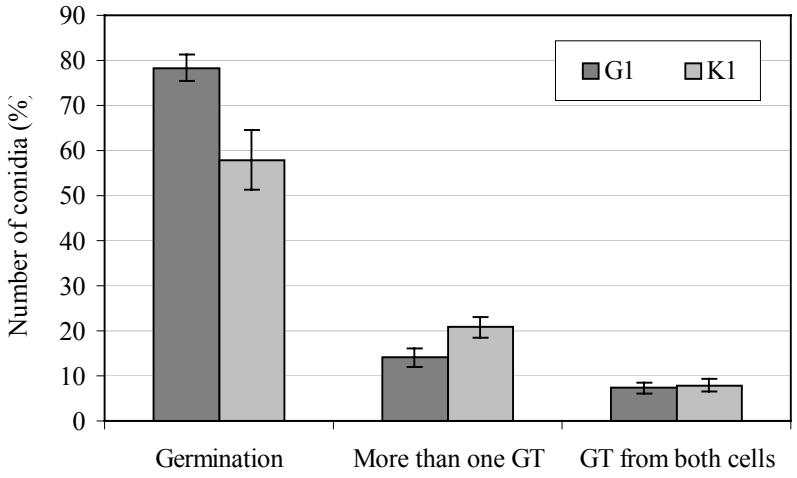

Figure 4: The germination rate (%) of conidia of *Diplocarpon rosae* isolates G1 and K1 on rose leaves: The percent of conidia that formed more than one germ tube (GT) and those that formed germ tubes from both cells of a conidium, (the data represent the mean values ± SD of 3 replicates n = 50, $P<0.05$, Duncan's multiple range test).

3.2.2 Process of symptom development

Disease symptom expression on intact plants inoculated with the representative isolates K1 and G1 were first observed 7 and 9 dpi respectively (Figure 5a, d). The symptoms formed on infected leaves were similar for both of the isolates and the only difference was that they appeared 2 d earlier for the isolate K1 (Figure 5b, e). On plants inoculated with K1, yellowing started on the 11th dpi (Figure 5c) but on those inoculated with G1 it started on the 14th dpi (Figure 5f). On all the infected plants the diseased leaves turned yellow and defoliation followed soon after. By the time of disease assessment (20 dpi) no noticeable differences were visible between the plants inoculated with isolate K1 and G1 (Figure 5c, f).

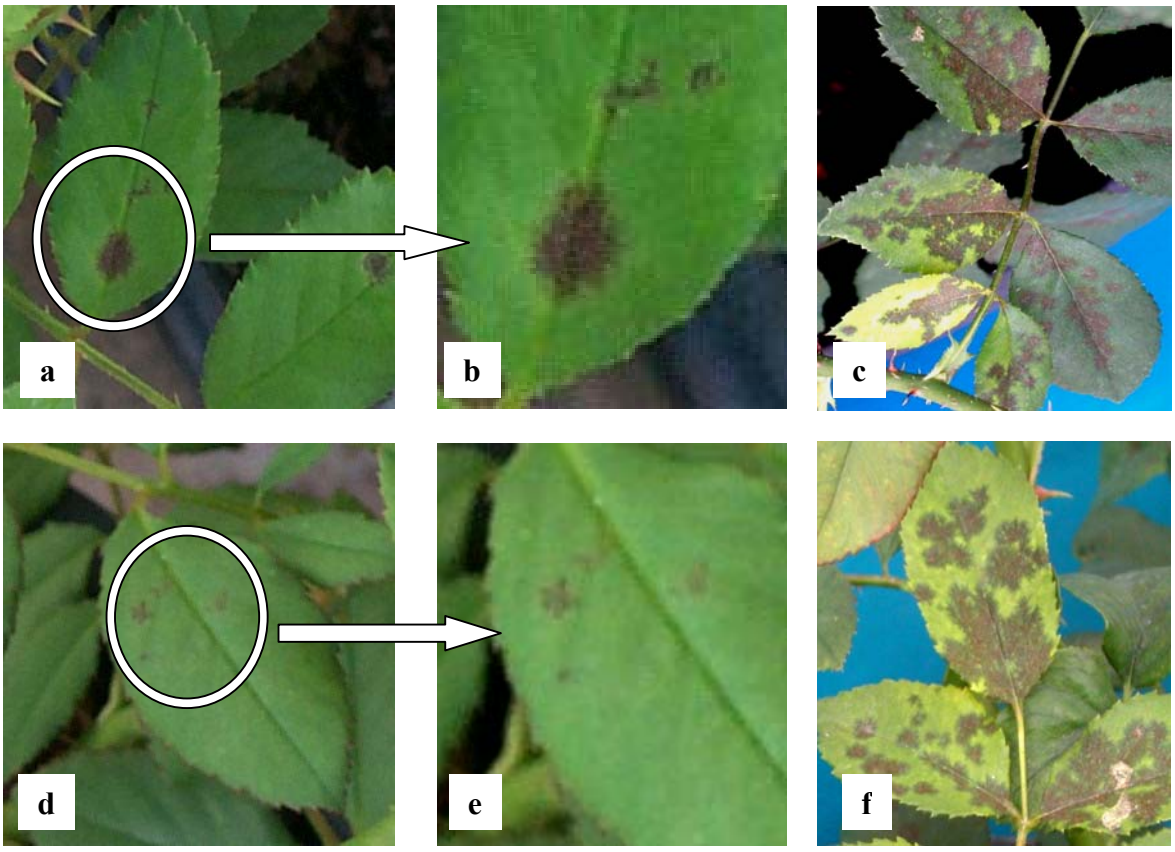

Figure 5: Photographs of rose leaves infected with *Diplocarpon rosae* isolates G1 and K1 showing the stages in disease symptom development: (a) a rose leaf inoculated with isolate K1 photographed 10 dpi, (b) a close up of (a), (c) the rose leaves inoculated with isolate K1 photographed 20 dpi, (d) a rose leaf inoculated with isolate G1 photographed 10 dpi, (e) a close up of (d), (f) rose leaves inoculated with isolate G1 photographed 20 dpi.

3.2.2.1 Effect of different temperatures

Detached leaves inoculated with the isolates G1 and K1 were incubated at different temperatures in order to monitor the effect of temperature on disease symptom development. At 25 °C the first symptoms appeared on the 4th dpi on leaves inoculated with either of the isolates (Table 3). These symptoms were small dark spots and an acervulus was visible when the spot was viewed under a hand lens. On the 5th dpi the spots were approximately average 1-2 mm and the number of acervuli per spot had also increased. On the 7th dpi a mass of white conidia could be observed on the spots on leaves inoculated with either of the isolates and the leaves had started to yellow (Table 3). On leaves inoculated with either of the isolates, the black spots with an average diameter of 10 mm by the 9th dpi. The leaves had also turned yellow except for the areas with the spots (Table 3).

At 20 °C the first disease symptoms were observed on the 5th dpi on leaflets inoculated with either of the isolates. 16 out of the 20 (80 %) leaflets inoculated with the isolate K1 had more than 5 spots per leaflet, while on those inoculated with the isolate G1 all the 20 leaflets had less than 5 spots per leaflet. On leaves inoculated with either of the isolates, all the spots had an average diameter of less than 1 mm. On the 7th dpi, all the leaflets inoculated with isolate K1 had more than 5 black spots with acervuli per leaflet and on the 9th dpi the average diameter of the spots was about 10 mm and yellowing had started. On the leaflets inoculated with isolated G1, 12 out of the 20 (60 %) leaflets had more than 5 spots per leaflet but no acervuli were observed on the 7th dpi. On the 9th dpi the spots were 7-8mm in diameter with some acervuli and only slight yellowing was

observed. By the 11th dpi many of the spots grew into each other making the spots suddenly larger (Table 3).

At 15 °C faint appearances of the symptoms were observed 7 dpi when the petri dishes containing the leaflets were held against the light. On the 9th dpi 10 leaflets per isolate had at least one black spot each. None of the leaflets had 5 or more spots per leaflet even after 20 dpi. No disease symptoms were observed on detached leaves inoculated with either of the isolates and incubated at 10 °C even after 20 dpi. This information is summarized in Table 3.

Table 3: The development of symptoms on detached rose leaves inoculated with *Diplocarpon rosae* isolates G1 and K1, incubated at different temperatures (n=20).

dpi	observed character	10 °C		15 °C		20 °C		25 °C	
		isolate G1	isolate K1	isolate G1	isolate K1	isolate G1	isolateK 1	isolate G1	isolate K1
5	average lesion diameter	no symptoms	no symptoms	no symptoms	no symptoms	1 mm	1 mm	1-2 mm	1-2 mm
7	acervuli formation	no symptoms	no symptoms	no symptoms	no symptoms	none	formed	formed	formed
9	average lesion diameter	no symptoms	no symptoms	1 mm	1 mm	7 mm	10 mm	10mm	10 mm
	yellowing of the leaflet	no symptoms	no symptoms	none	none	yellowed	yellowed	yellowed	yellowed
20	diseased leaf surface (%)	no symptoms	no symptoms	< 1	<1	60 %	60 %	60 %	60 %

3.2.3 Assessment of the aggressiveness *in vivo*: accumulation of reactive oxygen species.

Further test were carried out to confirm the differences in the aggressiveness of the two isolates. In plants under stress, the amount of reactive oxygen species (ROS) is higher than in plants undergoing no stress. In the following experiment the ROS, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), in plants inoculated with both isolates was estimated.

3.2.3 1 Superoxide anion (O_2^-) in the leaf tissue

The production of the superoxide anion in leaves inoculated with the isolate K1 increased steadily from 24 hpi to 48 hpi. The amount of superoxide anion produced in leaves inoculated with isolate K1 at 48 hpi was double that produced at 24 hpi in leaves inoculated with the same isolate. The production of superoxide anions in leaves inoculated with the isolate G1 increased from 24 hpi and peaked 36 hpi before gradually reducing with increasing length of time after inoculation for the given time span. The amount of superoxide anions produced in leaves inoculated with K1 at 48 hpi was more than double that produced in leaves inoculated with the isolate G1. The concentration of the superoxide anions correlated directly with the reduction of nitroblue tetrazolium (NBT), which was measured as the relative optical density of the solution in which the leaf discs from plants inoculated with the different isolates had been placed. The mean reduction of NBT reduction measured at 580 nm was plotted against the number of hour post inoculation as show in Figure 6.

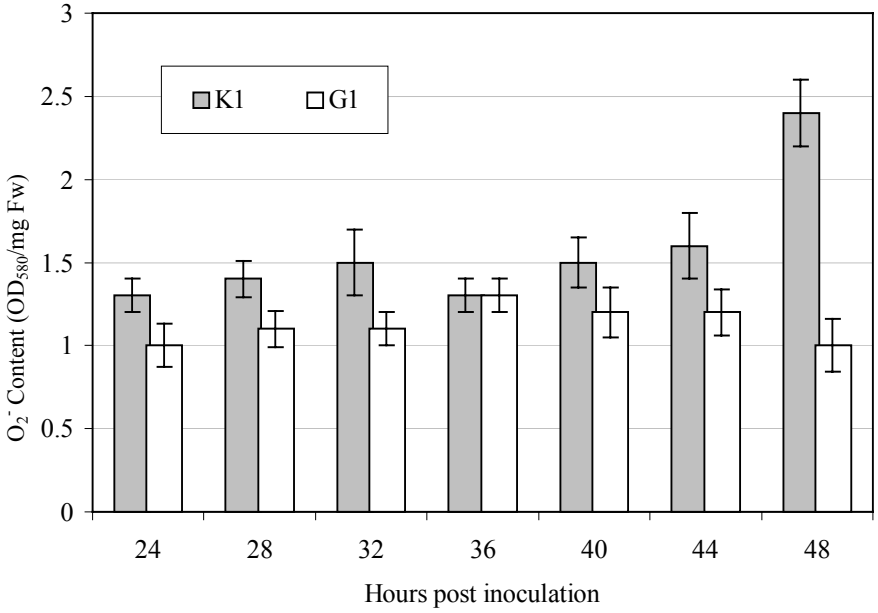

Figure 6: Reactive oxygen species (ROS) content in rose leaves of plants inoculated with *Diplocarpon rosae* isolates G1 and K1: The accumulation of the superoxide anions (O_2^-) with time (hpi) is expressed as the optical density at 580 nm/mg fresh weight, (the data represent the mean value ± SD of 3 replicates, n = 14, $P < 0.05$, Duncan's multiple range test).

3.2.3 2 Hydrogen peroxide (H_2O_2) in the leaf tissue

The hydrogen peroxide content in leaves inoculated with both isolates increased with time post inoculation (Figure 7). The H_2O_2 content in leaves inoculated with isolates K1 was higher than in those inoculated with isolate G1. The H_2O_2 content in leaves inoculated with isolate K1 reached its peak at 40 hpi and then remained relative constant until 48 hpi, while in leaves inoculated with isolate G1 the H_2O_2 production reached its peak at 36 hpi and remained constant until 44 hpi after which an increase was recorded.

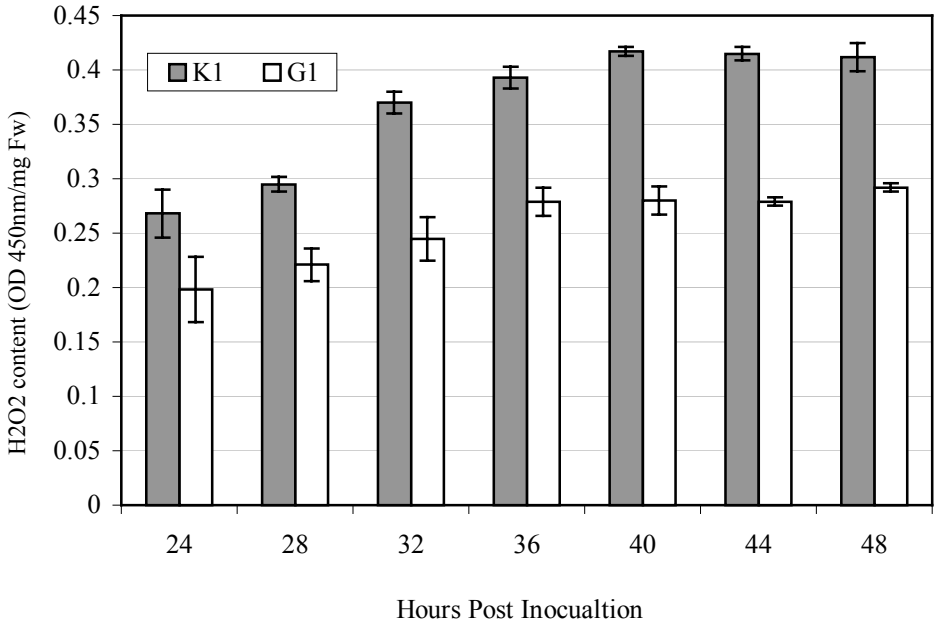

Figure 7: Reactive oxygen species (ROS) content in rose leaves of plants inoculated with *Diplocarpon rosae* isolates G1 and K1: The accumulation of hydrogen peroxide (H_2O_2) with time (hpi) is expressed as optical density at 450 nm/mg fresh weight, (the data represent the mean value ± SD of 3 replicates, n = 14, $P < 0.05$, Duncan's multiple range test).

3.3 Microscopical studies of the infection process

The purpose of this section of the study was to make detailed observations of the growth cycle of the fungus. A detailed study of the growth pattern of the fungus carried out with only one isolate, G1, is described in this section. All further investigations were carried out with only this one isolate.

3.3.1 Growth on the host leaf surface

3.3.1.1 Germination of conidia

The inoculum was composed of two-celled conidia. The average size of the conidium was 20.48 µm (± 1.2) by 5.87 µm (± 0.51). The two-celled conidia germinated to form one or several germ tube(s) (Figure 8a and b), from the 9th hour post inoculation

The germ tube was sometimes separated from the conidium by a septum (Figure 8a). The length of the germ tube varied from very short to very long ones growing over several cells. Where the germ tube was very short it was seen an extension of the conidium cell wall (Figure 8e and 9b) or it was found directly beneath the conidium (Figure. 8c and 9a). Such germ tubes were difficult to observe under a light microscope. Germination occurred at any place on the conidia surface.

3.3.1.2 Appressorium formation

The distal end of the germ tube became enlarged and rounded off to form an appresorium (Figure 8a) or the germ tube penetrated the cuticle directly (Figure 8b and 9b), i.e. an appressorium was not always formed. Where an appressorium was formed it was sometimes separated from the germ tube by a septum. The appressorium did not have an appressorial core. On the surface of the appressorium that was in contact with the cuticle, a penetration peg with a very small diameter was formed. On Figure 8f one can see the penetration pore through which the penetration peg grows. The fungus penetrated the host cuticle using this penetration peg. The wall of the appressorium in contact with the leaf surface and that of the penetration pore were observed to be thicker than the conidial cell wall. The walls of the conidium and the appressorium stained with fuchsin acid (0.05 %), but where penetration had occurred the conidium did not stain with fuchsin acid (Figure 9c). Brown rings were observed around the penetration pore (Figure 8c and d). The thickness of the cuticle at the penetration site was cleanly severed (Figure 9f). Only conidia germination, germ tube growth and appressorium formation took place on the surface of the leaf, further fungal growth after penetration of the cuticle occurred between the upper and lower leaf cuticles. No hyphae were found on the leaf surface.

3.3.2 Penetration of the host cuticle and growth in the host tissue

Upon penetration the penetration peg gave way to an infection vesicle (Figure 9b-d). The infection vesicle was found in the subcuticular space and it partly encroached on the periclinal wall of the epidermal cell directly below. At the point of penetration the epidermal cell wall below reacted by forming a papilla on the inner side of the cell wall. At the site of penetration the cuticle was cleanly severed with no inward deformation and host cell wall around the penetration peg was different from the rest of the host cell wall (Figure 9f). The periclinal wall of the underlying epidermal cell was dissolved ahead of the fungal structure but the plasma membrane appeared intact. The effects of the penetration peg on the periclinal cell wall were both lateral and vertical as can be observed on Figure 9f. Electron opaque layers were observed between the appressorium and the cuticle, in the upper layers of the host cell wall below the point of penetration, and in the lower host cell wall layers just above the plasma membrane of the epidermal cell (Figure 9f). The host cell wall below the point of penetration increased in diameter and changed in its staining properties (Figure 9e). Where the fungus was growing the wall stained lighter with toluidine blue than the surrounding wall. Hyphae grew from the infection vesicle. The hyphae grew in the subcuticular space and some few branches in the intercellular region between the epidermal cells and rarely in the periclinal wall of the epidermal cells. The hyphae were observed to have septa arising at short intervals from each other and their cells appeared to be almost rectangular in shape under the light microscope.

3.3.3 Subcuticular growth

Most of the primary hyphae grew in the subcuticular space and in the upper layers of the periclinal wall of the epidermal cell, while some grew in the intercellular spaces between the epidermal cells. The subcuticular hyphae multiplied and rapidly spread in a radial pattern from the point of penetration towards the periphery of the leaf. These subcuticular hyphae initially spread laterally without invading the epidermal cells below them. The periclinal cell wall in or near where the mycelia grew was observed to be wider and stained light violet unlike that in the uninfected parts of the plant where the cell wall stained dark violet when stained with toluidine blue (Figure 9e). The cuticle above each strand of primary subcuticular hyphae was pushed slightly upwards. The subcuticular stromata were one cell thick except where an acervulus was formed and here the subcuticular stromata were two to five cells thick. At such points the cuticle was pushed upwards to form a dome shaped structure.

The subcuticular primary hyphae grew in close association with each other in pairs, or groups of three or more parallel hyphae that were tightly pressed together (Figure 10a). Although the hyphal strands grew in a radial pattern away from the point of penetration, they met at several points to form what appeared to be a network of hyphae under the cuticle. Where two fascicles met, they combined to form a bigger fascicle i.e. one fascicle changed its direction of growth by separating its constituting hyphae. These hyphae separated to grow in two opposite directions but parallel and in close association with the other fascicle had they joined (Figure 10c). On very rare occasions did one fascicle cross another by dipping under it temporarily, and then coming back to the old level after the passage was completed (Figure 10c). A new hyphal branch arose by forking of the parent

cell at the distal end of the growing hypha to produce two new hyphae, which continued to grow tightly pressed together side by side, while new fascicles arose by hyphae that were originally growing side by side separating to grow at an acute angle to each other and to the main fascicle (Figure 10a). Each hypha in a fascicle branched independently.

As the primary subcuticular hyphae grew away from the point of penetration, they gave rise to new intercellular hyphae at intervals along their length. These intercellular hyphae grew into the lower parts of the host tissue. The wall of the subcuticular hyphae was initially smooth along the whole length when observed under a compound light microscope, but as the disease progressed, the wall formed folds or was convoluted at several points along the outer walls of the hyphae. At these points the diameter of the hyphae was increased, and from the resulting larger cell of the subcuticular hyphae a narrow hypha was formed. The narrow hypha (Figure 10f) penetrated the periclinal wall of the epidermal cell. This narrow hypha either penetrated into the epidermal cell directly below it to form a haustorium (Figure 11b) or grew as a hypha in the periclinal cell wall (Figure 10f). A narrow hypha formed from a normal sized subcuticular hypha enlarged just outside the epidermal cell wall and then penetrated the cell wall (Figure 10d) to form a haustorium on its inner side. Therefore the subcuticular mycelia also directly invaded the epidermal cells to form haustoria. Many haustoria arose from a point on the hyphae of one fascicle and were formed in a single epidermal cell (Figure 11a). The haustoria formation was at intervals along the length of the subcuticular mycelia. The haustoria were formed from sections of the fascicle growing over the periclinal wall of an epidermal cell and never at points where the subcuticular mycelia grew over the anticlinal cell wall.

3.3.4 Invasion of the host tissue

3.3.4.1 Intercellular hyphae

At the point of penetration some of the primary hyphae that arose from the infection vesicle grew into the middle lamella between the epidermal cells (Figure 11c and d). These intercellular hyphae were septate. The septa were observed to have woronin bodies. The intercellular hyphae grew both downwards towards the lower parts of the leaf tissue and also laterally between the epidermal cells, such that an epidermal cell was almost enclosed by intercellular hyphae. Growth of the intercellular hyphae was observed to be associated with partial or complete dissolution of the surrounding anticlinal walls as they progressively grew towards the lower parts of the leaf tissue (Figure 11c). Occasionally a branch from the intercellular hyphae invaded the adjacent epidermal cell wall to form a haustorium (Figure 11e, f and 12a) or continued to grow as a hypha on the inner side of the cell wall but exterior to the plasma membrane (Figure 13a). The intercellular hyphae and those growing on the inner side of the epidermal cell wall grew progressively towards to the palisade mesophyll cells. Upon contact with the palisade cell wall the intercellular hypha invaded the palisade mesophyll cell wall to form a haustorium (Figure 13a and b).

At the point of invasion of the mesophyll cell the diameter of the host cell wall around the penetration point was less than that of the surrounding wall (Figure 12f). The hypha growing on the inner side of the epidermal cell wall penetrated the double cell wall (epidermal and the palisade cell wall) to form a haustorium on the inner side of the palisade cell wall (Figure 13a). At the point of cell invasion the penetrating hypha

drastically reduced in diameter and the host cell wall surrounding the penetrating hypha was very thin. Upon penetration the section of the penetration hypha that grew on the inner side of the wall formed the neck of the haustorium. The host palisade cell reacted to this invasion by forming a thick callose (Figure 12b and f) through which the penetrating hypha grew before expanding to the haustorium body at the distal end.

3.3.4.2 Intramural mycelia

In the advanced disease state where palisade mesophyll layer had been invaded and the subcuticular mycelia had spread to some extent away from the point of penetration, branches of intramural hyphae were actively formed from the subcuticular hyphae in the periclinal walls of epidermal cells (Figure 13c). The cell of the subcuticular hypha from which the intramural hypha was formed enlarged before invading the periclinal wall to form the intramural hypha (Figure 10d). These intramural hyphae were formed at intervals along the length of the subcuticular hyphae. Initially the intramural hyphae were observed in the walls of a few epidermal cells but two days after they had started forming they were observed to be in almost all periclinal walls of the of the cells in the area covered by the subcuticular mycelia. The first intramural hypha was formed in the walls of epidermal cells near the penetration point. The intramural hyphae were formed at variable angles to the subcuticular hyphae.

The growth of each intramural hypha was limited to the region between the anticlinal walls of a single cell i.e. each intramural hypha extended only over a single cell. Upon coming into contact with the anticlinal wall, it formed haustoria in each of the adjacent epidermal cells (Figure 13e). Rarely were haustoria subtended into the underlying

epidermal cell from the intramural hyphae as it grew in the periclinal wall before coming into contact with the anticlinal wall (Figure 13d). The terminal cell of the intramural hypha increased in size just outside the host cell wall before penetrating it (Figure 13f).

As already mentioned the intramural hypha was limited to each cell and on very rarely did it grow through the double anticlinal cell wall to the periclinal wall of the neighbouring cell. Where this happened the diameter of the intramural hypha reduced drastically as it grew through the double walled anticlinal cell wall and then expanded to original size on reaching the periclinal wall of the neighbouring cell.

3.3.4.3 Haustorium

Haustorium-like structures were formed in the epidermal cells and palisade cells invaded by the fungus, these structures are referred to as haustoria in this study. The initial haustoria were formed in the epidermal cells from the intercellular mycelia. These were formed very early in the disease development i.e. in the first 24 hours post inoculation. Subcuticular hyphae that grew progressively away from the point of penetration formed the next batch of haustoria in the epidermal cells. The subcuticular mycelia formed haustoria at intervals along their length into the epidermal cells below them. Hyphae of a single fascicle in most cases formed haustoria at about the same time into a single epidermal cell below them. Therefore many haustoria of different shapes and sizes could be found in a single epidermal cell (Figure12a).

In an advanced disease state, haustoria were formed in the epidermal cells from the intramural mycelia growing in the periclinal wall of the epidermal cell. This batch of

haustoria from the intramural hyphae was formed just before the beginning of the reproduction process.

The subcuticular runner hyphae progressively colonised new areas in the plant tissues, therefore the whole pattern of haustoria formation kept repeating itself. In a newly colonised area the haustoria from the intercellular hyphae were formed first followed by those from the subcuticular hyphae and then by those from intramural hyphae. In an infection site, the disease was at different levels of advancement in the various cells. Haustoria were being formed in host cells by hyphae growing in different parts of the host tissue.

The hyphal cell from which the penetrating hypha was formed enlarged just before host cell invasion. This enlarged cell was delimited from the rest of the hypha by a septum with woronin bodies and was closely appressed to the host cell wall. A penetrating hypha was formed from the portion of the hypha in contact with the host cell. The penetration hypha was used to invade the host cell. The tip of the penetrating hypha had a very small diameter compared to the rest of the hypha. The host cell wall directly below the tip of the penetrating hypha was cleanly severed, with no inward wall deformation. The plant cell reacted to the invasion by forming a thick callose on the inner side of the cell wall directly below the point of invasion. The penetrating hypha grew through this callose and later expanded into the haustorium body on appearing on the other end of it (Figure 12b and f). The callose was in form of a hemispherical papilla beneath the penetration points where the penetrating hypha had not fully penetrated the host cell wall or in form of collar surrounding the neck of the penetrating hypha when penetration was complete. The

callose fluorescent brighter than the other structures in leaves stained with aniline blue and was easily distinguishable from the fungal mycelia. The penetrating hypha became the haustorium neck, such that the haustorium appeared to be subtended into the cell by a narrow and long neck.

Therefore a haustorium was formed when the hyphal cell in contact with the host cell wall enlarged outside of the host cell wall before penetrating it. The portion of the hyphal cell in contact with the cell wall formed a penetrating hypha whose diameter was drastically reduced in comparison to that of the normal intercellular hypha. This penetrating hypha penetrated the host cell wall and pushed the host cell plasma membrane away from the cell wall. The penetrating hypha expanded to a haustorium body on the inner side of the host cell wall but exterior to the host cell plasma membrane. The haustorium was enclosed by part of the host plasma membrane, which convoluted around it. The part of the host plasma membrane surrounding the haustorium body was called the extrahaustorial membrane (EHM) (Figure 12c). Between the haustorium body and the EHM was an extrahaustorial matrix (EHX). The EHM was very close to the collar ensheathing the neck of the haustorium and in the neck region no EHX was observed (Figure 12b). A section of the haustorium body was sometimes covered by a very thin layer of the EHX and had the EHM lying very close to the haustorium cell wall, but the EHX around the distal end of the haustorium was substantial. The haustoria were therefore formed on the inner side of the cell wall but exterior to the plasma membrane. The haustoria were also observed to lie close to the host nucleaus of the epidermal cells.

The haustoria were of different shapes and sizes depending on their developmental stage. Within the epidermal cell walls the shapes varied from pyriform for the very young ones through ventricose to oval for the older ones. Some of the haustoria had the shape of two or three ventricose sections joined to each other by their distal ends (Figure 11f). The ventricose sections were separated by sections of very narrow diameter. Haustoria formed in the palisade mesophyll cells were clavate shaped (Figure 12f). Some of the haustoria were very long (> 30 μm). The very long haustorium had an appearance of an intracellular hypha, but the two could be differentiated from each other in that the intracellular hyphae had no neck regions and no collar material. The young haustoria were small in size but later enlarged to occupy the whole length of the host cell. Where further growth of the haustorium was hindered by the host cell wall the haustorium grew in a curved manner away from the host cell wall.

3.3.4.4 Intracellular hyphae

During the advanced state of the disease development, hyphae with a small diameter were formed from the intercellular mycelia and they grew through the cell lumen. These intracellular hyphae were distinguished from the other hyphae in that they had a much smaller diameter and they were intracellular. They were distinguished from the haustoria in that they did not have a neck region like the haustoria. The initial intracellular hyphae formed in a cell were enclosed in a callose sheath (Figure 14c) but those formed later were surrounded by an electron opaque layer (Figure 14d). They were also not enclosed in the extrahaustorial membrane. These intracellular hyphae run from one end of a cell to the other and several intracellular hyphae were found in a single cell (Figure 14b). The intracellular hyphae also grew from one cell to another (Figure 14d). At the point where

they penetrate the neighbouring cell the tip of the penetrating hypha was greatly reduced in diameter and the host cell wall was locally degraded. No haustoria mother cell was formed. Longitudinal sections showed that in these advanced disease state the host cells lost their integrity as they became more heavily invaded by intracellular hyphae. When an epidermal cell was fully occupied by intracellular hyphae the normal cell contents were no longer visible. The cell had been reduced to a mere outline with the cell wall beginning to slowly disintegrate or be digested away (Figure 16f).

Intracellular hyphae were mainly found in the epidermal cells and very few were found in the palisade cells. In the older infections more intracellular subepidermal hyphae were formed. In fallen leaves the number of intracellular hyphae per host cell increased (Figure 16d).

3.3.5 Fructification

In the advanced disease state after the formation of the intracellular hyphae had begun finger-like projections were formed at intervals along the length of the subcuticular hyphae (Figure 14e). These projections had shorter cells than the hyphae, grew in a radial pattern from a point on the hyphae, and branched dichotomously to give a fan-shaped appearance. This fan shaped structure became the base of an acervulus. The tip of each of these finger-shaped projections curved upwards and became conidiogeneous cutting off a two- celled or a single celled conidium (Figure 15c and f). The two-celled conidium was separated from the conidiophore by a septum. The conidium ontogeny was observed to be thalloblastic and the septum separating the conidium from the conidiophore was formed before that separating the two cells of the conidium. The narrower and more or less

pointed cell of a conidium was attached to the conidiophore, while the cell with a more rounded end pointed upwards (Figure 15c). The conidiophore was very short and only a single conidium was cut off from each conidiophore. In the cytoplasm of each the cells of the two-celled conidia, were what appeared like vacuoles (Figure 15c and f), each cell had at least one of these vacuole-like structures, but in some cells two were found. In the same acervulus containing the two-celled conidia were found single celled microconidia (Figure 15c and f). The microconidia were clavate shaped, were borne singly on conidiophores in the same acervulus as the two-celled conidia. The microconidia were shorter and narrower compared to the two-celled conidia and were borne on a separate conidiophore from those bearing the two-celled conidia. The microconidia were distinguishable from other round cells that were also found in every acervulus. These round cells were not borne on conidiophores and their formation preceded conidia formation.

The roof of the acervuli was the plant cuticle. Upon formation of the conidia in the acervuli was the cuticle pushed upwards to form a dome shaped structure that was more or less symmetrical (Figure 15a). The acervuli had an appearance of small dark points when viewed with the naked eye or dark blisters when viewed with a hand lens. Under the acervulus roof were found the conidia and conidiophores, the acervulus base composed of the finger like projecting mycelia and part of the subcuticular hyphae from which the acervulus base stroma was formed. Soon after conidia formation the acervuli stromata become dark brown (Figure 15d). This browning was responsible for the further darkening of the infected spot on the leaf to give the typical black spot symptoms. This

brown pigmentation could not be removed with saturated chloral hydrate solution regardless of how long the samples are left to stand in this clearing solution.

The browning of the mesophyll cells upon invasion by the fungus was responsible for the initial browning at the infection site. It was not however established if only the invaded cells turned brown. When the roof of the acervuli tore open, and the mass of mature conidia oozed out (Figure 15e and 16a). The acervuli roof had opening slits running down it whole middle length but some were observed to open at other points (Figure 15e and 16a).

3.3.6 Growth through the lower side of leaf

On leaves collected from the institute garden where the infection occurred under natural conditions, germinated conidia were observed on the lower surface of the leaves. TEM micrographs of these leaves revealed that the fungus had penetrated the lower cuticle of the leaf and into the epidermal cells. In the epidermal cell a thick callose was formed around these hyphae such that they did not manage to grow beyond the lower epidermal cells (Figure 17d).

On the other hand, on leaves of garden plants that had been trapped by thorns into an almost vertical position, were several brown spots observed on the lower side. When these leaves were fixed and discoloured with chloral hydrate, stained in aniline blue and then observed under the light microscope, it was established that *D. rosae* was responsible for the black spots on the lower side of the leaf. The fungal conidia on the lower side of the leaf had germinated and the germ tube had penetrated the cuticle and caused infection on the lower side of the leaf. The parallel subcuticular hyphal strands

similar to those normally observed on the upper side of the leaf were seen to form underneath the lower cuticle (Figure 17a). Haustoria were formed in the epidermal cells and in the guard cells (Figure 17b). Subcuticular acervuli were also formed on the lower side and they released their conidia when the lower cuticle was burst open (Figure 17c).

3.3.7 Overwintering

The state in which state it overwinters under the weather conditions in Germany is unknown. Likewise the plant parts in which the fungus overwinters is unknown.

3.3.7.1 On fallen leaves

Infected rose leaves fall relatively easily with a slight touch of a finger or gentle air movement from wind. Some diseased leaves remained attached to the rose plants in the garden during the winter. From the leaves collected in September 2003 and stored outside on a tray of soil, a sample picked in October had many clearly visible subcuticular mycelia that stained well with aniline. Anastomosis of the subcuticular hypha was observed. Haustoria could also be made out in the lifeless epidermal cells. Subcuticular acervuli containing conidia were also present in the overwintering leaves. The subcuticular hyphal walls were brown in colour and were thicker than the walls the normal hyphae i.e. they were heavily melanized (Figure 17e). Dark brown round to oval structures, with heavily ornamented outer surface, were formed from the dark thick-walled subcuticular mycelial and the fungal mycelia growing in the epidermal cells. These structures seemed empty in October and had very thick walls which were difficult to crush (Figure 18a).

Observation of the leaf samples collected at the end of December 2003 from those leaves that had been stored outside in September, showed that the heavily melanized subcuticular hyphae had cut off two celled conidia-like structures (Figure 17f). When compared to the normal conidia these two-celled conidia like structure were shorter (average length 9.69 μm), double walled, and heavily melanized. In these leaves the round brown-black coloured heavily ornamented structures were also observed, but by this time a small opening could be seen at one end of these structures. Small oval shaped cells could be seen inside these round heavily ornamented structures (Figure 18d).

Semi-thin sections made from sample picked in April 2004 showed that the plant tissue had heavily degenerated, and in the epidermal cells were found many of the round heavily ornamented structures. These structures were filled with small cells (Figure 18b). In a few of the leaf sections made from the leaves incubated on soil trays from September 2003 to April 2004, subepidermal apothecia-like structures were observed but the internal structures were not mature (Figure 18c).

3.3.7.2 On the canes

Canes picked on the 20th of February 2004 from the garden belonging to the Institute for Plant Disease at the University of Bonn were found to have conidia of *Diplocarpon rosae* on their bark. On the bark of the canes picked in March 2004 were observed germinated conidia, subcuticular hyphae (Figure 18e), haustoria in the cells and opened acervuli from which conidia oozed (Figure 18f). The rose plants in the garden started to produce new leaves in mid February 2004 and those young leaves found on the plants in March were still symptom free. The first disease symptoms in the year appear at the beginning of

May. Any diseased plant material found on the plants March and February was what had over wintered on the plants from the previous year autumn infections.

3.4 Investigations of the effect of nutrient source on the fungal development.

3.4.1 Development of *D. rosae* on artificial growth media

D. rosae conidia germinated from the 9th hour on all the artificial growth media used. **Water Agar**: The fungal colonies were not visible to the naked eye even after 3 weeks of growth. Very few hyphae were formed and conidia formation started on the 8th day of growth (Figure 19a).

Potato dextrose agar: Growth on potato dextrose agar (PDA) and ½ PDA was very similar. The mass of fungal mycelia could be seen as a speck with the naked eye on from the 14th day of growth. The top and edge of the fungal colony was made up of white mycelium but the bottom of the colony and the reverse side were yellowish orange in colour. When viewed under the binocular microscope the lower mycelia at the centre of the colony were orange. The fungus formed extensive hyphal network on these growth media growth and the first conidia were identified on the 10th day of growth. However, after 3 weeks of growth the ratio of conidia to hyphae was still very low i.e. the fungus had formed very extensive mass of hyphae but very few conidia (Figure 19d).

Malt extract agar: The growth pattern of the fungus on malt extract agar and ½ MEA was very similar. The fungal mycelia could be seen with the naked eye from the 14th day of growth as small white points scattered on the surface of the growth medium. The top and the bottom of the fungal colony were white. A closer look with the binocular microscope revealed that some of the colonies had a beige wet appearing mat at the top.

The beige mat was a mass of conidia. There was little hyphal network and the conidia were formed from the mycelia at the centre of the colony. The conidia held together in loosely packed mat. Conidia formation started on the 8th day of growth on the fungal colonies with very short hyphae. Few conidia and hyphae were formed (Figure 19b).

Biomalt: The fungus showed a very similar growth patterns on biomalt and ½ biomalt agar. The fungal colony could be seen with the naked eye as whitish points scattered on the growth media from the 14th day of growth, but when the colonies grew together the top mycelia was white and the reverse side appeared yellowish orange. The old mycelia and the dead conidia appeared orange in colour. The fungus formed extensive hyphal network and equally many conidia. Conidia formation started on the 7th day on the short hyphae. An abundant mass of conidia was formed and the ratio of conidia to hyphae was relatively high. Conidia were formed even on two adjacent hyphal cells (Figure 19c).

After 4 weeks of growth all of the colonies were observed to be pale brown in colour on all the growth media. The inoculum obtained on these artificial growth media could be rapidly multiplied on the artificial growth media but it had an infection rate of 10 % (± 5) on rose leaves.

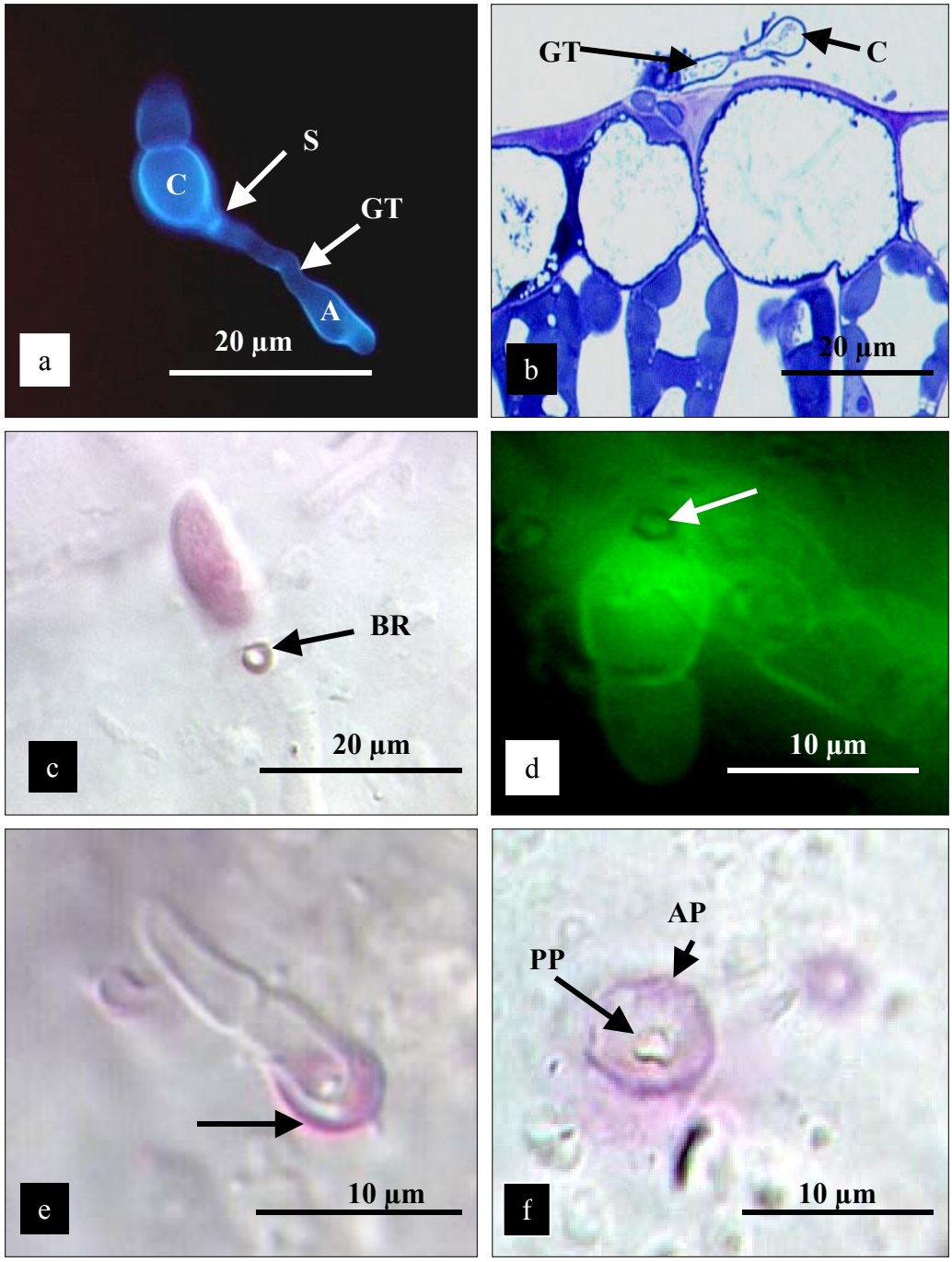

Figure 8: Light microscope photographs of *Diplocarpon rosae* infecting rose leaves showing, (a) a germinating conidium (C), a germ tube (GT), a septum (S) and an appressorium (A), (b) a conidium (C) and a short germ tube (GT) penetrating the host without forming an appressorium, (c) a brown ring (BR) formed at the point of penetration, (d) a brown ring (arrow) formed at the point of penetration where an extremely short germ tube is formed, (e) a very short germ tube formed directly below the conidium note the reinforced walls of the appressorium (arrow), (f) a penetration site showing print of an appressorium (AP) and a penetration pore (PP).

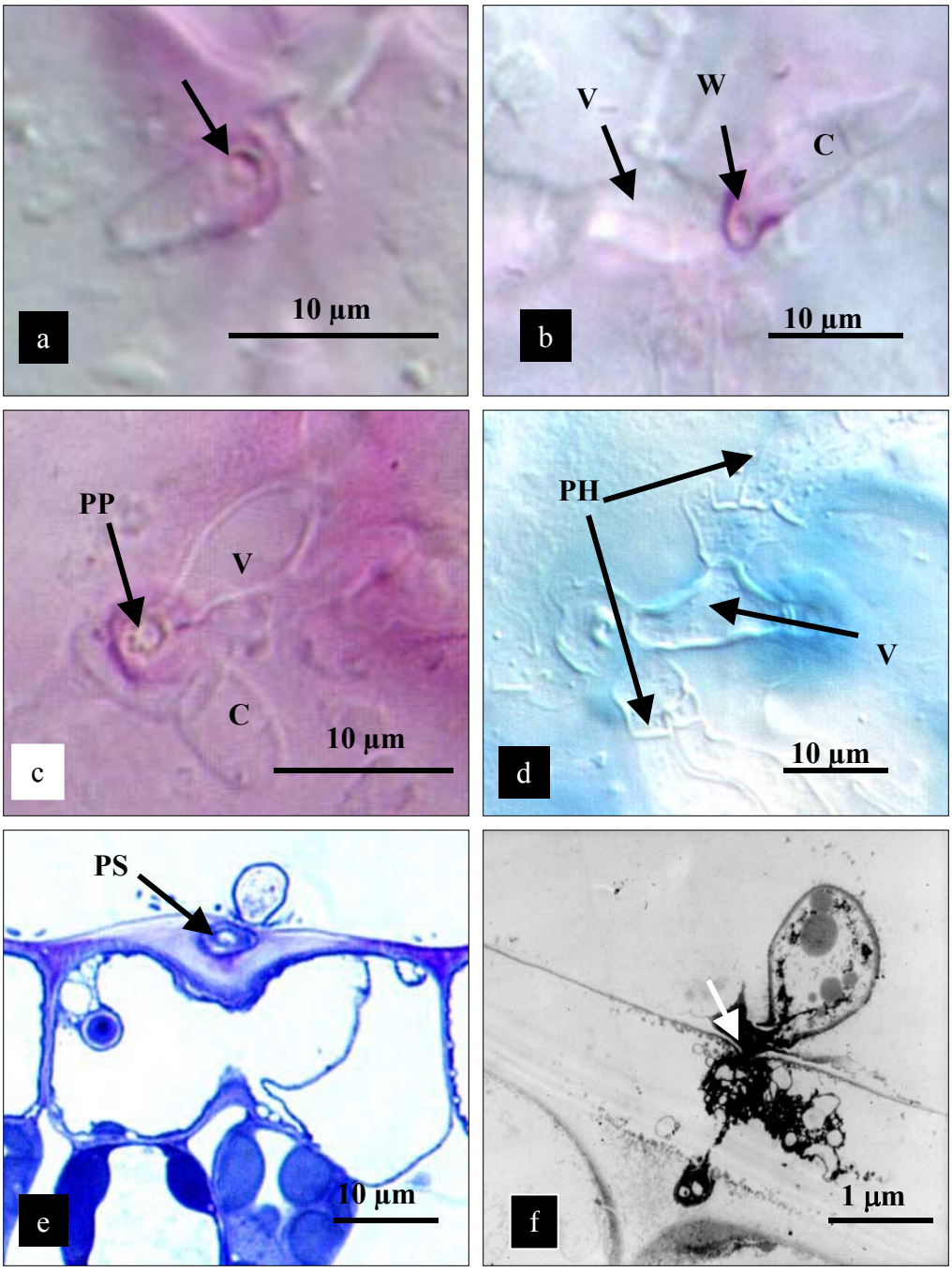

Figure 9: Light microscope photographs and an electron microscope micrograph (f) of *Diplocarpon rosae* infecting rose leaves showing, (a) a penetration point (arrow) just below the conidium, (b) a conidium (C) and a direct penetration of the host without a well defined appressorium, the walls of the short germ tube and penetration pore (W) are reinforced and stain with fuchsin acid, a vesicle is (V) formed upon penetration, (c) a penetration site below a conidium, with a penetration pore (PP), infection vesicle (V), (d) an infection vesicle (V) and primary hyphae (PH) formed from it, (e) a penetration site with the penetrating hypha (PS) in the periclinal cell wall, note the change in the host cell wall thickness and its staining properties, (f) a penetration site with cuticle dissolution (arrow) and cell wall disintegration around the penetration hypha.

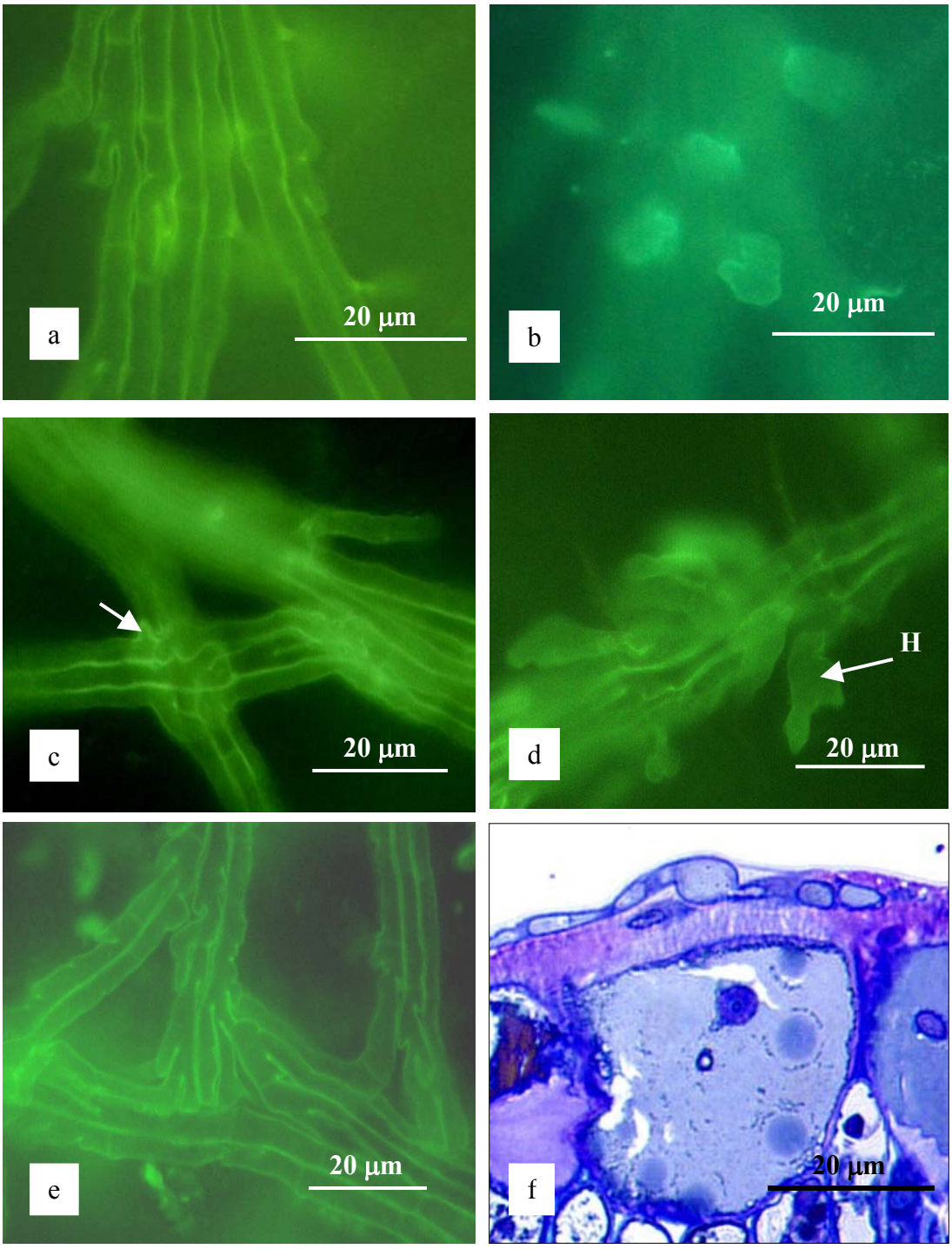

Figure 10: Light microscope photographs of *Diplocarpon rosae* in rose leaves showing , (a) subcuticular hyphae growing in a fascicle of parallel hyphae, which branch independently at acute angles, (b) enlarged cells formed just before penetration of the epidermal cells below the subcuticular hyphae in (a), (c) two fascicles crossing each other by one of them dipping temporarily below the other (arrow), (d) a hypha (H) formed by the subcuticular hyphae into the periclinal wall and enlarges just before penetrating the epidermal cell wall, (e) two fascicles crossing each other by one separating its constituting hyphae to join the other and the two form one big fascicle, (f) a subcuticular hypha invading the periclinal cell wall, the terminal cell expands just outside the cell wall.

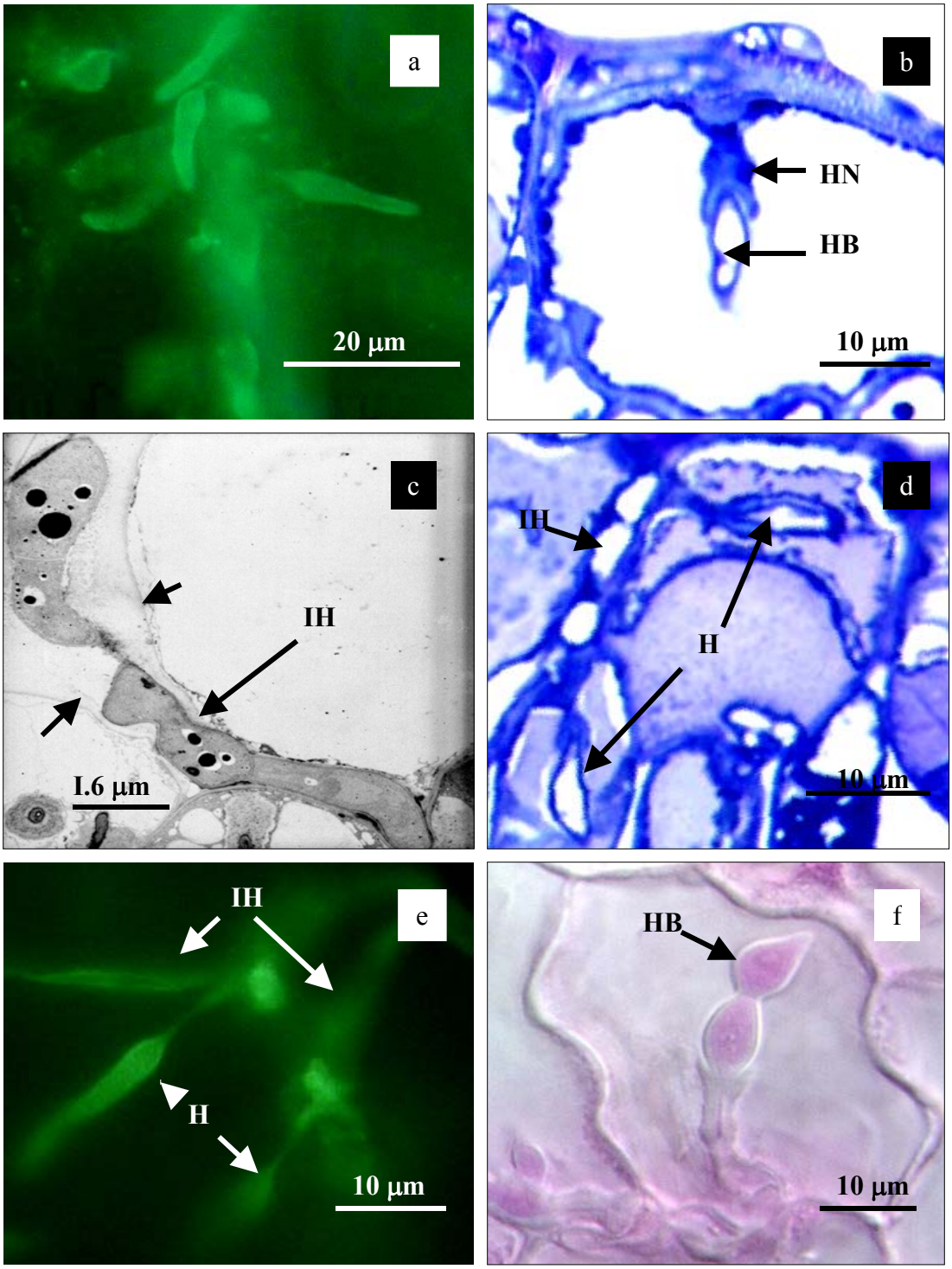

Figure 11: Light microscope photographs and an electron microscope micrograph (h) of *Diplocarpon rosae* in rose leaves showing, (a) haustoria formed from a fascicle of subcuticular hyphae in the epidermal cell below, (b) a haustorium formed by the subcuticular hyphae in the epidermal cells below it, haustorium body (HB), haustorium neck (HN) cover with a callose collar, (c) an intercellular hypha (IH) growing between two epidermal cell, note the particial dissolution of the cell wall (arrows), (d) haustoria (H) formed in an epidermal and a mesophyll cell from the intercellular hyphae (IH), (e) intercellular hyphae, (IH), that have formed haustoria, (H), in epidermal cells, (f) a haustorium formed in an epidermal cell from the intercellular hyphae, note the shape of the haustorium body (HB).

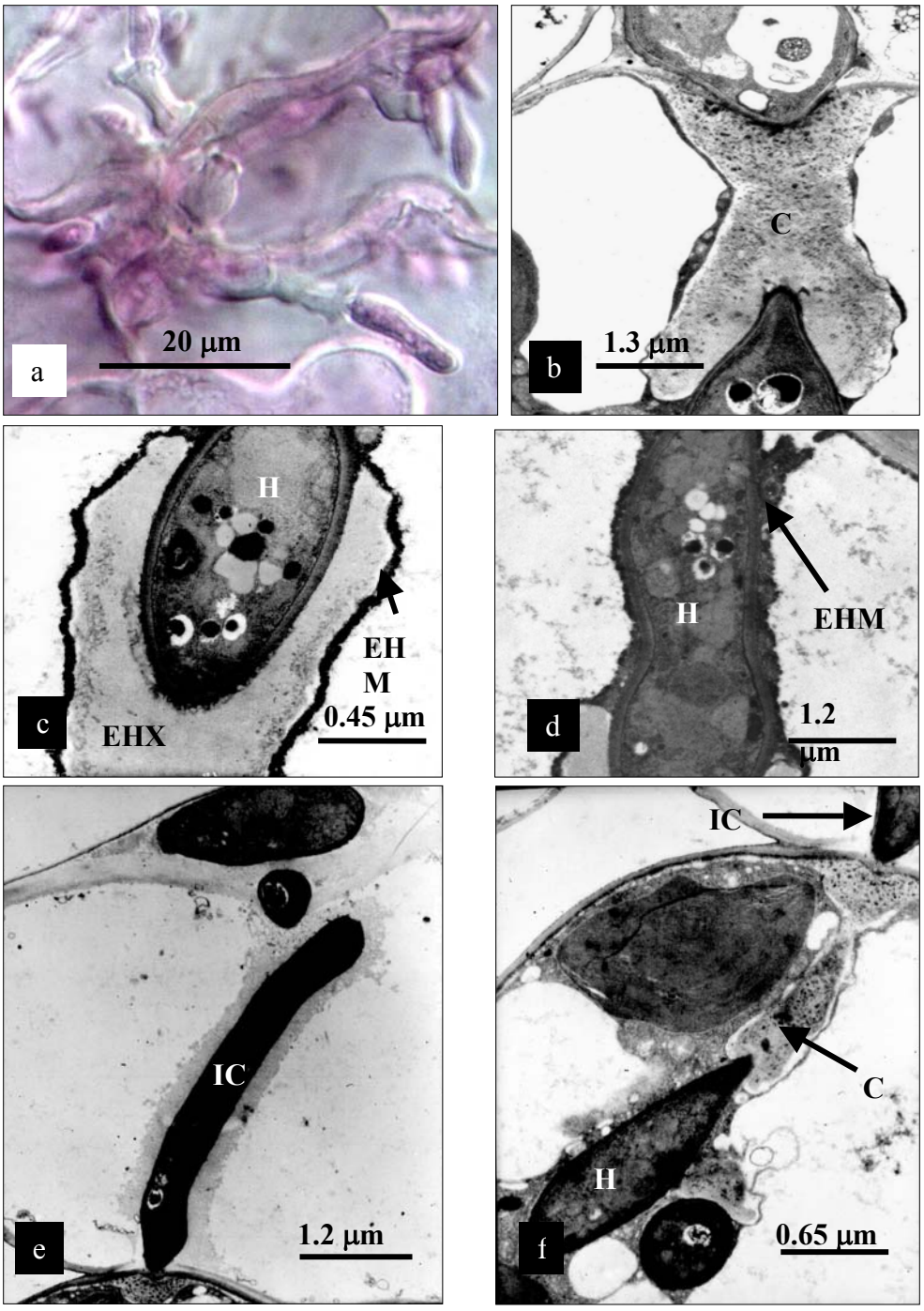

Figure 12: A light microscope photographs (a) and electron microscope micrographs (b, c, d, e, f,) of *Diplocarpon rosae* growing inside roses leaves showing, (a) haustoria of different shapes and sizes formed in epidermal cells by hyphae growing in different parts of the host, (b) the neck of a haustorium enclosed by a callose material (C) and the extrahaustorial membrane (EHM) lies close to the callose plug with no EHX being formed in this part of the haustorium, (c) a haustorium body (H) surrounded by an extrahaustorial matrix (EHX) that is enclosed in EHM, (d) EHM lies close to the proximal end of the haustorium (H), (e) an intracellullar hyphae growing through an epidermal cell, (f) a haustorium formed in a mesophyll cell by the intracellular hyphae in (e), note the haustorium neck in enclosed by a callose plug (C).

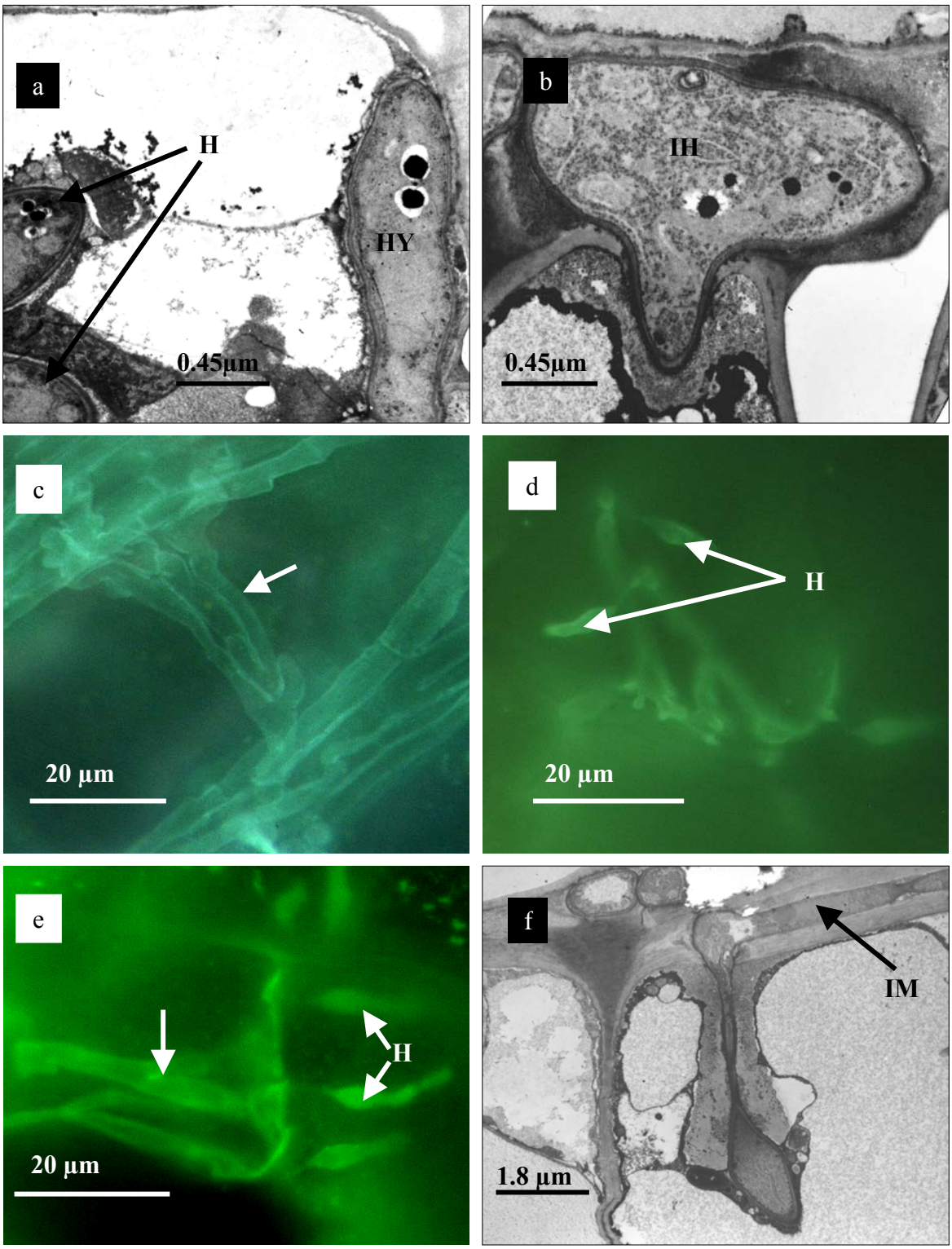

Figure 13: Light microscope photographs (c, d, e) and electron microscope micrographs (a, b, f) of *Diplocarpon rosae* growing inside roses leaves showing, (a) a hypha (HY) growing inside the epidermal cell wall but exterior to the plasma membrane invades the underlying mesophyll cell, the lower sections of the bodies of two haustoria (H) in the same cell can be seen, (b) an intercellular hypha, (IH), invades a mesophyll, (c) intramural hyphae (arrow) formed from the subcuticular hyphae, (d) intramural hyphae form haustoria in the underlying epidermal cells, (e) intramural hyphae form haustoria in an epidermal cell upon coming in contact with the anticlinal wall, (f) an intramural hypha, (IM), swells before forming a haustorium, (H), in the epidermal cell below, cell wall dissolution is observed at the point of invasion.

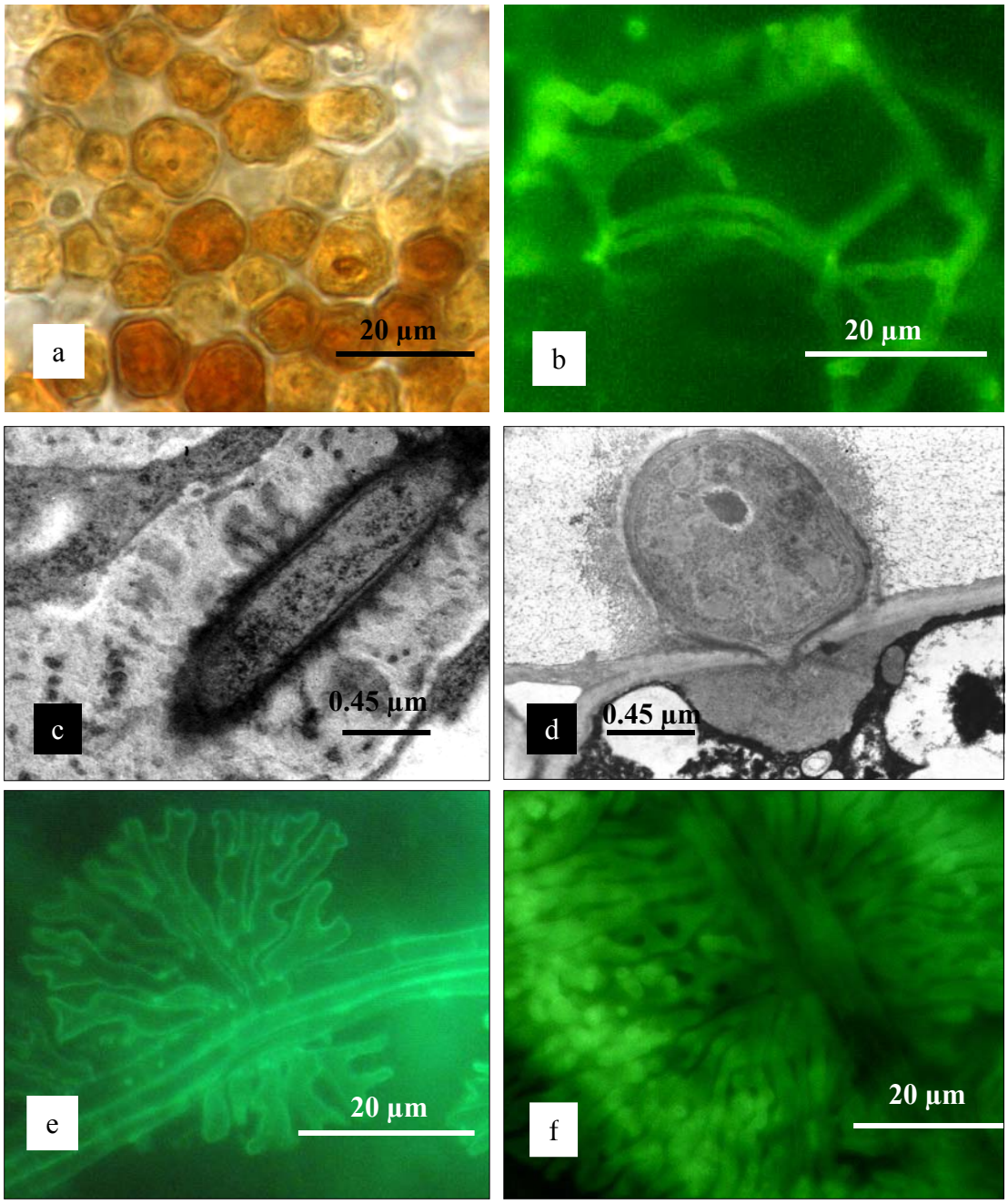

Figure 14: Light microscope photographs (a, b, e, f) and electron microscope micrographs (c, d) of *Diplocarpon rosae* in rose leaves showing, (a) browning of some mesophyll cells upon invasion of the mesophyll layer by the fungus, (b) intracellular hyphae in an epidermal cell, (c) one of the initial intracellular hyphae in the epidermal cells enclosed in a callose plug, (d) an intracellular hypha in an epidermal cell penetrates the double cell wall to invade the underlying mesophyll cell. Note the electron opaque layer around the intracellular hyphae and the cleanly severed host cell wall below the penetrating hypha, (e) fan-shaped finger-like projections growing from the subcuticular hyphae to form the base of the acervuli, (f) the acervuli base stroma formed a close-knight mat on both sides of the subcuticular hyphae.

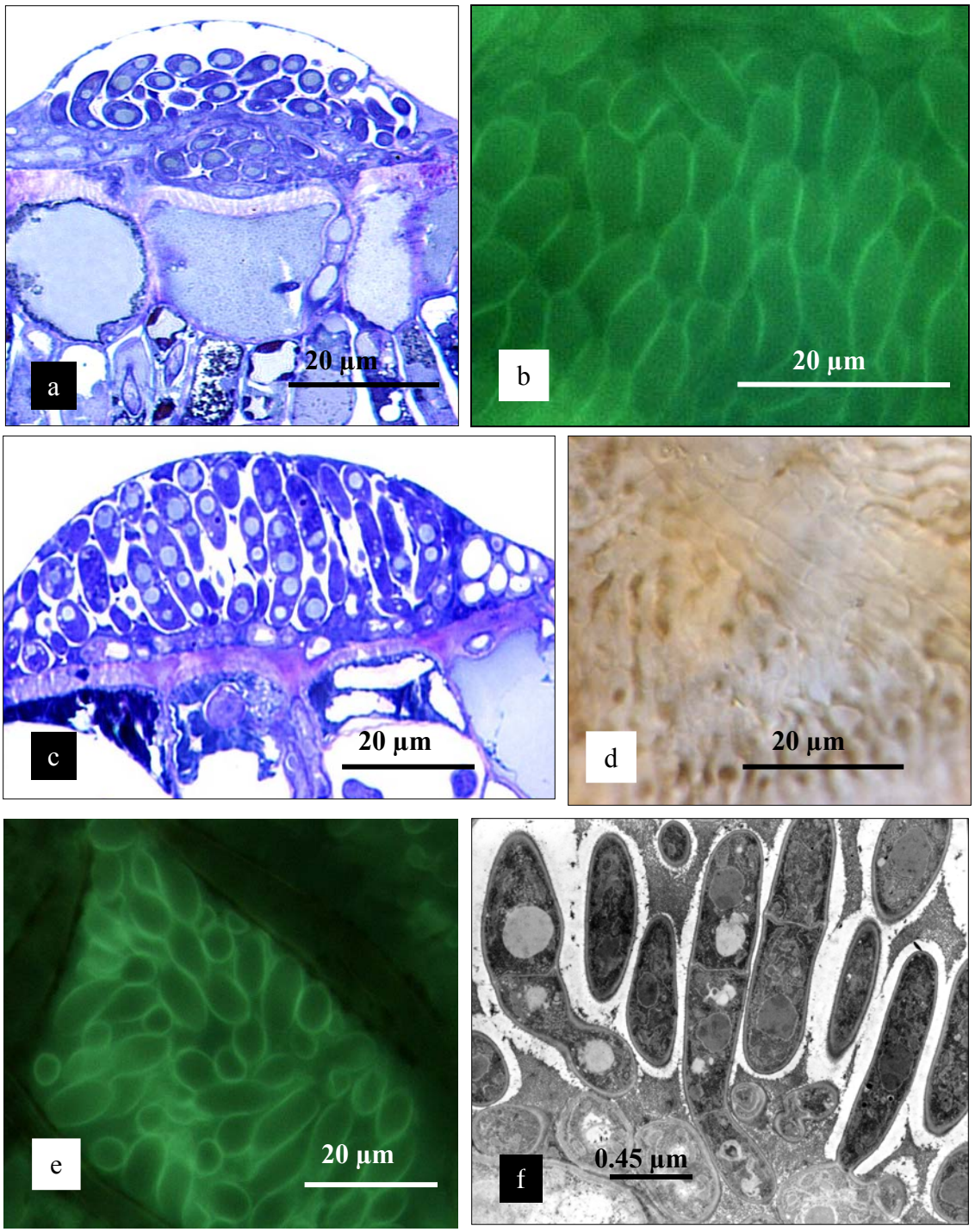

Figure 15: Light microscope photographs and an electron microscope micrograph (f) of *Diplocarpon rosae* in rose leaves showing, (a) a developing acervulus with the cuticle above it slightly pushed upwards, (b) conidia in an acevulus, (c) a mature acervulus with both double and single celled conidia, round structures can be seen in each of the conidium cells, (d) browning of an acervulus, (e) a mature acervulus that has opened from the top, (f) mature conidia with a septum dividing the conidium cells, single celled conidia arising from a different conidiophore

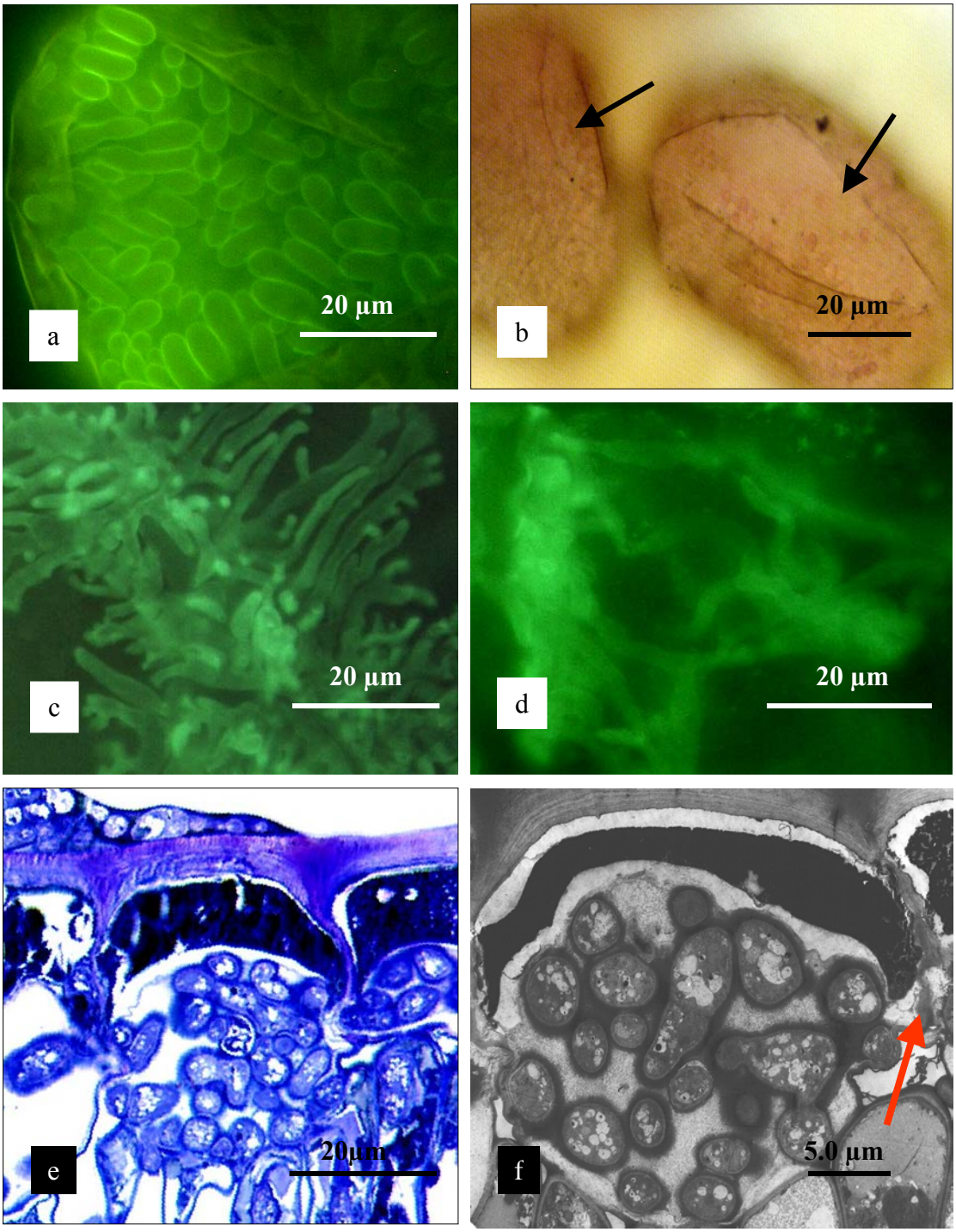

Figure 16: Light microscope photographs and an electron microscope micrograph (f) of *Diplocarpon rosae* in rose leaves showing, (a) a mature acervulus that has opened from the top, (b) two empty acervuli that opened at different points (arrows), (c) an empty acervulus with the base stroma continuing to increase in size, (d) sub-epidermal intracellular mycelia formed at old infection sites, (e) intracellular hyphae in an epidermal cell, (f) intracellular hyphae in an epidermal cell with slight dissolution of the cell wall (arrow).

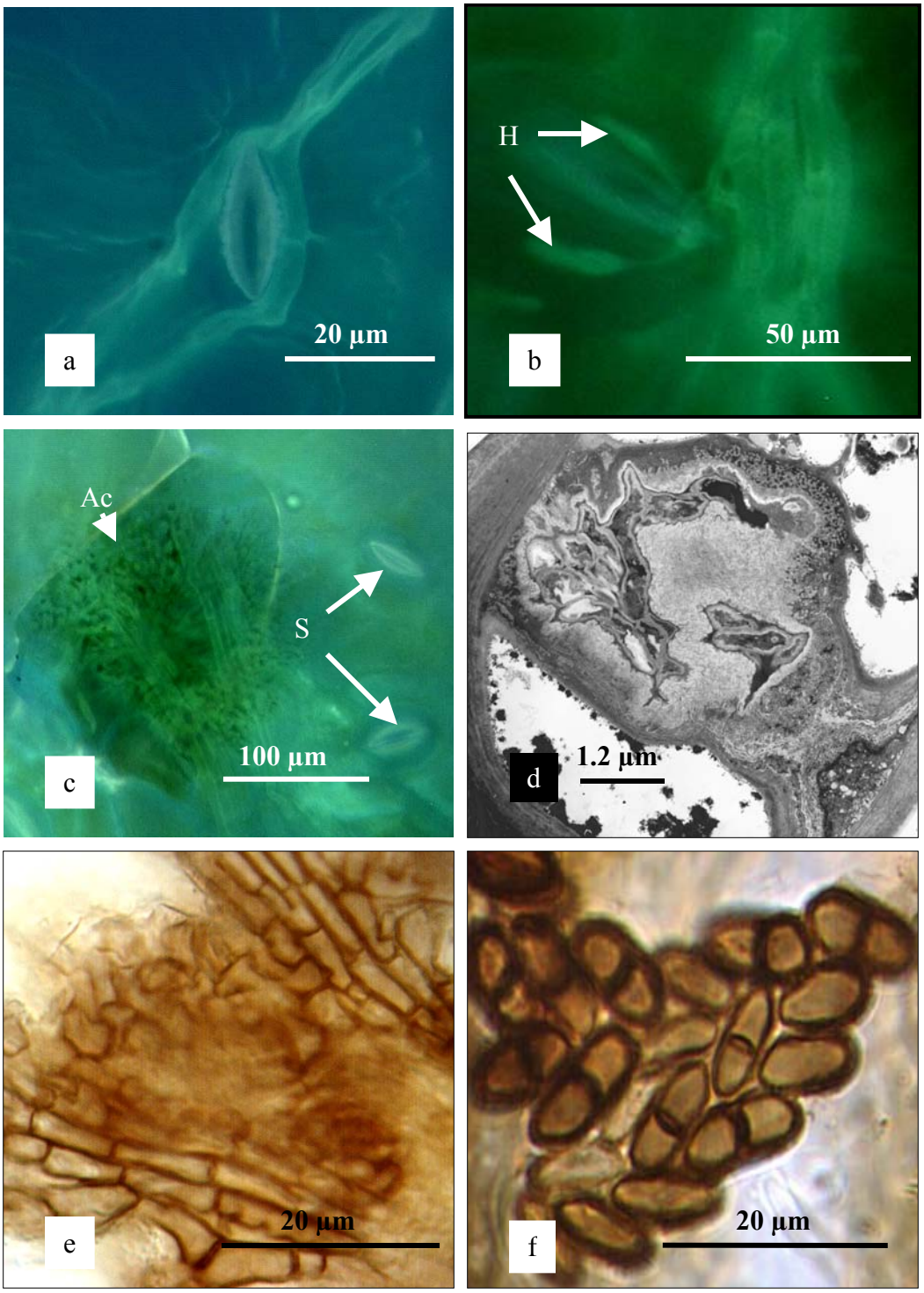

Figure 17: Light microscope photographs and an electron microscope micrograph (d) of *Diplocarpon rosae* in rose leaves showing, (a) subcuticular mycelia growing around a stomata on the lower leaf surface, (b) haustoria (H) in stomatal guard cells, (c) an open acervulus (Ac) near stoma (S) on the lower side of a leaf, (d) callose plug enclosing fungal hyphe in the lower epidermal cell, (e) browned subcuticular mycelia on leaves collected in September 2003 and kept on a tray in the garden until December 2003, (f) conidia–like structures cut from the browned mycelia on the overwintering leaves kept in the garden from September till December 2003.

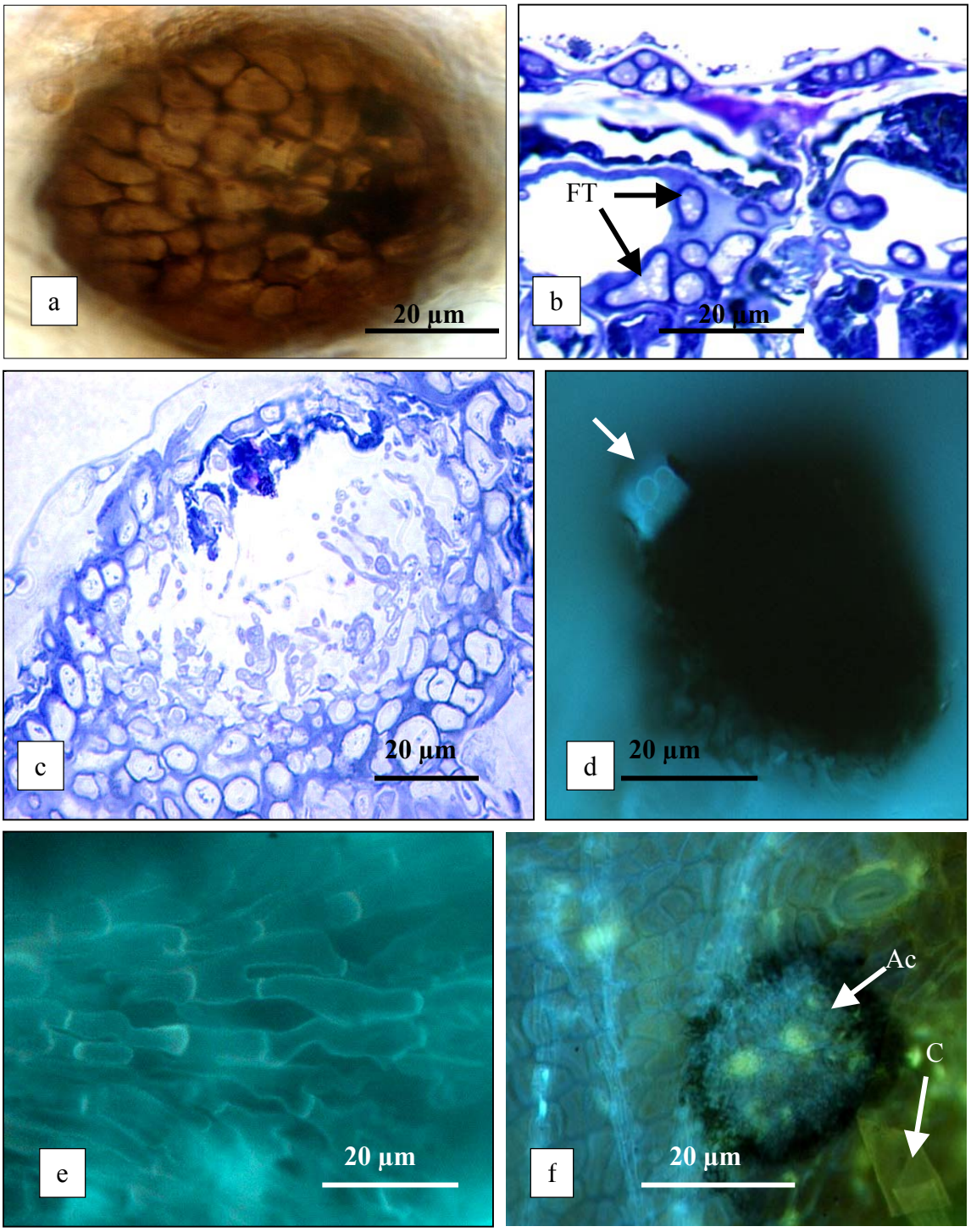

Figure 18: Light microscope photographs of *Diplocarpon rosae* infecting rose leaves showing, (a) a heavily ornamented structure found in overwintered leaves kept in garden from September 2003 until April 2004, (b) fungal structures, FT, observed in rose leaves that were collected in September 2003 and let to stand on a soil tray until April 2004, (c) a sub-epidermal structure formed in leaves that had overwintered in the garden from September 2003 to March 2004, (d) the heavily ornamented structure with an opening at one end (arrow), (e) parallel hyphae formed under the cuticle of the rose stem, (f) the cuticle (C) peels back to exposed an acervulus (Ac) formed on the stem.

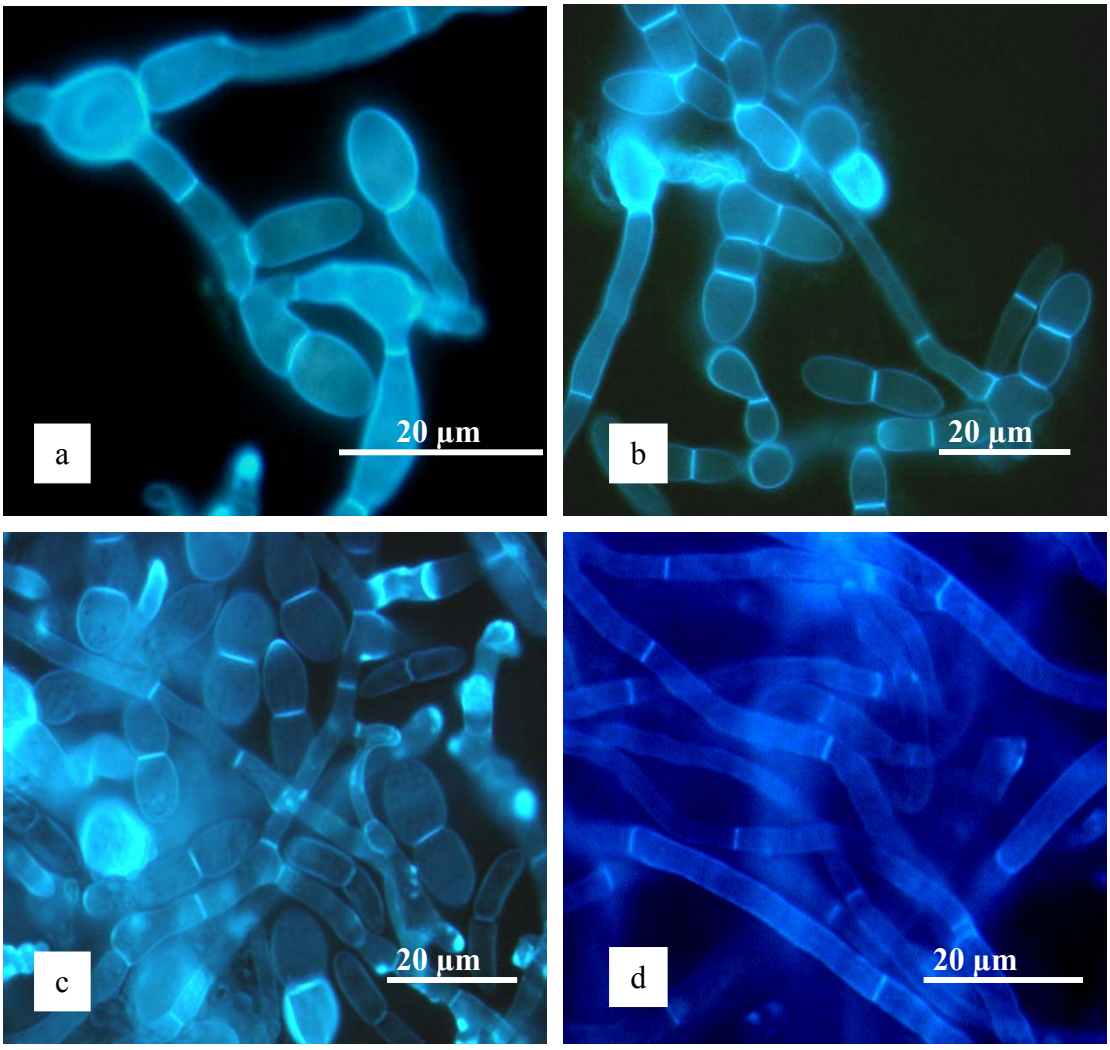

Figure 19: Light microscope photographs of *Diplocarpon rosae* growing on artificial media: (a) 10 days old fungal growth on water agar, (b) 10 days old fungal growth on malt extract agar (c) 10 days old fungal growth on biomalt agar (d) 10 days old fungal growth on potato dextrose agar.

3.4.2 Fungus development in detached leaves and intact plants

The detached leaf technique has been recommended in the study of the blackspot disease because similar symptoms are formed on both the detached leaves and the whole plants. But the differences likely to occur in disease development when the two techniques of study are used have not been looked into. On detached leaves the first disease symptoms were observed between the 4^{th} to the 5^{th} dpi and on the intact plants the symptoms first appeared between the 7^{th} and the 10^{th} day. Microscopic observations revealed that subcuticular hyphae, intercellular hyphae, intracellular hyphae, haustoria were formed in infected intact plants and as well as on the detached leaves. In the detached leaves the first intracellular hyphae were observed on the 4^{th} dpi and the first acervuli formed from the 5^{th} dpi. On the intact plants the intracellular hyphae were formed from the 7^{th} dpi. The base of the acervulus was formed from the 7^{th} dpi but the first acervulus appeared on average from the 10^{th} dpi (Table 4).

Table 4: The development of *Diplocarpon rosae* in the leaves of intact rose plants and in detached rose leaves.

	days post inoculation (dpi) to the formation of			
plant part infected	symptom	intracellular hyphae	acervuli base	acervuli
detached leaves	3	4	4	5
intact plants	7	7	8	10

3.5 Investigations into the effectiveness of fungicides on the development of *D. rosae*

3.5.1 Effect on symptom development

The effectiveness of four fungicides' active ingredients, trifloxystrobin, pyraclostrobin, myclobutanil and tebuconazole, on disease symptom development was investigated. The disease symptom development if any, on detached leaves treated with the fungicides before inoculation and on plants treated with fungicides at different times post inoculation was compared with the untreated control plants. The disease severity based on the size of the black spots, yellowing or lack of it, and premature defoliation were used to assess the degree of disease severity. The disease symptom development was assessed on the 20^{th} dpi.

Untreated

The untreated control plants were taken to represent 100 % of disease severity and they were used as a reference point in the assessment of the effect of the fungicide active ingredients on disease symptom development. These untreated control plants showed symptoms of black spots with a diameter of more than 10 mm. The leaf area around the black spots remained green but the rest of the leaf surface turned yellow, leading the "green island formation". There was premature defoliation.

Trifloxystrobin

On detached leaves treated with trifloxystrobin on the same day they were inoculated with the conidial suspension and on those treated one week before inoculation, no disease symptoms developed. On plants treated with the fungicide at different intervals post inoculation, the observed symptoms were similar to those observed on the untreated

control plants. The black spots observed on the leaves had an average diameter of more than 10 mm, yellowing of the leaves was observed, formation of "green islands" and premature defoliation were also observed. The symptoms observed on the 20th dpi on plants treated with the fungicides on the 3rd, 7th and 10th dpi were not noticeably different from each other (Figure 20 and 24).

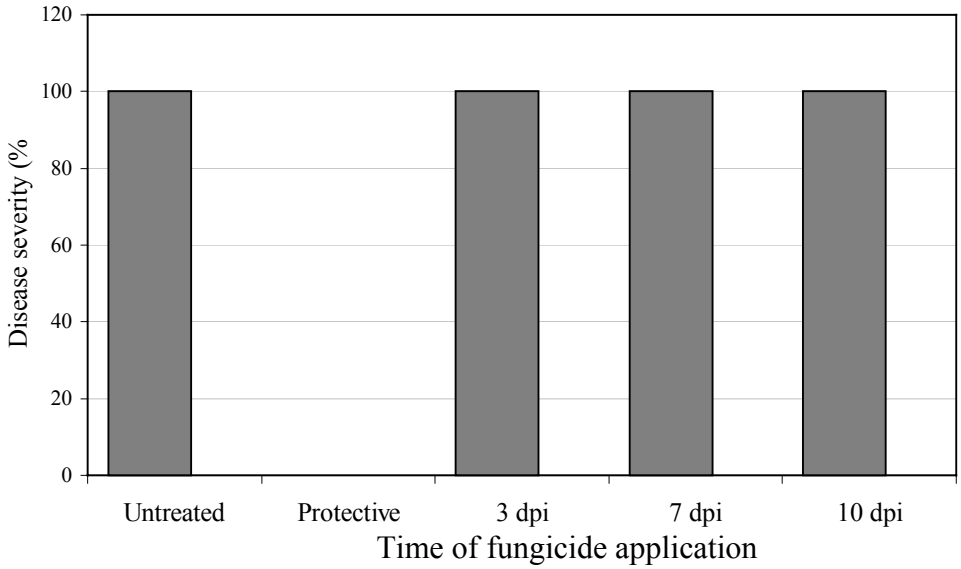

Figure 20: The effectiveness of 100 ppm trifloxystrobin on symptom development when applied both protectively and curatively on rose leaves inoculated with *Diplocarpon rosae,* (the data represent the mean value of 3 replicates, n = 4, P<0.05, Duncan's multiple range test).

Pyraclostrobin

On the detached leaves treated with pyraclostrobin on the same day they were inoculated with the conidial suspension and one week before inoculation, no disease symptoms developed. However, on plants treated with the fungicide at different times post inoculation, there were differences in the degree of disease severity observed. On plants treated with pyraclostrobin 3 dpi the black spots formed were between 5 and 10 mm in

diameter, there was no yellowing, no green island formation and no premature defoliation observed. The disease severity was rated at an average of 36.7 %. On plants treated with pyraclostrobin 7 dpi black spots had with an average diameter of 10 mm, there was no yellowing, but there was premature defoliation and the average disease severity was rated at 70 %. On plants treated with fungicides 10 dpi, the black spots were more than 10 mm in diameter and there was yellowing, "green island" formation and premature defoliation. The average disease severity was rated at 83.3 % (Figure 21 and 24).

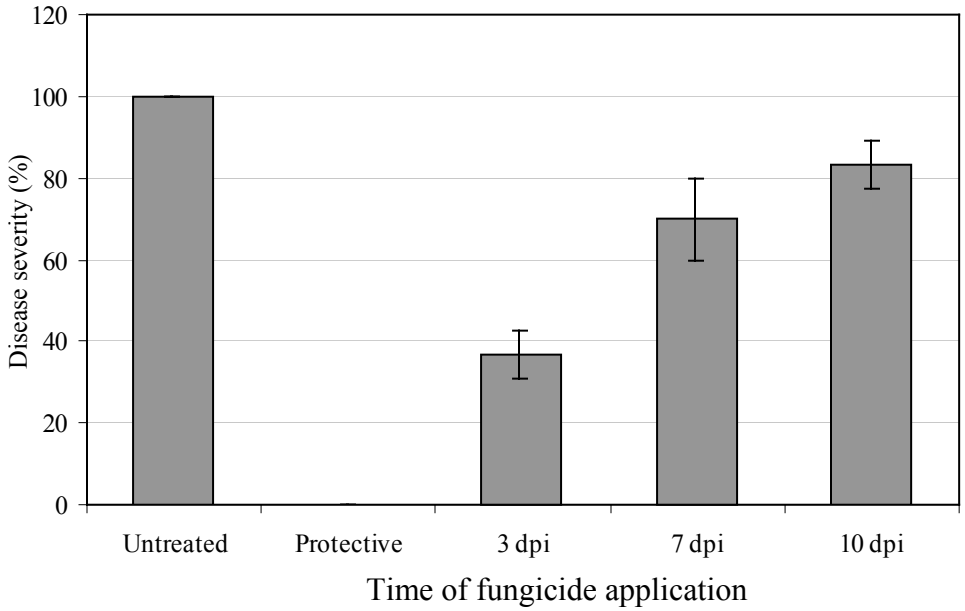

Figure 21: The effectiveness of 100 ppm pyraclostrobin on symptom development when applied both protectively and curatively on rose leaves inoculated with *Diplocarpon rosae,* (the data represent the mean ± SD of 3 replicates, n = 4, P< 0.05, Duncan's multiple range test).

Myclobutanil

On detached leaves treated protectively with myclobutanil no disease symptoms developed. On plants treated with myclobutanil at different intervals post inoculation were the disease symptoms different. On plants treated with the fungicide 3 dpi no

disease symptoms developed but some small yellow spots were observed on the leaves. On plants treated with myclobutanil 7 dpi, the spots formed were less then 5 mm in diameter and with very small halos formed around them. There was no yellowing and no premature defoliation observed. The disease severity was rated at an average of 6.7 %. On plants treated with the fungicide 10 dpi, the black spots formed had an average diameter of 5 mm and they were accompanied by "green island" formation, yellowing and premature defoliation. The disease severity was rated at an average of 30 % (Figure 22 and 24). The applications of myclobutanil left heavy chemical residues on the leaves.

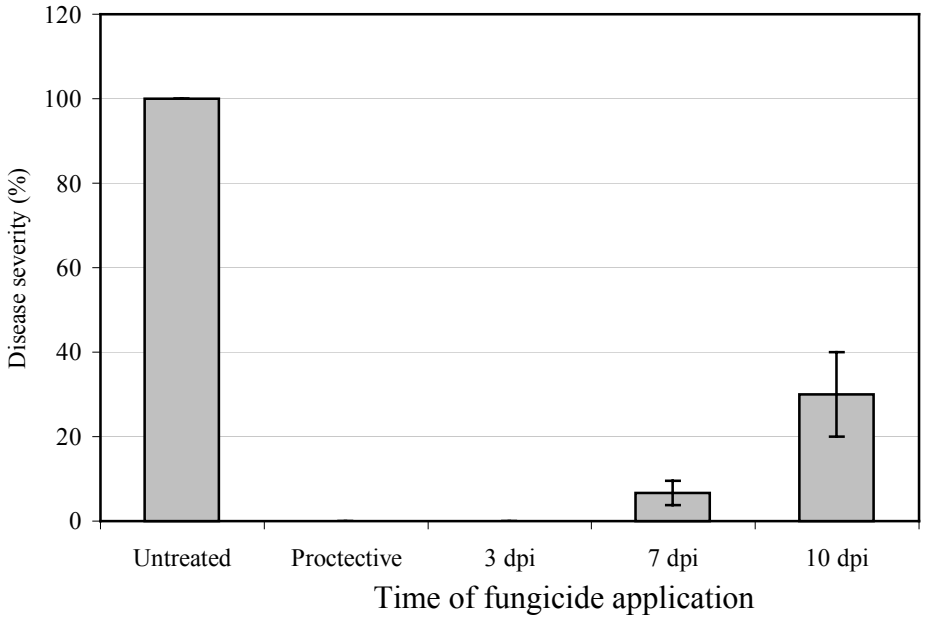

Figure 22: The effectiveness of 100 ppm myclobutanil on symptom development when applied both protectively and curatively on rose leaves inoculated with *Diplocarpon rosae,* (the data represent the mean ± SD of 3 replicates, n = 4 and $P < 0.05$, Duncan's multiple range test).

Tebuconazole

On detached leaves treated protectively with tebuconazole no disease symptoms developed. On plants treated with tebuconazole 3 dpi, no disease symptom developed but yellow specks were observed on the leaves. On those plants treated with tebuconazole 7 dpi flecks similar to those observed in hypersensitive reactions developed but no typical black spot symptoms developed; no defoliation and the typical yellowing was also absent. The disease severity on plants treated on the 3rd and 7th dpi was rate at 0 %. On plants treated with the fungicide 10 dpi the typical black spot symptoms developed and the spots had an average diameter of 5 mm, with no yellowing, no 'green island' formation and no premature defoliation. The disease severity was rated at an average of 10 % (Figure 23 and 24).

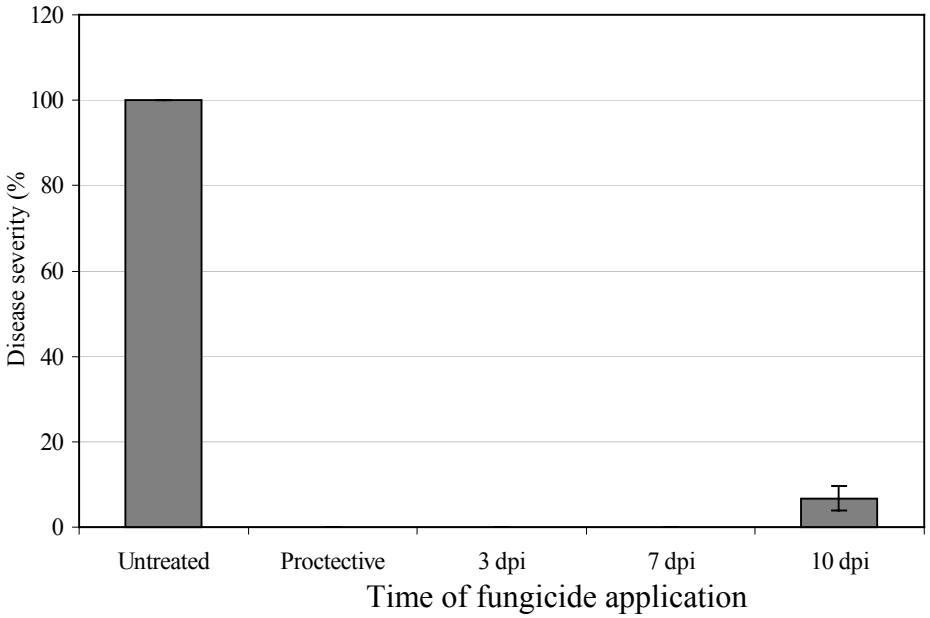

Figure 23: The effectiveness of 100 ppm tebuconazole on symptom development when applied both protectively and curatively on rose leaves inoculated with *Diplocarpon rosae,* (the data represent the mean ± SD of 3 replicates, n = 4 and $P < 0.05$, Duncan's multiple range).

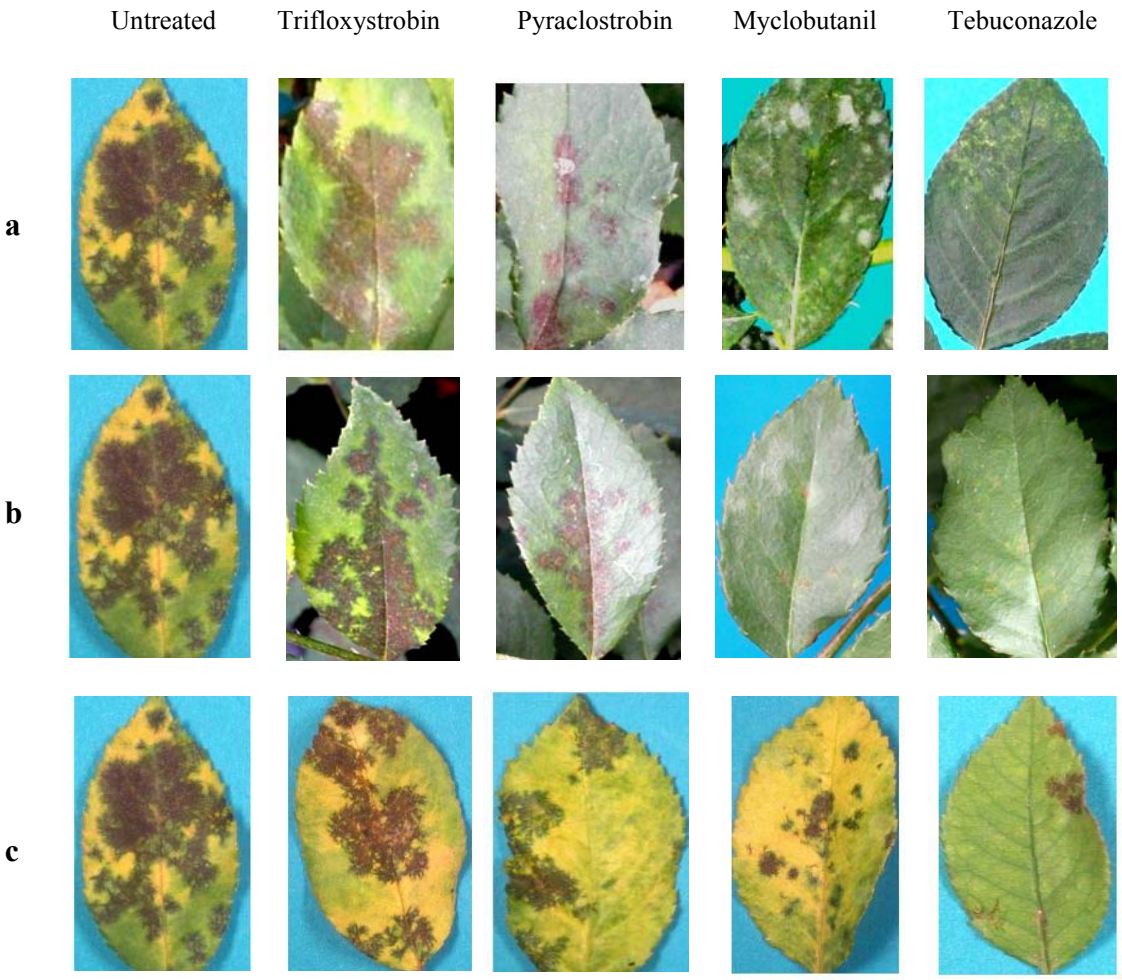

Figure 24: Photographs of rose leaves taken from plants inoculated with *Diplocarpon rosae* and treated with different fungicides at different intervals post inoculation. (a), (b), and (c) mark rows of leaves taken from plants treated with 100 ppm of fungicides active ingredients 3, 7 and 10 dpi respectively. Trifloxystrobin, Pyraclostrobin, Myclobutanil and Tebuconazole mark columns of leaves taken from plants treated with the fungicide active ingredients, trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, while untreated marks the for untreated control plants. All photographs were taken on the 20th dpi.

3.5.2 Effect on pre-infection structures

The effect of the fungicide active ingredients (trifloxystrobin, pyraclostrobin, myclobutanil, and tebuconazole) on the germination and the formation of subsequent pre-infection structures on artificial growth media and on the detached leaves were investigated.

3.5.2.1 *In vitro* analysis.

Observations made on different water agar blocks stained with diethanol revealed that the conidial did not germinate on water agar containing 10 ppm of the active ingredient of any of the fungicides tested. The germination on the untreated control was 82.1 %.

3.5.2.2 *In vivo* analysis

i) Analysis on leaves treated with fungicides on the same day they were inoculated

The conidia germination percentage on leaves treated with fungicides on the same day that they were inoculated was determined on the 3rd dpi. The germination rate was 14.7 %, 10.7 %, 0.0 %, 0.0 %, and 80 % for tebuconazole, myclobutanil, pyraclostrobin, trifloxystrobin and untreated control respectively. The conidia did not germinate on leaflets treated with trifloxystrobin and pyraclostrobin. The percentage germination on the untreated leaflets was significantly different from that on the leaflets treated with fungicides. The germination percentage on the leaflets treated with trifloxystrobin and pyraclostrobin was not significantly different but was significantly different from that on the leaflets treated with myclobutanil and tebuconazole ($p< 0.05$) (Figure 25).

ii) Analysis on leaves treated one week before inoculation

The percent germination rate on the 3rd dpi was 9.34 %, 6.7 %, 0.0 %, 0 %, and 80 % for tebuconazole, myclobutanil, pyraclostrobin, trifloxystrobin and the untreated control respectively (Figure 25). The germination percentage on the untreated leaflets was significantly different from that on fungicides treated leaflets, but the percentage germination on the leaflets treated with the fungicides was not significantly different from each other ($P< 0.05$).

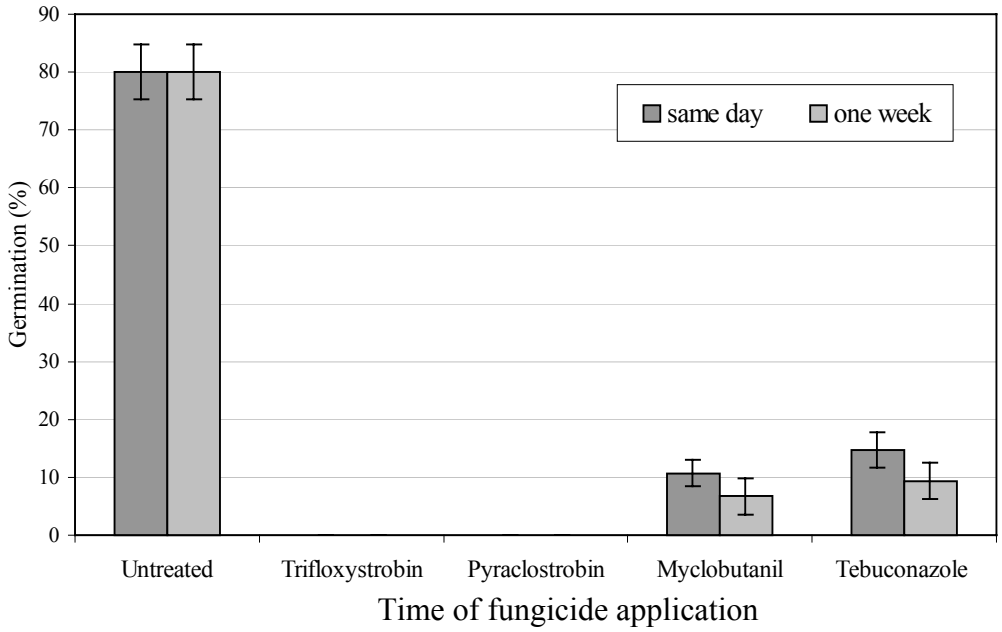

Figure 25: The germination rate (%) of *Diplocarpon rosae* conidia on rose leaves treated with 100 ppm either of the fungicide active ingredients: trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, one week before and on the same day they were inoculated, (the data represent the mean ± SD of 3 replicates, n = 4, $P< 0.05$, Duncan's multiple range test).

3.5.3 Effectiveness on the different fungal structures after curative applications

3.5.3.1 Effectiveness of application on the 3rd dpi

The fungal structures that were already formed in the host at this point of fungicide application were the subcuticular mycelia, intercellular hyphae and haustoria. Germinated conidia and their germ tubes were present on the leaf surfaces.

(i) Samples collected 7 dpi

Untreated

The subcuticular hyphae were observed and haustoria could be clearly identified on the inner side of the wall of the epidermal cells. Intercellular hyphae from which haustoria were formed could be easily identified. The cytoplasm of some of the epidermal cells at the infection site appeared crystalline. The acervulus base had started to form at few points along the length of the subcuticular hyphae. Intracellular hyphae were also observed in the some epidermal cells.

Trifloxystrobin

Subcuticular runner hyphae were reduced to mere fragments that were scattered in the subcuticular region. Haustoria were observed in some epidermal cell but no intercellular hyphae to which such haustoria are normally attached were observed. The germ tubes of several conidia joined together and a haustorium-like structure was formed directly from this common germ tube. The contents of most of the fungal structures were crystalline appearance. No intracellular hyphae were observed (Figures 26 and 27).

Pyraclostrobin

The subcuticular mycelia had been destroyed and only fragments of them were observed. Haustoria were observed in the epidermal cells but no intercellular hyphae were observed. Some of the haustoria had a crystalline appearance and fluorescent very brightly. Germinated conidia bore only stubs of the germ tubes that had been destroyed. Some conidia germinated directly to form a bulbous haustorium-like structure upon germination. No intracellular hyphae were observed (Figures 26 and 27).

Myclobutanil

Subcuticular hyphae were destroyed and only fragment of them were observed. These mycelia fragments did not fluorescent evenly i.e. parts of a single hyphal fragment in close proximity with each other fluorescent with different intensities to give alternating bands of brightly fluorescence parts and non-fluorescence parts. Geminated conidia bore only stubs where the germ tubes were previously attached. No intracellular hyphae were observed. The applications of myclobutanil left heavy chemical residues on the leaf surfaces (Figures 26 and 27).

Tebuconazole

Small fragments of the subcuticular mycelia could be seen scattered at an infection site. These fragments were torn and shrivelled up, and sometimes lacked a cell wall, such that the cell contents spilt out. There were distortion fungal structures to a point where one could no longer identify them. Germinated conidia with torn up stubs of the germ tubes were observed. Few haustoria identified in the epidermal cells but had no attaching

intercellular hyphae. Intercellular hyphae were absent from all infection sites observed (Figures 26 and 27).

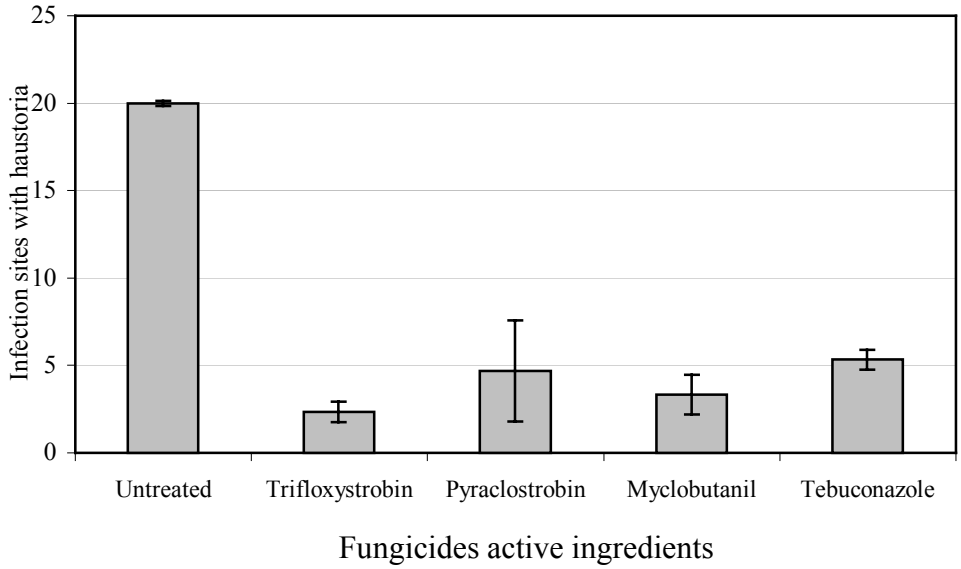

Figure 26: The number of infection sites on rose leaves that had been treated with different fungicides ingredients: trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 3rd dpi, in which haustoria were observed on the 7th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, P< 0.05, Duncan's multiple range test).

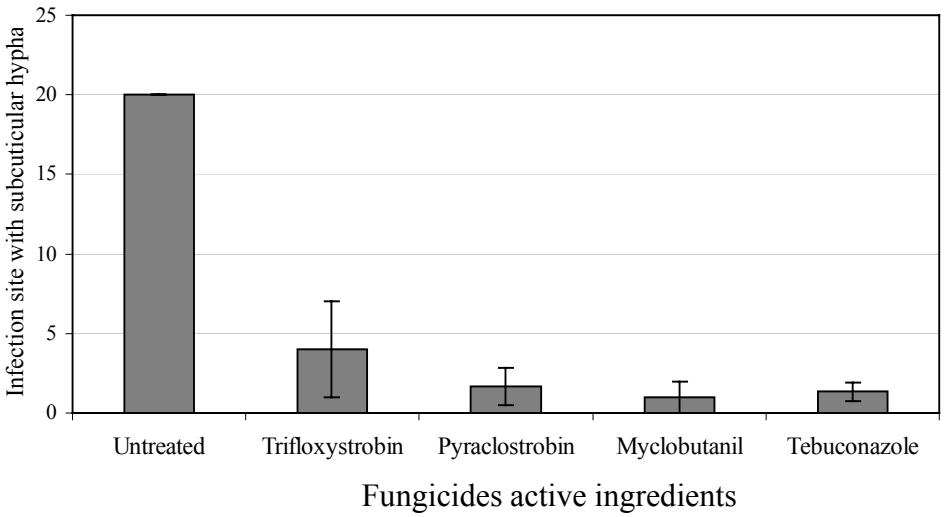

Figure 27: The number of infection sites on rose leaves that had been treated with different fungicides ingredients: trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 3rd dpi, in which subcuticular hyphae were observed on the 7th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, $P < 0.05$, Duncan's multiple range test).

(ii) Samples collected 10 dpi

Untreated

There was a network of subcuticular runner hyphae, haustoria were visible in the epidermal cells, and at least one acervulus per infection site had been formed. Intramural and intracellular hyphae were also observed. Some epidermal cells were almost fully enclosed by intercellular fungal hyphae. The cellular contents of the invaded epidermal cells fluorescent brightly. Haustoria were observed in epidermal cells. Each haustorium had a long thin neck that attached it to the intercellular hyphae.

Trifloxystrobin

In some infection sites only fragments of the subcuticular mycelia were observed but in others the fungus seemed to be growing in the normal way or just like in the untreated control plants. The subcuticular hyphae in some parts did not fluorescent. The contents of

the torn subcuticular hyphae had spilt out. The hyphal fragments bore small normal looking haustoria in the epidermal cells below them. Conidia also bore haustorium-like structures. Haustorium-like structures were also formed on short fragments of the subcuticular hyphae that were attached to the conidia. Haustoria observed in the epidermal cells were not attached to any intercellular hyphae. Where the growth of the fungus was similar to that in the untreated plants at least one acervulus was observed per infection site. Some mature acervuli were isolated with no surrounding subcuticular mycelia. No intracellular hyphae were observed (Figures 28-32).

Pyraclostrobin

Subcuticlular mycelia were thoroughly torn up into disjointed fragments. These fragments formed normal looking haustoria in the epidermal cell directly below the fragments. Some germinated conidia bore only stubs where germ tubes were previously attached. On short germ tube found attached to the conidia were haustoria-like structures formed. These haustoria-like structures were formed soon after germination of the conidia. Haustoria were also found in the epidermal cells with small traces of intercellular mycelia attached to them. In some epidermal cells, the haustoria were crystalline in appearance and fluorescent very brightly. No intracellular hyphae were observed. Acervuli were observed in some of the infection sites (Figures 28-32).

Myclobutanil

Fragments of the torn up subcuticular hyphae with broken cell walls and crystalline like cell contents spilling out of them were observed. Haustoria in the epidermal cells were not connected to any intercellular hyphae. Some of the haustoria parts had broken cell

walls such their contents spilt out or only disjointed parts of a haustorium were observed. The contents of the haustoria appeared crystalline and fluorescent brighter than the surrounding. No intercellular or intracellular hyphae were observed. No acervulus or acervulus base were observed. The applications of myclobutanil left heavy chemical residues (Figures 28-32).

Tebuconazole

The subcuticular mycelia were almost completely destroyed except for a few small fragments that were found scattered at the infection site. These mycelia fragment were also deformed to the extent that it was difficult to recognise them, therefore no photographs of the torn subcuticular hyphae were made. Some of the germinated conidia bore deformed structures that were difficult to identify. The haustoria found were fewer than in infection sites on untreated leaves. Haustoria-like structures were formed directly from the conidia. A very short hypha sometimes connected the conidium to a haustorium. The wall of this short hypha was destroyed and its contents spilt out. No intercellular or intracellular hyphae were observed. No acervuli were observed (Figures 28-32).

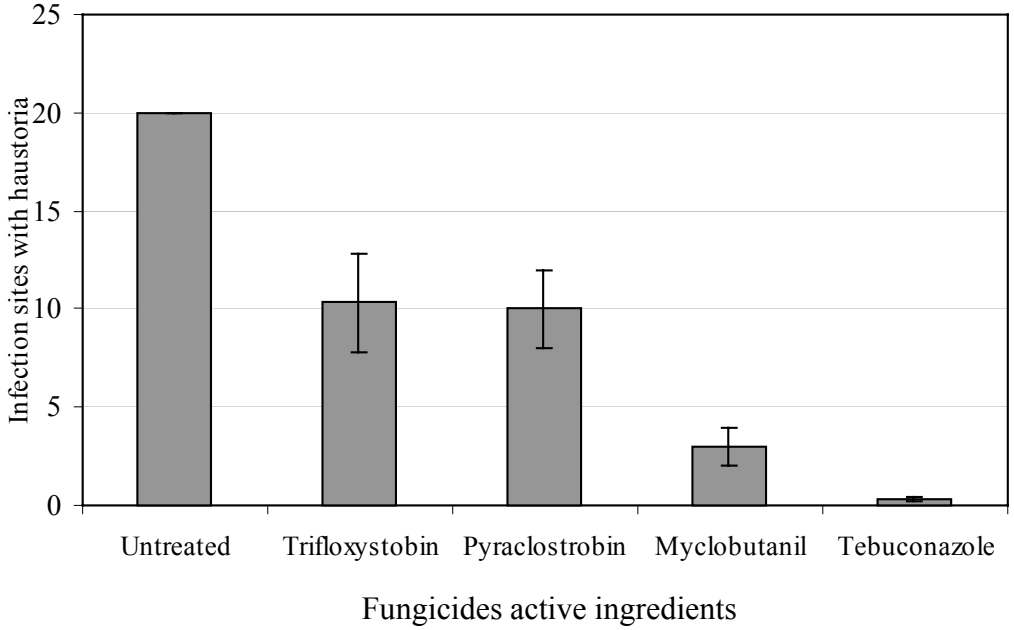

Figure 28: The number of infection sites on rose leaves that had been treated with different fungicides ingredients: trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 3rd dpi, in which haustoria were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, $P < 0.05$, Duncan's multiple range test).

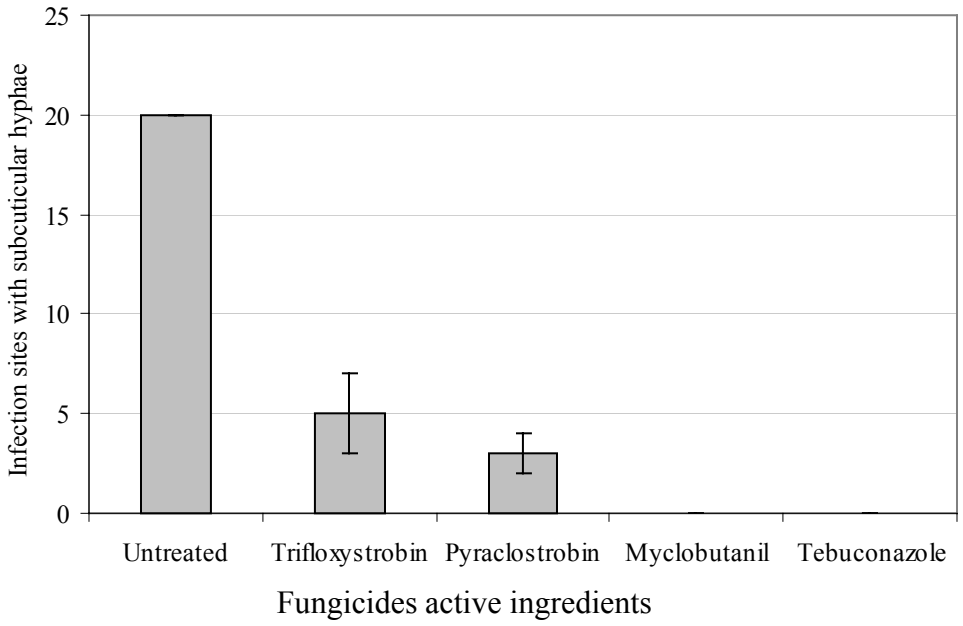

Figure 29: The number of infection sites on rose leaves that had been treated with different fungicides ingredients: trifloxystrobin pyraclostrobin, myclobutanil, tebuconazole, on the 3rd dpi in which subcuticular hyphae were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, $P < 0.05$, Duncan's multiple range test).

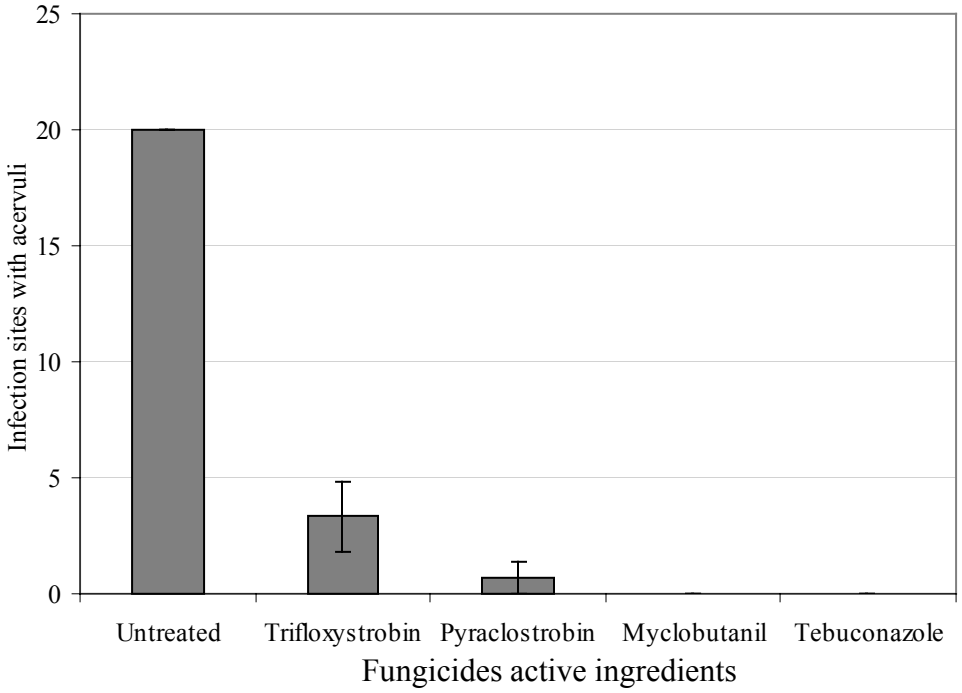

Figure 30: The number of infection sites on rose leaves that had been treated with different fungicides ingredients: trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 3rd dpi, in which acervuli were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, $P < 0.05$, Duncan's multiple range test).

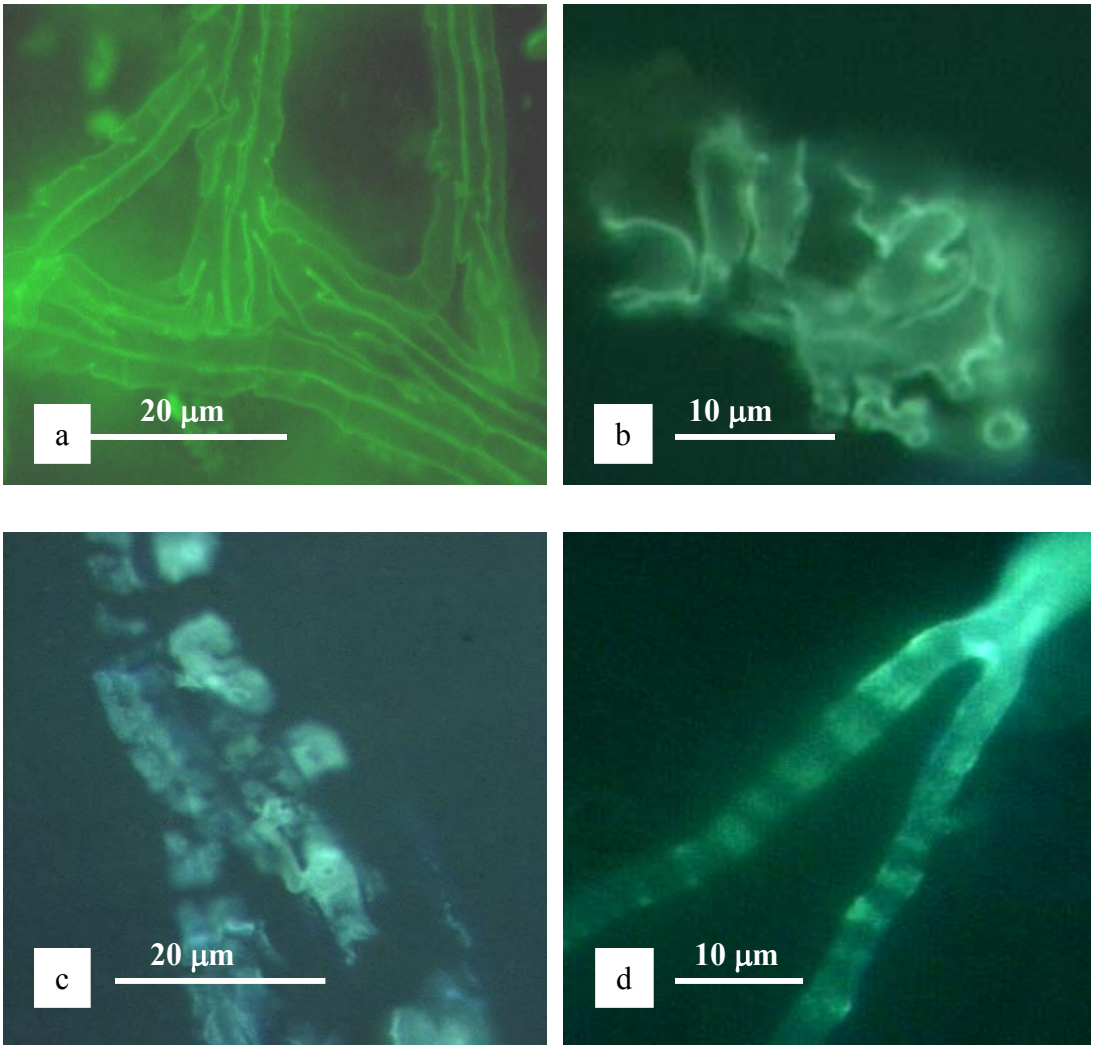

Figure 31: Light microscope photographs of *Diplocarpon rosae* in rose leaves showing subcuticular hyphae in leaves of: (a) an untreated control plant, (b) a plant treated with 100 ppm trifloxystrobin, (c) a plant treated with 100 ppm pyraclostrobin, (d) a plant treated with 100 ppm myclobutanil.

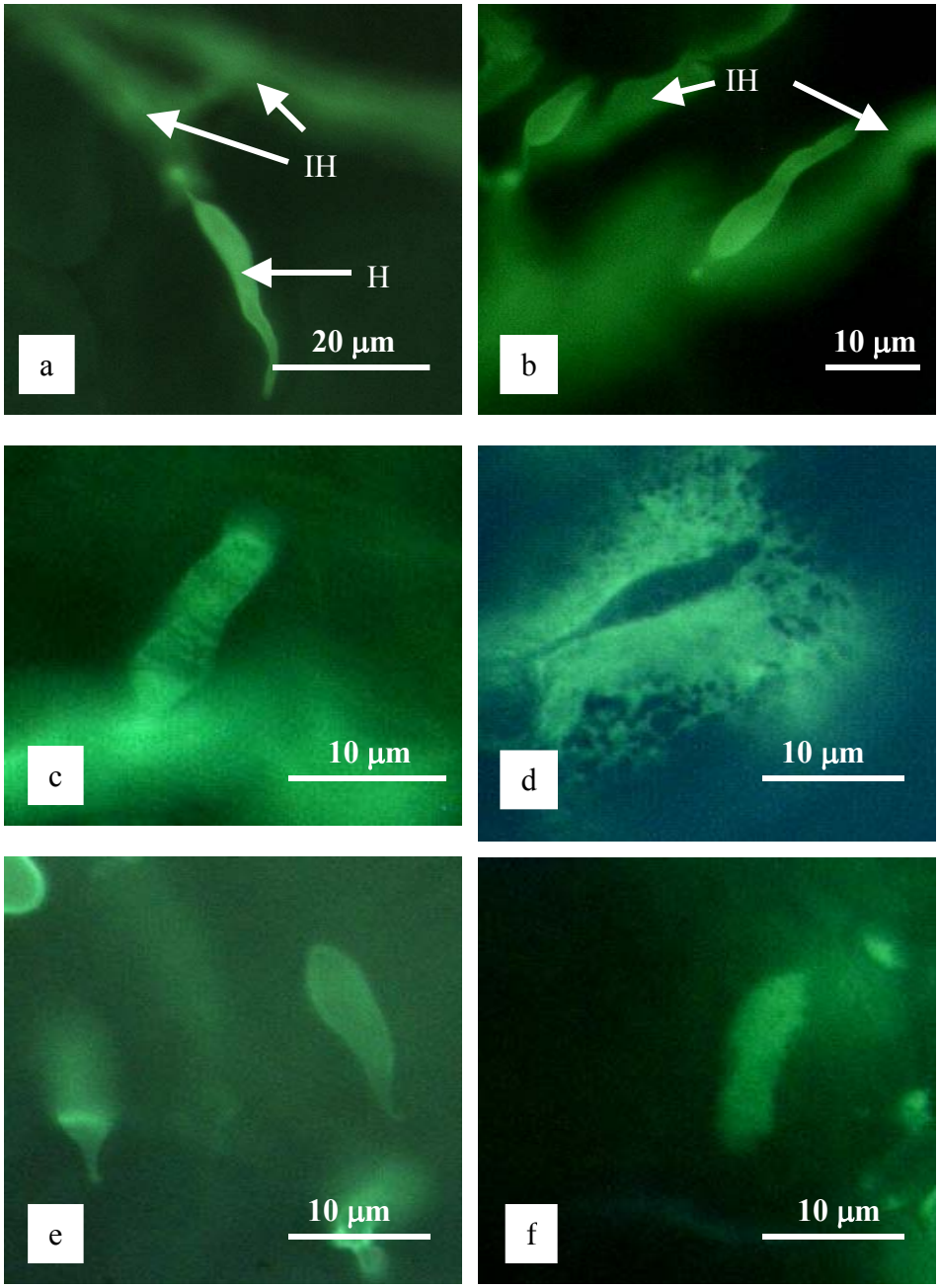

Figure 32: Light microscope photographs of *Diplocarpon rosae* in rose leaves showing haustoria in leaves from: (a) and (b) untreated control plants, note the intercellular hyphae (IH), (c) a plant treated with 100 ppm trifloxystrobin, (d) a plant treated with 100 ppm pyraclostrobin, (e) a plant treated with 100 ppm myclobutanil, note the absence of intercellular hyphae, (d) a plant treated with 100 ppm tebuconazole, note the absence of intercellular hyphae

3.5.3.2 Effectiveness of application on the 7th dpi

The fungal structures already present in the host at this point of fungicide application were subcuticular, intercellular, intramural and a few intracellular hyphae. Haustoria had also been formed and the base of the acervuli had started to form at several points along the subcuticular hyphae.

(i) Samples collected 10 dpi

Untreated

There was a network of subcuticular runner hyphae under the cuticle. Haustoria were observed in the epidermal cells, and at least one acervulus was found per infection site. The intramural hyphae in the epidermal periclinal walls and intracellular hyphae in the epidermal cells were observed. Some epidermal cells were almost fully enclosed by intercellular hyphae. The epidermal cells below the runner hyphae had a crystalline-like cytoplasm that fluorescent and in which haustoria were found. The haustoria had a long thin neck that attached to the intercellular hyphae.

Trifloxystrobin

Some of the subcuticular hyphae network was still in place but it did not fluorescent, while in other places the subcuticular hyphae were torn into fragments that were scattered at the infection site. On other infection sites the fungus grew in a similar manner as in the untreated plants. Haustoria were found at some infection sites but no intercellular hyphae were observed.

The haustoria formed were fewer compared to those in the untreated leaves. Fewer acervuli were formed compared to those in the untreated leaves. Some of the acervuli observed were not connected to any subcuticular hyphae (Figures 33-35).

Pyraclostrobin

Fragments of the subcuticular hyphae were seen scattered at an infection site and where the network of the subcuticular hyphae was still in place, the hyphae did not fluorescence. No intercellular or intracellular hyphae were observed at any infection site. Few haustoria were observed in the epidermal cells but they were not connected to any intercellular hyphae. Few acervuli were formed but no subcuticular mycelia were connected to them (Figures 33-35).

Myclobutanil

No subcuticular hyphae were observed except for a few small fragments that were scattered in the subcuticular region. There were few haustoria in some epidermal cells. These haustoria were not connected to any intercellular hyphae. No intracellular and intercellular hyphae were visible. In some epidermal cells the cytoplasm appeared crystalline. No acervuli were formed. The applications of myclobutanil left heavy chemical residues (Figures 33-35).

Tebuconazole

No intact subcuticular hyphae were observed but a few short fragments were occasionally found. These hyphal fragments however had a discontinuous or broken cell wall. Haustoria were observed in some epidermal cells. The haustoria were not connected to

any visible fungal mycelia. No intracellular, intramural or intercellular hyphae were observed at any infection site. No acervuli were formed (Figures 33-35).

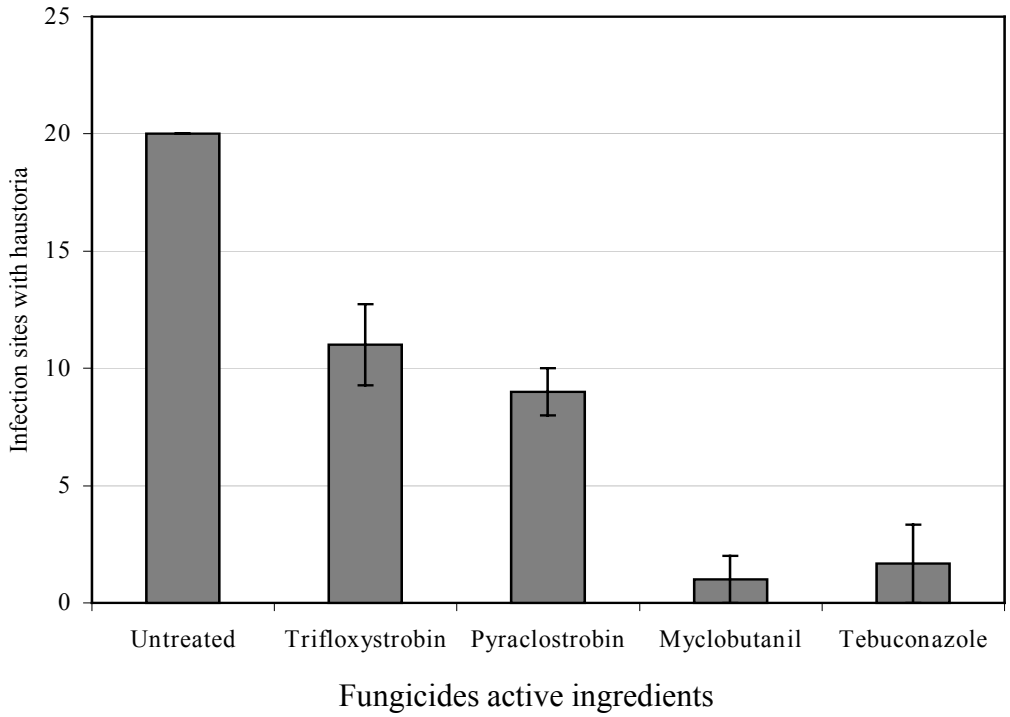

Figure 33: The number of infection sites on rose leaves that had been treated with different fungicides ingredients; trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 7th dpi, in which haustoria were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, P< 0.05, Duncan's multiple range test).

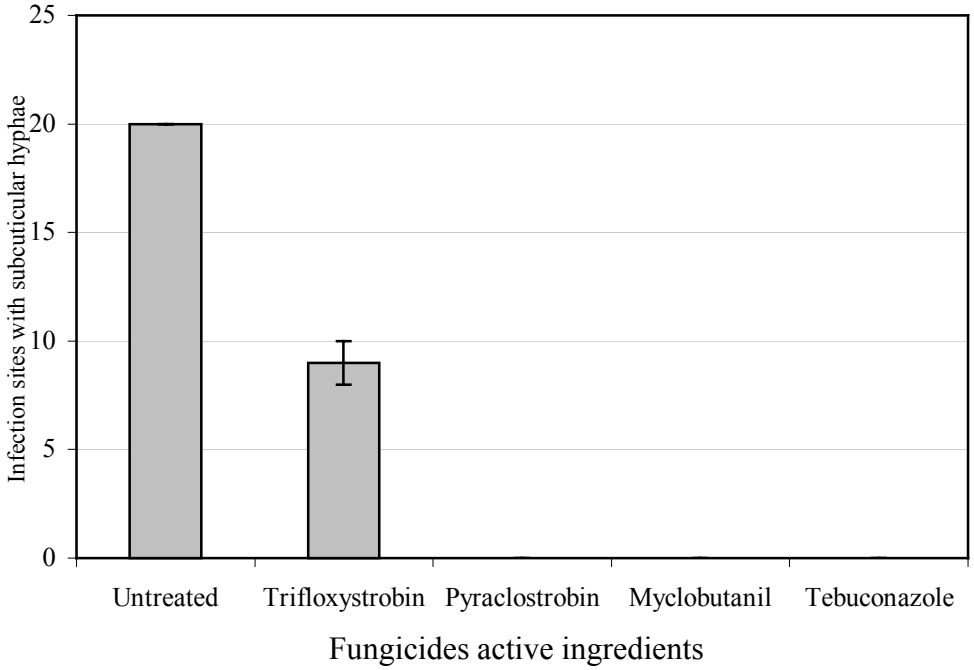

Figure 34: The number of infection sites on rose leaves that had been treated with different fungicides ingredients; trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 7th dpi, in which subcuticular hyphae were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates. n = 20, P< 0.05, Duncan's multiple range test).

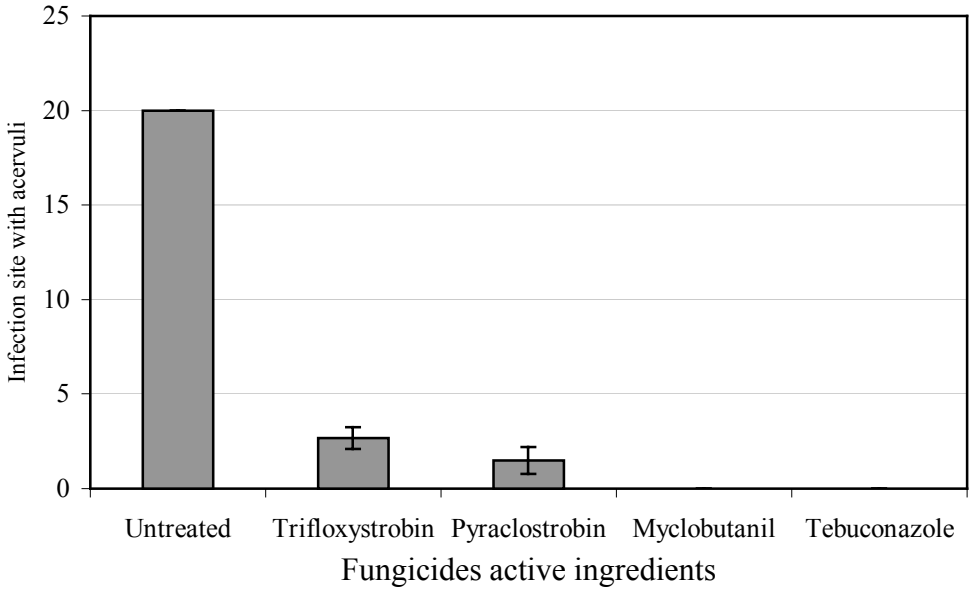

Figure 35: The number of infection sites on rose leaves that had been treated with different fungicides ingredients; trifloxystrobin, pyraclostrobin, myclobutanil, tebuconazole, on the 7th dpi, in which acervuli were observed on the 10th dpi, (the data represent the mean ± SD of 3 replicates, n = 20, P< 0.05, Duncan's multiple range test).

3.5.3.3 Effectiveness of application at the 10th dpi

The fungal structures that were already formed in the host at this time of fungicide application were subcuticular, intercellular, intramural, and intracellular hyphae, haustoria, and several fully developed acervuli containing conidia. The disease symptoms had started to appear at the infection sites.

(i) Samples collected 14 dpi

Untreated

There was a massive subcuticular network. Intercellular, intracellular and intramural hyphae were observed. Haustoria formed in the epidermal cells from the intercellular, intramural and subcuticular hyphae were observed. The haustoria were connected to the subtending hyphae. Infection sites had grown into each other and therefore the size of an individual infection sites was difficult to determine. Several acervuli were observed near each other at an infection site.

Trifloxystrobin

Subcuticular hyphal network was observed. The intercellular, intracellular and intramural hyphae were also observed. Some of the epidermal cells were almost fully enclosed by the intercellular hyphae. Haustoria formed by the intercellular, intramural and subcuticular hyphae were observed in the epidermal cells. In some infection sites the acervuli appeared similar to those in the untreated but in others the acervuli contents (base, conidiophores and conidia) had been reduced to a mass of small indistinguishable fluorescence pieces. In some of the acervuli, the conidia were deformed i.e. the cells of the conidium were separated from each other and seemed to have lost their turgidity. In

some other infection sites the destruction was minimal and one could observe a few conidia in the mass of fluorescence pieces (Figure 36).

Pyraclostrobin

Subcuticular hyphal network was still in place, intercellular, intramural and intracellular hyphae were also observed but they were fewer than in the untreated. Haustoria formed from intercellular, intramural and subcuticular hyphae were observed in the epidermal cells. Acervuli with normal looking conidia are found at each infection site but the acervuli formed were fewer than those in the untreated control. The viability of these normal looking conidia was not investigated (Figure 36).

Myclobutanil

Only fragments of subcuticular hyphae were observed. There was increased branching of the subcuticular hyphae in that a single fascicle had more hyphae than in the untreated control. There were no intercellular and intracellular hyphae. Very few intramural hyphae were observed. There was extensive destruction the acervuli; the bases of the acervuli were reduced to a mass of indistinguishable fluorescence pieces. The conidia were torn up into pieces or deformed; the 2 cells of the conidia had been separated from each other. Individual conidium cells seemed to have burst open (Figure 36). The application of myclobutanil left heavy chemical residues.

Tebuconazole

Fragments of the subcuticular hyphae were present at each of the infection sites. A single fascicle had more hyphae than in the untreated leaves i.e. there was increased branching on each hyphae strand compared to the untreated. Fewer haustoria were observed in the

epidermal cells compared to the untreated leaves. At each infection site there were reduced intercellular and intramural hyphae compared to the untreated leaves, but there were no intracellular hyphae. The acervulus base and most of the conidia were torn up, although a few of the conidia appeared normal (Figure 36).

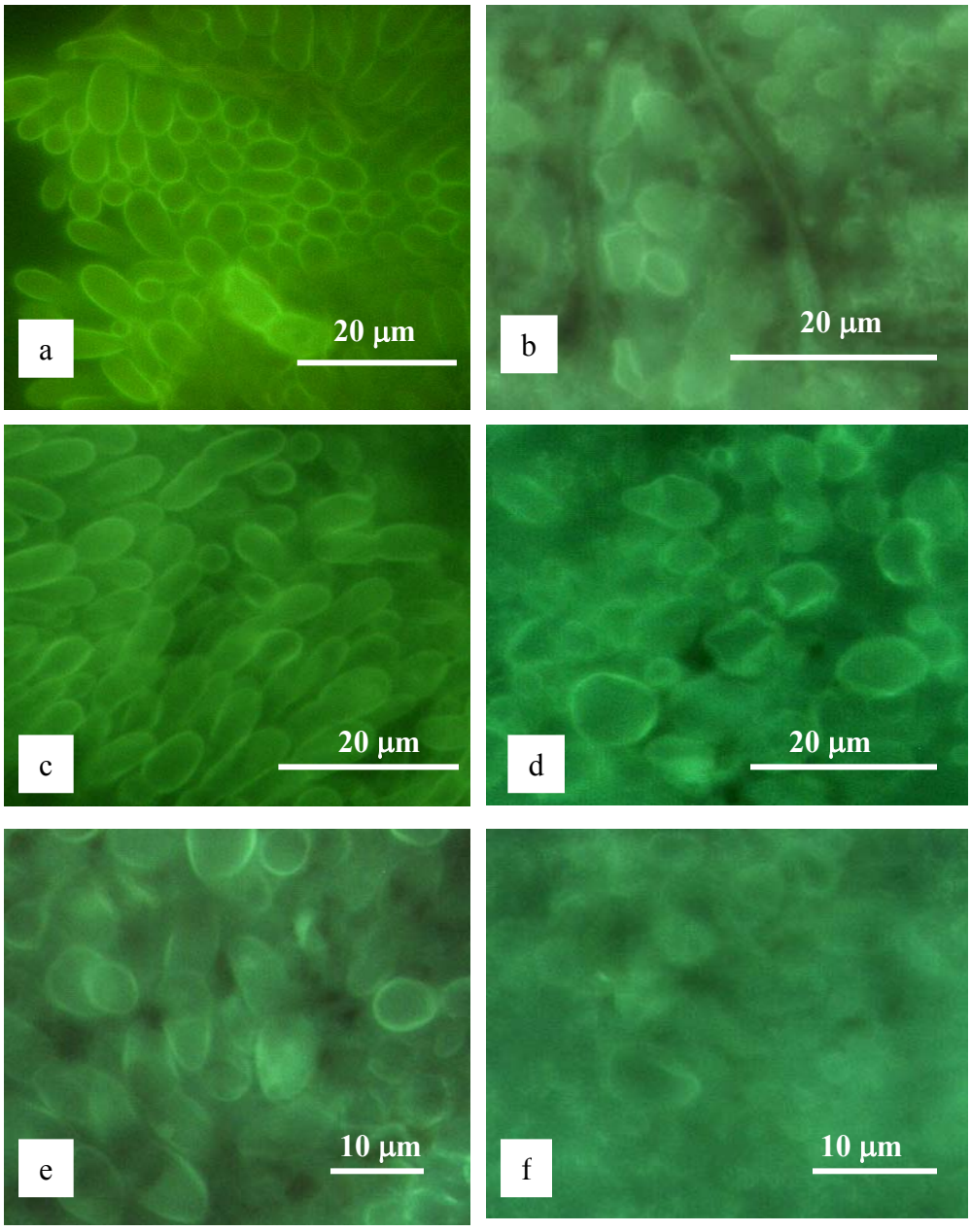

Figure 36: Light microscope photographs of *Diplocarpon rosae* in rose leaves showing conidia in an acervulus in a rose leaf from: (a) an untreated control plant, (b a plant treated with 100 ppm trifloxystrobin, (c) a plant treated with 100 ppm pyraclostrobin, (d) a plant treated with 100 ppm myclobutanil, (e) a plant treated with 100 ppm tebuconazole, (f) a plant treated with 100 ppm myclobutanil.

4 Discussion

The understanding of a host-pathogen interaction is an important prerequisite for the control of plant diseases. Only when one knows how the pathogen interacts with the host can one chose the management strategies to employ. Different isolates of a pathogen may interact differently with the host and respond differently to the control methods in use. Therefore the understanding of a pathogen's life cycle and how the different isolates interact with the host is paramount to disease control. This in turn will determine which methods of control are more appropriate.

The invasion of plants by pathogens is perceived as biotic stress in the plant cells. Under stress conditions there is excess production of reactive oxygen species (ROS), which leads to disruption of the electron transport systems in the plant (Kovtun et al 2000, Arora et al 2002). Measuring the relative accumulation of such molecules can be a good indicator of the stress in the plant cells. Reactive oxygen species (ROS) is a collective term used to for oxygen radical and the non-radical hydrogen peroxide (von Tiedemann 1997). The production of ROS is termed as oxidative burst and it is a rapid and transient response of plant cells to elicitors or plant pathogens in the early phase of interactions (Doke 1982, Baker et al 1995, Mehdy et al 1996). Elicitors include plant propagules or products such as *Phytophthora infestans* hyphae (Doke 1983), chemicals such salicylic acid (Milosevic and Slusarenko 1996) or mechanical stress (Legendre et al 1993). Localized pressure and enzymatic degradation of the plant cell wall elicits generation of H_2O_2 (Gus-Mayer et al 1998, Mellersh 2001). Many fungal pathogen gain access to the plant tissues by penetrating the plant cell walls through various mode of penetration which include use of mechanical force or use of cell wall degrading. The plant cell wall is

the first line of defence and plants have evolved means to perceive and respond defensively to the physical or chemical events associated with such penetration. The destructive effects of a pathogen are also perceived as stress in the plant cells and the greater the damage caused to the plant cells the greater the stress and amount ROS produced. The reactive oxygen species are able to interconvert into each other through several reactions and H_2O_2 has the longest half-life and provides a good estimate of the relative ROS in the plant cells (Elstner 1987, Baker et al 1995). Measuring the relative accumulation of ROS can be a good indicator of the aggressiveness of different isolate.

In this study the Kenyan isolate K1 caused more stress to the plant cells within studied time span compared to the German isolate G1. K1 can therefore be said to be more aggressive. This was also confirmed by the fact that the K1 lead to symptom expression earlier than G1. The differences observed in the isolates used in this study were not enough to group them into races but were concluded to be differences in aggressiveness. G1 however had a higher germination rate than the K1. Further investigations using plant differentials similar to those used by Debener (1996) need to be done to check if the disease expressions caused by these isolates on other rose cultivars are different. It necessary to standardise the differentials used worldwide in order to obtain comparable data. In addition, carrying out molecular studies will shed more light into the genetic diversity of the fungal isolates.

At 20 ^{o}C isolate K1 lead to symptom expression earlier than G1 but at other incubation temperatures the effect of the temperature was higher than the differences between the isolates. The development of disease symptoms was much slower at 15 ^{o}C than at 20 and

25 ^{o}C, and no disease symptoms developed at 10 ^{o}C. This indicates *D. rosae* was able to germinate and penetrate the host cuticle at 15 ^{o}C, but it developed very slowly compared to the development at 20 and 25 ^{o}C. Studies by Palmer et al. (1978), and Horst (1983) led to the conclusion that the conidia do not germinate if they have not been wetted for a minimum of 6 hours at relative humidities below 90 % and for at least 5 min even at 100 % RH. The percent infections increase with length of leaf wetness. Horst (1983) had also reported that the pathogen tolerates a wide range of temperatures from 15-27 ^{o}C, mainly through an inverse relation of humidity and moisture. With this in mind, one can then presume that the pathogen has evolved a mechanism whereby it can germinate at low temperatures when free water is more readily available than at high temperatures. The pathogen then remains in the host until the temperatures are favourable for mycelial development. The optimum temperature for conidium germination is 18 ^{o}C and it is lower than the optimal temperatures for mycelia and disease development, which are 21 ^{o}C and 24 ^{o}C respectively (Horst 1983). This compares very well with *Peronospora sparsa*, the causal agent of downy mildew of roses. *P. sparsa* infects roses even at 5 ^{o}C or lower depending on the isolate, but the optimum temperature for infection and disease development are 15 to 20 ^{o}C and 20 to 25 ^{o}C respectively (Aegerter et al 2003, Breese et al 1994). The infection and colonization of many hosts by their respective pathogens depends on the temperature, length of leaf wetness and relative humidity among many other factors.

The colour and size of conidia of *D. rosae* observed in this study were within the range observed by other researchers (Ali et al 2000, Wenefida and Spencer 1993). Germination started within eight to nine hours after inoculation and within 12 hpi appressoria

formations had started and in some cases the penetration had taken place. Commonly only one of the cells of the conidia germinated but even up to three germ tubes per conidium were observed About 7 % of the germinated conidia formed germ tubes from both cells of the conidium. The germination pattern observed in this study was very similar to that described by Aronescu (1934), Palmer et al (1978), and Wiggers et al (1997). Formation of more than one germ tube per conidium has also been observed in other fungal pathogens like *Colletotrichum* species, where the germ tubes also have variable lengths (Smith et al 1999). As observed in this study and also recorded by Aronescu (1934), the very long germ tubes of *D. rosae* fail to form appressoria and probably never succeed in penetrating. These long germ tubes may have depleted their energy reserves and were therefore not able to penetrate the host cuticle. Niks (1990) reported that increase in germ tube number and length decreased the amount of energy available for *Puccinia hordei* sporelings to reach the stomatal entry points during infection of barley. He believed that the sporelings were exhausted due to depletion of endogenous energy reserves before completion of the infection. Thines et al (2000) demonstrated that appressorium mediated host penetration by *Magnaporthe grisea* involves many metabolic reactions and requires much energy.

In this study, well-defined appressoria did not always form at the ends of the germ tube but many of the germ tubes were simply swollen at their tips, a fact that had also been reported by Aronescu (1934), Palmer et al (1978) and Wiggers et al (1997). As opposed to other fungi like *Colletotrichum* species where the appressoria are well defined and delimited from the germ tube by a septum (Emmett and Parbery 1975, O'Connell et al 1985, Wharton et al 2001), the appressorium formed by *D. rosae* were extensively

variable, some were just swellings on the germ tube, while others were well defined rounded and delimited swellings at the end of a germ tube, but sessile structures were not uncommon. On some germ tubes no changes in their morphology were observed prior to penetration or the changes may have been too subtle to be noticed. Therefore, in this study the term appressorium was the used to define a structure formed on the germ tube for purposes of adhering firmly to the host to achieve penetration.

The reason(s) why much variability with respect to appressorium formation was observed is not known, and it could have been due to host factors as well as fungal factors or a combination of the two. In many plant-fungal interactions where well-differentiated appressoria are formed, factors such as chemical signals (for example potassium and calcium ions, simple sugars), pH gradient and temperature shifts have been said to induce appressorium formation (Hoch and Staples 1991). Host related factors such as hydrophobicity (Lee and Dan 1993), hardness (Xiao et al 1994), components of host surface such as wax (Podila et al 1993) and topographical properties, like the correct dimensions of a ridge formed by the stomatal lip of the guard cell (Hoch and Staples 1991) are recognized as major signals. Tucker and Talbot (2001) recently reviewed the factors that influence pre-penetration stages in various fungal pathogens. Principally physical and chemical factors of host origin govern germ tube growth and the formation of appressorium but the fungi respond to other environmental factors such as light and temperature (Emmett and Palberry 1975).

Nair and Ellingboe (1965) argued that spore (or conidium) populations of the same species might be composed of different genotypes that require different environmental

conditions for germination. It is therefore possible that the conidia in a population will not be homogenous in the conditions required for appressorium formation (Emment and Parbery 1975). The variation could also be attributed to observation of premature structures or incompletely formed structured due to the environment being unsuitable. Given that the population used in this study was not a single conidium population, this argument could also be acceptable.

None of the earlier reports on *D. rosae* mentioned the presence of the brown rings, presumed to be melanin rings, formed at the point of penetration. Melanization of the appressoria has been well studied in fungi that form fully melanized appressoria such as *Magnaporthe* and *Colletotrichum* species. Howard et al (1991) indicated that fungi with melanized appressoria such as *Magnaporthe* species might be able to penetrate the host cuticle and cell wall mainly by means of turgor pressure. Melanization of the appressoria has been found to be essential for penetration of the host by *M. grisea* (Mendgen et al 1996), and treatment of its conidia with chemicals that inhibit melanization or generation of mutants by targeted disruption of genes involved in the melanin biosynthesis resulted in formation of non-pigmented appressoria and the mutants did not penetrate the host surface (Woloshuk and Sisler 1982, Chumley and Valent 1990, Howard and Ferrari 1989, Kubo and Furusawa 1991, Kimura et al 2001). Turgor pressure of melanin deficient mutants is only 30-70 % of that measured in fully melanized appressoria (Dean 1997). Melanization has been estimated to reduce porosity of appressorial cells of *M. grisea* to less than 1 nm from approximately 2 nm and be sufficient to block efflux of cytosolic solutes responsible for generating appressorial pressure (Howard et al 1991). Although

much is known about the role of melanin in completely melanized appressoria, the role of melanin rings is yet to be elucidated.

In addition to their formation by *D. rosae* in its interaction with rose leaves, melanin rings have been reported at the host pathogen interface of Venturia *inaequalis* and apple leaves (Steiner and Oerke 2001). In *V. inaequalis*–apple interaction, the rings are thought to play a role in the adhesion of the pathogen to the cuticle. Pre-inoculation applications of melanin biosynthesis inhibitors to the apple leaves largely inhibited melanization of the rings and reduced infection by 50-80 %. This indicated that melanization of the rings was important for the penetration of apple leaves by *V. inaequalis*. The melanin rings are assumed to play a similar role in the penetration of rose leaves by *D. rosae*, and preliminary studies (data not shown) are pointing in this direction.

In *D. rosae* the penetration pore wall was reinforced suggesting that turgor pressure may also play a role in the penetration of intact host walls. This reinforcement of the wall may ensure that the pressure generated by turgor and by the cytoskeleton was exerted over a restricted area of the pore by the growing penetration hypha. The turgor pressure generated in appressoria of different pathogens varies e.g. in *M. grisea* its 20-80 bars (Howard et al 1991) and in *Uromyces appendiculatus* 3-5 bar (Terhune et al 1993). *M. grisea* forms fully melanized appressoria, while the appressoria of *U. appendiculatus* have no melanin. Terhune et al (1993) reported that with the 3-5 bars turgor pressure the *U. appendiculatus* appressorium base can distort polystyrene ridges and the emerging penetration hypha can curl the stomata lip inward. This shows that even in non-melanized appressoria an enormous pressure is generated, therefore it would be logical to presume

that turgor pressure build-ups in appressoria of *D. rosae*. Although the force generated by the cytoskleton may only be of importance if the turgor pressure is very low (Mendgen et al 1996), the cytoskeleton plays a role in stabilizing the tip of the penetration hypha.

The importance of turgor pressure in penetration of rose leaves by *D. rosae* is not known and other mechanisms of penetration other than mechanical force seem to be also involved. The host cell wall at the point of penetration was observed to be locally degraded with no evidence of inward deformation or material being dislodged, a factor that Pring et al (1995) in their study of the *Colletotrichum* species associated with enzymatic action. The localized changes in staining properties of the host cell wall adjacent to the penetration peg implicated enzyme action as also reported by Wharton et al (2001) in their study on *Colletotrichum sublineolum*.

In fungi that establish an intimate relationship with their host, such as those causing rust diseases, the differentiation of appressoria and subsequent penetration is accompanied by a complex pattern of hydrolytic enzyme secretion (Dean 1997). As reported by Fernando et al. (2001), Bucheli et al (1990) and Sweigard et al (1992a and b), cell wall degrading enzymes are also associated with penetration of host by fungal pathogen that form well differentiated melanized appressoria such *Colletrotrichum* and *Magnaporthe*. Pryce-Jones et al (1999) demonstrated that non-melanized appressoria of *Erysiphe graminis* f. sp *hordei* generate a turgor of 2-4 Mpa. Extracellular enzymes have been shown to be present during the appressorium penetration by *E. graminis*, indicating a combination of physical force and use of cell wall degrading enzymes working together (Francis et al 1996, Pryce-Jones et al 1999). Therefore in the case of penetration of rose leaves by *D.*

rosae it is also likely that both modes of penetration may be used singly or together at a single penetration site.

The involvement of enzymes in host penetration and further development of *D. rosae* has been implicated for various additional reasons; the electron opaque substances in the host cell wall below the point of penetration were associated with the host cell wall dissolution and the epidermal cell wall below the point of penetration was also dissolved ahead of any visible fungal structures. Growth of the intercellular mycelia was associated with partial or complete dissolution of the host cell wall, and penetration of the palisade mesophyll cell was accompanied by local degradation of the host cell wall at the point of penetration without inward deformation or displacement. The electron opaque layers near the appressorium-cuticle interface, in the cell wall can be explained in three possible ways. These electron opaque layers could be made up of materials resulting from cell wall such as waxes, cuticle, cellulose, and pectin degradation (Becker 1993). These electron opaque layers could be enzymes themselves that have been preserved by glutaraldehyde since glutaradehyde preserves protein like fungal enzymes during fixation (Hayat 1989a and b, Park et al 2000). A third possibility is that the electron opaque layers are made up of a mixture of the enzymes and the degraded products. Changes in electron density of epidermal cell walls in contact with fungal stroma observed in apple leaves infected with *V. inaequalis* were thought to be due to enzymatic degradation (Valsangiacomo and Gessler 1988). The electron dense zones observed below the appressorium in early infections of apples leaves by *V. inaequalis* were also attributed to enzymatic degradation of the cuticular membrane. The ability of crude enzymes produced

by *V. inaequalis* to degrade apple leaf cell walls has been demonstrated (Valsangiacomo et al 1992).

Upon penetration of the host cuticle by *D. rosae* an infection vesicle is formed in the host cell wall. In previous studies on *D. rosae* there is no mention of the infection vesicles formed upon penetration. This infection vesicle seems to serve as a holding centre for all fungal cellular contents immediately after penetration. The initial subcuticular and intercellular hyphae are formed from this vesicle. The subcuticular hyphae grow in bands of at least 2 or more parallel hyphae. New subcuticular hyphal strands are formed at acute angles. The acute branching angle can be observed in Frick's (1944) drawings although she did not comment on its size. Palmer et al (1978) reported that the subcuticular hyphal strands arise at right angles; but they could have been interpreting a meeting point of two hyphal strands as a branching point or the formation of intramural hyphae, which are formed at variable angles. Dodge (1931) observed that the two new hyphal branches formed upon division of a parent cell at first widely diverged before converging to become tightly pressed together, this initial diverging and converging of new branches was not observed by the writer.

The growth of the subcuticular hyphae of *D. rosae* can be compared to V*enturia inaequalis* and *V. nashicola*, the causal agents of scab on apples and Asian pears respectively. These two pathogens grow between the epidermis and the cuticular membrane without penetrating the cells or invading the intercellular spaces (Park et al 2000, Valsangiacomo et al 1989). The mycelia of *V. nashicola* only grow subcuticularly within the pectin layers of the periclinal walls of epidermal cell without invading the

epidermal cells themselves (Park et al 2000). *V. nashicola* is suspected to produce enzymes, cutinase and pectinase to enable it to inhabit these pectin layers. In apple leaves infected with *V inaequalis*, translucent zones observed in host cell walls were associated with local degradation of the host cell wall (Valsangiacomo et al 1989). In rose leaves infected with *D. rosae*, the host wall surrounding the subcuticular or intramural hyphae stained lighter with toluidine blue and was wider in diameter. This indicated a probable change in the composition of the wall due to extracellular fungal products such as enzymes. The intramural hyphae probably used enzymes to grow through the host cell wall. Aronescu (1934) attributed the swellings in the host cell wall to enzymatic action.

As *D. rosae* continues to develop in the host tissue it leads to the development of brown or black spots on the leaf surface. According to Dodge (1931), the brown or black spots of discolouration were confined to the upper side of the leaf for some time and occasionally he found sori on the lower side of the leaf directly below the brown or black discolouration. He presumed that the infection sometimes occurred naturally on the lower side. Aronescu (1934) clearly stated that spots on the lower surface were formed by the fungus infection on the upper side growing through the leaf. In this study however, observations made on naturally and artificially infected leaves revealed that the fungal mycelia growing from the upper side of the leaf penetrated up to the palisade mesophyll cells only. Infections established on the upper side of the leaf did not lead to symptom development on the lower side of the leaf and vice versa. Infections on the lower side were rare under natural conditions.

The initial browning of the leaf surface, during symptom development, was due to browning of the palisade mesophyll layer, followed by the browning of the subcuticular hyphae and the acervuli. The writer assumed that the browning of the mesophyll cells was due to formation of phenolic compounds, while browning of the subcuticular hyphae and the acervuli was due to pigmentation. Phenolic compounds are produced as a host defence mechanism to the invasion by pathogens as summarized by Agrios (1997). The pigmentation in the subcuticular hyphae and acervuli was assumed to be due to melanin formation. Melanin production by fungal pathogens has been well researched as reported by Butler and Day (1998), and Henson et al (1999) in their reviews. Although some fungi produce melanin constitutively, others have been observed to undergo melanogenesis in response to environmental stresses, such as exposure to toxic metals, desiccation, hyperosmotic conditions, temperature extremes, limited nutrients and UV radiation to mention but a few. In general, melanin is important for the survival and longevity of fungal propagules (Bell and Wheeler 1986). Wolf (1912) thought that the black discoloration was due to disorganization of the cells of the leaf and not due to the mycelia of the fungus, which are colourless. Dodge (1931) was however convinced that the browning was due to the collapse of the infected cells, while Aronescu (1934) attributed the browning to the colour of the older mycelia. Frick (1944) thought the browning was due to the changes in the contents of the infected epidermal cells but the aged thick walled parallel hyphae contributed to the typical black spot symptoms.

The scope of the tools that previous researchers on the life cycle of *D. rosae* used was limited and could have led to erroneous conclusions. Palmer et al (1978) observed that few intercellular hyphae were formed and that the most of them were often between the

palisade cells, but in this study extensive intercellular growth was evident and almost all of the intercellular growth took place in the epidermal layer. Wolf (1912) reported that the acervuli stromata were connected to the internal mycelia below by hyphae growing between or in the epidermal cells. In this study the stromata of the initial acervuli were connected only to the subcuticular mycelia from which they were formed. As opposed to the findings of several authors, the writer observed extensive plant tissue disorganization in fallen or detached infected leaves that were disintegrating. Wolf (1912) observed extensive disorganization and break down of mesophyll tissue directly below the acervulus stroma during the acervulus development but in Dodge's (1931) report this disorganization and break down was said to be limited. According to Palmer et al (1978), at the disease developmental stage when the visible symptoms were present, there was extensive destruction of the host tissue such that the upper epidermis, upper portions of the palisade and xylem parenchyma could not be histologically distinguished. This is not surprising because they used detached leaves for their studies. Frick (1944) however saw no disorganization. In this study there was minimal destruction of host tissue during the biotrophic phase. However, in old infections sites with advanced necrotrophy there was massive destruction of the epidermal cells, with the whole epidermal cell being filled with intracellular hyphae and the cell wall disintegrating. Since Wolf (1912) does not state how old the infection sites he observed were, it is possible that he observed very old infection sites with abundant secondary hyphae, hence the erroneous conclusion.

In this study, the intracellular mycelia were observed from about the 7-8th dpi on leaves still attached to the plant and about the 4th to the 5th dpi on detached leaves, which was just before the formation of the acervuli in both cases. The number of intracellular

hyphae was higher in older infections and in fallen leaves. Although Wolf (1912) reported the presence of intracellular mycelia in both the epidermal and mesophyll cells, Dodge (1931) in his studies about 20 years later did not observe any intracellular hyphae in sections of material fixed 2dpi through two months post inoculation and even in overwintered leaves. Dodge thought that Wolf mistook the haustoria, haustoria mother cells and intercellular hyphae for intracellular hyphae. Frick (1944) on the other hand observed intracellular mycelia only in fallen disintegrating leaves but never on living plant tissue attached to the plant, indicating that the fungus never caused plant cell death but was able to survive in dead plant tissue. However the writer observed intracellular hyphae in plant cells at the beginning of reproduction. Palmer et al (1978) did not mention the presence of the intracellular hyphae but concluded that the histological host-pathogen relationship between *D. rosae* and roses was similar to that of the rust pathogens and their hosts. The writer clearly showed that *D. rosae* is a hemibiotrophic fungus, with the biotrophic phase in the host cells being marked by the formation of haustoria and the necrotrophic phase by the formation of the intracellular hyphae.

The length of the biotrophic phase in different pathogen-host relationships is very variable. It can last for a few hours, like in *Colletotrichum lindemuthianum* where it lasts 24 hours (O'Connell et al 1985) or several days like in the case of *D. rosae*. In the biotrophic phase the host cells remain alive and all fungal structures in the host cells are enclosed by the host plasma membrane that invaginates around them. There is an interfacial matrix between the fungal cell wall and the invaginating plasma membrane, and this interfacial matrix is thought to play a role in the maintenance of biotrophy and suppression of host defence reaction (Gay et al 1984). Studies done on other

hemibiotrophic pathogens have shown that the intracellular hyphae mark the onset of the necrotrophic phase (Perfect et al 2001). During the biotrophic phase of *C. lindemuthianum* the host and fungal plasma membranes are separated by an interfacial matrix (Perfect and Green 2001), which is not formed in the necrotrophic phase. Formation of secondary hyphae by *C. lindemuthianum* is associated with secretion of large amounts of cell-wall degrading enzymes (Centis et al 1997). In *D. rosae*, the haustoria and the large hyphae that formed on the inner side of the host cell wall were surrounded by part of the host plasma membrane and are therefore part of the biotrophic phase. The intracellular hyphae were not enclosed by part of the host plasma membrane but were surrounded by a moderately electron opaque layer. The intracellular hyphae are also associated with extensive host cell wall dissolution suggesting secretion of cell wall-degrading enzymes. The intracellular hyphae therefore marked the onset of the necrotic phase. The penetration of neighbouring cells by the intercellular and intracellular hyphae probably involves use of cell wall degrading enzymes, because the cell wall thickness is reduced at the point of penetration compared to the surrounding wall and the wall microfibrils are cleanly severed.

The initial haustoria were formed very early in the disease development, Aronescu (1934) observed them as early as 15 dpi. All haustoria were formed inside the host cell wall but exterior to the plasma membrane a fact that the reports of early writers like Palmer et al (1978) disputed. In this study the haustoria formed were of varying sizes and shapes, from pyriform for the very young ones through ventricose to oval and clavate for the older ones, but Palmer et al (1978) had observed all haustoria to be only belled shaped. The haustoria of *D. rosae* grow in size after their formation. Haustoria are specialized

hyphae for nutrient absorption and metabolism and are recognized as one of the distinguishing characteristics of a biotrophic fungi (Mendgen and Hahn 2002) or the biotrophic phase of a hemibiotroph (Perfect and Green 2001). The size and shape of the haustoria in fungi like powdery mildew (Bushnell 1972) and possibly in *D. rosae* have been thought to play a role increasing the surface area of the haustoria–host plasma membrane interface, hence the surface for nutrient absorption.

Haustoria of the rust fungi have been well researched on and much can be gleaned from these studies. The extrahaustorial matrix has been reported to consist mainly of carbohydrates and proteins that are primarily of plant but partly of fungal origin (Harder and Chong 1991) and serves as a point of transfer of nutrients and signals between the plant and the pathogen (Murdoch 1998, Stark-Urnau and Mendgen 1995, Hu and Rijkenberg 1998, Heath and Skalamera 1997). Studies by Mendgen and Hahn (2002) revealed that the bulk of the sugars and amino acids are taken up by haustoria. The haustoria are therefore the feeding organ of the fungus in the biotrophic phase. Voegele and Mendgen (2003) convincingly argued that since upon switching of the hemibiotrophic fungus from the biotrophic to the necrotrophic phase the plasma membrane surrounding the hyphae disintegrates, it seems like the interfacial matrix plays a role in maintaining the biotrophy. An overall view of the growth pattern of the *D. rosae* reveals that it forms very many haustoria as it continues to grow away from the point of penetration. This leads to the assumption that the above arguments concerning the role of the haustoria as a feeding organ associated with biotrophy hold true also in *D. rosae,* and the haustoria therefore provide the food need for the subcuticular hyphae to reach parts far removed from the infection point and for the fungus to ramify the host tissue

intramurally. Intramural mycelia grow in the spaces between the hyphal fascicles thus helping the fungus to absorb nutrients from the epidermal cells between the hyphal fascicles. In the end haustoria are found in very many of the host cells and more than one haustorium can be found in a single cell. Heath (1990) showed that haustoria formation is under signals from the host that either induce or complete the differentiation of haustoria. This explains *D. rosae* does form haustoria in artificial grow media.

The end of an asexual cycle of *D. rosae* is signified by the formation of bicelled conidia in subcuticular acervuli. In this study two-celled conidia and microconidia (spermatia) were formed in the same subcuticular acervuli just as already described by Dodge in 1931. He observed that in many of the wintered leaves either wholly spermatial pustules or in mixed ones with normal two cell conidia. However in this study no subcuticular acervuli consisting of only microconida were observed. The role of the microconidia in the life cycle of *D. rosae* has not been established and further investigations need to be done to establish their role in propagation of the pathogen.

From the growth pattern observed the following conclusions can be drawn: *D. rosae* penetrates the cuticle either by mechanical force or enzymatic degradation of the cell wall or both. After that it establishes by an initial subcuticular and intramural necrotrophy that is associated with swelling and dissolution of the host cell wall. The fungus spreads and ramifies the host tissue subcuticularly and intercellularly. Parallel to this the fungus invades the host cell to form haustoria that are enclosed by part of the host plasma membrane. Large hyphae are formed on the inner side of the cell wall but exterior to the plasma membrane thereby establishing a biotrophic phase in the host cells. There is an

increase in intramural ramification, which is associated with haustoria formation in the epidermal cells and it is followed by intracellular necrotrophy. Therefore the necrotic and biotrophic phases occur at the same infection site in different parts of the host tissue. Host defence reaction is induced by the invasion of the cells to form haustoria, but the formation of callose as part of the host cell defence does not stop the formation of the haustorial body. The callose material is therefore formed as a collar around the haustorial neck and the proximal end of the haustorial body, but never around the whole of the haustorial body. In infections caused by *E. graminis* the formation of the callose is stopped soon after the emergence of the young haustorium (Bushnell 1972). Renewed growth or expansion of haustoria in host cells also leads to formation of callose where the new growth takes place. The callose is a host cell wall wound reaction (Agrios 1997). When the biotrophic phase of *D. rosae* is compared to biotrophic life cycle of a biotroph like the bean rust fungus, *Uromyces appendiculatus* (Harding et al 1999) where minimal collar material is formed and the host defence reaction is suppressed during the development of the haustoria, then one can conclude that *D. rosae* is a hemibiotrophic fungus whose biotrophic phase has not fully evolved, since the host defence reaction is not fully suppressed.

For the survival of the fungal population, the fungus has to have a mechanism to survive the unfavourable weather conditions. The findings in this study confirmed earlier reports that *D. rosae* overwinters in fallen leaves and on the cane of rose plants. Some diseased leaves remained attached to the plants during winter and can also serve as source of inoculum in spring. Lyle (1943) reported on the ability of this fungus to overwinter on canes, while Cook (1981) reported on its ability to overwinter on canes and in bud scales.

Frick (1944) demonstrated that the saprophytic mycelia found in overwintered leaves formed conidia when placed in a damp chamber at room temperature. These conidia germinated and caused blackspot lesions when inoculated on rose leaves.

Wolf (1912) described the ascospores of *D. rosae* for the first time. Aronescu (1934) observed them also in the USA some years later and they have since been reported in Great Britain (Knight and Wheeler 1977, Cook 1981) and in Russia (Dudin 1972) The formation of these ascospores is rare and their role in genetic recombination is not fully elucidated (Walker et al 1995). According to Wolf (1926) mycelium invades the deeper layers of a fallen leaf to form stroma between the epidermis and the palisade parenchyma. If the weather is favourable for the formation of ascocarps, then apothecia with asci are expected in spring, but if the conditions for the formation of the asci are unfavourable, the deep-seated fruiting bodies are filled with apothecial conidia (Dodge 1931). In this study, heavily ornamented structures were observed in overwintering leaves from December. By April these structures had an opening through one could see small oval shaped cells. It is however unclear what these structures are and the writer assumed that they were fruiting structures in which apothecial conidia (the small oval shaped structures) were found. The deep-seated fruiting bodies with a shape similar to that of the apothecia described by Wolf (1912) were also observed in this study but no asci had developed. The writer assumed that the weather in Bonn during the winter of 2003 was not favourable for the complete development of the ascocarps and the conidia formed from the overwintered saprophytic mycelia on cane or leaves was the main source of primary inoculum in spring. In the experiments carried out by Frick (1944) with infected rose leaves collected from various parts of Switzerland and overwintered in Zurich, she

was not able to find any apothecia. With respect to overwintering, *D. rosae* can be poorly compared with *V. inaequalis*, another ascomycete that grows subcuticularly and forms ascospores as in overwintered plant material. In *D. rosae* the conidia formed from the overwintered saprophytic mycelia are the main source of primary inoculum in spring (Frick 1944), but in the case of *V. inaequalis* the ascospores formed in the overwintered leaves are the main source of primary inoculum (MacHardy 1996). How *D. rosae* survives the dry spells in the tropical countries is yet to be studied.

D. rosae grew in detached leaves. Although the fungal structures that developed in the detached leaves were similar to those that developed in leaves of the intact plants, the fungal structures were formed earlier in detached leaves than in leaves on intact plants. The biotrophic phase of the fungus was shorter in detached leaves than in leaves on intact plants. This is thought to be because of the degeneration of the detached leaves after some time therefore causing the fungus to switch to a saprophytic way of feeding. The ability of the fungus to form many haustoria in the detached leaves within such a short time was attributed to the fact the plant defence reaction on the detached leaves is minimal, therefore fungus is able to grow unhindered.

The saprophytic competence of the fungus demonstrated by its ability to live on dead or senescent plant tissue is further confirmed by its ability to grow on artificial media. The fungus grew and reproduced on all artificial growth media tested: water agar, PDA, MEA and biomalt agar. However the highest numbers of conidia were formed on biomalt-based media followed by malt extract agar based medium and the least formed on PDA. Both biomalt agar and malt extract agar contain malt extract. Malt extract agar also contains

peptone from soja-bean meal, while biomalt may be enriched with other additives such iron, calcium, vitamins etc which may have been responsible for the added advantage in biomalt in comparison to MEA. The fungus sporulates better on malt extract based products than on potato dextrose agar, an observation that had also been made by Shirikawa (1955) and Drewes-Alvarez (1992). Although no measurements of the biomass produced were carried out, *D. rosae* produced more mycelia on PDA than on MEA. The differences in the amount of biomass formed on PDA and biomalt agar were not evident and need to be measured. However, fungal cultures of *D. rosae* maintained on artificial media have been reported to lose pathogenicity within a period of three, five, and six to nine months by Frick (1944), Drewes-Alvarez (1992), and Stewart and Semeniuk (1965) respectively.

Accurate knowledge of the lifecycle of a pathogen provides the opportunity for better control in the sense that one knows when to the pathogen is most likely to be sensitive to the given control methods. In the case of fungicide application this knowledge leads not only to timely applications but also to more economical fungicide use. Timely applications lead to fewer fungicide applications in a season (Bowen and Roark 2001).

The disease control aspect of this study focused on the effectiveness of the new class of fungicides, the strobilurins, in the control of *D. rosae* in comparison to those fungicides that are already in the market. In general, pre-inoculation applications of the strobilurins led to 100 % conidia germination inhibition but post-inoculation applications did control the disease. None of them was able to stop the development of disease symptoms at all post-inoculation intervals tested. Despite the fact that an early application of

pyraclostrobin significantly reduced the disease severity compared to the untreated control, it did not stop the development of the disease symptoms. The application of the strobilurins when the fungus was fully established in the host led to minimal or no reduction in the disease severity. This can be explained by the fact although the strobilurins led to the destruction of the subcuticular mycelia and haustoria within four days after application, new subcuticular hyphae and haustoria were formed with time presumably from the deep seated mycelia, hence the further development of the fungus leading to disease symptom expression. The growth of the fungus in the treated plants was still so robust that the disease symptoms were formed on the same day in both the fungicide treated and the untreated plants. Pyraclostrobin was more effective than trifloxystrobin in all the post inoculation applications, which maybe due to the higher sytemic activity of pyraclostrobin in comparison to trifloxystrobin (Bartlett et al 2001).

The DMI fungicides on the other hand allowed some germination of the *D. rosae* conidia in all their pre-inoculation applications, but none of the germinated conidia were able to lead to disease development. Their application early in the pathogen development led to an almost complete inhibition of symptom development and there was no yellowing or premature defoliation. However, their application just at the onset of symptom development arrested symptom development but in the case of myclobutanil there was yellowing and premature defoliation. This indicates that the DMI fungicides destroyed the fungal structures in the host tissues in such a way that the fungus could not recover. This can be explained by the fact the remnant fungal structures observed after treatment of the plants with the DMI fungicides were scattered with no connection to each other and therefore could not develop further. In addition in plants treated with tebuconazole,

the fungal structures were destroyed beyond recognition, Noegel (1998) had reported on the ability of tebuconazole to deform fungal structures due to its interference with the fungal cell membranes. Tebuconazole was therefore more effective than myclobutanil.

In all post inoculation applications the DMIs were significantly more effective in inhibiting mycelial and haustoria development and in reducing disease severity than the strobilurins. This is maybe because DMIs are more systemic (Kuck et al 1996, Newmann and Jacob 1996, Bartlett et al 2001). Trifloxystrobin and pyraclostrobin have very low movement into the leaf, low translaminar movement and are not xylem or phloem mobile (Bartlett et al 2001), while the DMIs can either be translocated in the apoplast or symplast (Newmann and Jacob 1996). In the post-inoculation treatments all the fungicides were more effective in destroying the subcuticular hyphal network than the haustoria. This is most likely due to the accessibility of the subcuticular hyphae in comparison to the haustoria.

The strobilurins interfere with energy production by blocking ATP synthesis (Ammermann et al 1992, Becker et al 1982, Zheng et al 1997). The inhibition of spore germination by strobilurins is related to the spore's dependency on mitochodrial respiration during the germination process (Gold et al 1996). The two fungicide active ingredients, trifloxystrobin and pyraclostrobin, were however not able to completely stop the development of the *D. rosae* once it was inside the plant. This may be due to the weaker dependency of the mycelia on respiration for vegetative growth and/ or the possibility that an alternate pathway for electron transport may be inducible in the mycelia (Gold et al 1996, Mizutani et al 1995). Despite the fact that the DMIs

(myclobutanil and tebuconazole) allowed germination and germ tube formation, they strongly inhibited the development of and destroyed subcuticular mycelia. The effect of the DMIs can be explained by the fact that during the initial phase of infection, fungi sensitive to sterol inhibitors contain enough ergosterol in the germinating spores to produce germ tubes and infection structures and to penetrate into the host tissue (Noegel 1998). When the fungus has depleted its ergosterol and has absorbed the fungicides a.i. from the plant tissue the effects the chemical become obvious. In DMI sensitive fungi, the take up of the chemical inhibits the production of ergosterol (vital in stabilizing the cell membranes in the fungi) by the fungus and the fungus is killed by the depletion of the sterol building blocks necessary for the cell membrane.

Trifloxystrobin has in previous experiments showed excellent protective as well as curative activities against apple scab (Knauf-Beiter et al 2000). Protective application of 6 mg a.i./l inhibited spore germination by 80% and reduced penetration frequency by 85%, and no visible macroscopic symptoms developed. Absence of macroscopic symptoms led to the assumption that the development of all subcuticular stromata formed at the successful penetration site were completely hindered. At the rate of 20 mg a.i/l spore germination reduced by 85 %, penetration frequency by 98 % and the development of visible disease symptoms completely inhibited. In post inoculation treatments, the application 6mg a.i./l 24 hours post inoculation almost completely inhibited penetration, application 3 dpi led to such great reductions in the stroma development that the disease development was suppressed. Eradicative treatment led to collapse of the already developed fungal structures such as runner hyphae, conidiophores and conidia. The apple scab fungus, *Venturia inaequalis*, develops only subcuticular stromata and their

destruction inhibited further development of the fungus. A single application of trifloxystrobin led to destruction of the subcuticular mycelia of *D. rosae* as well, but did not limit the fungus from further development because the deep seat mycelia formed new subcuticular stromata. This can also be explained in terms of the low systemic properties of trifloxystrobin, therefore the active ingredients is not accessible to the haustoria in the host cells. The fungus therefore continues to absorb nutrients that supports further growth and replacement of the destroyed structures.

In field trials with trifloxystrobin in combination with DMI fungicides, it was found to control major ear and leaf diseases on cereals at the rate of 125-187.5 g a.i./ ha and it provided long lasting protection (Margot et al 1998). On grapes, trifloxystrobin has been found to be effective in controlling the oomycete fungal pathogen *Plasmopara viticola*, which causes downy mildew of grapes and the ascomycete *Uncinula necator*, which causes powdery mildew of grapes (Reuveni 2001). Trifloxystrobin was very effective against conidial germination of *U. necato*r in vitro and it inhibited mycelia growth and sporulation of both fungi in-vivo. Trifloxystrobin had a partial effect on zoospore discharge and suppressed zoospore motility and formation of germ tubes by *P. viticola*. The fungicide a.i. strongly inhibited germination of *U. necato*r at low concentrations and had excellent prophylactic activity against both diseases of grapes. It suppressed sporulation and further development of *U. necator* on grape leaves bearing sporulating colonies or on mildewed clusters in the field. Trifloxystrobin controlled apple diseases, scab and powdery mildew, at relatively low rates; scab at 3.75-5 g a.i /hl in protective or curative schedules and powdery mildew at 5-7.5 g/hl. It controlled the foliar disease of peanut (early and late leaf spots, and rust) at 70-105 g a.i./hl or in combination with

propiconazole at 62.5 + 62.5 g a.i./ hl. Control of black sigatoka of banana was achieved with 70-90 g a.i./hl.

In this study the pre-inoculation application of the strobilurins completely inhibited conidia germination and germ tube formation. A single application of 100 ppm of trifloxystrobin or pyraclostrobin led to limited destruction of the subcuticular mycelia. Studies were carried out by Wojdyla and Orlikowski (1999) to determine the effectiveness of two other strobulurins, kresoxim-methyl and azoxystrobin, on germination of the conidia of *D. rosae* revealed that when the two fungicides were applied 14 d pre-inoculation the inhibition was 86% and less than 40% respectively. Kresoxim-methyl inhibited the germination of *D. rosae* conidia by 86%, while with azoxystrobin the germination inhibition varied from 9-38%. However weekly sprays of the two fungicides on plants with black spot symptoms reduced the disease very significantly compared to the untreated control plants (Wojdyla and Orlikowski 1999). In their study they showed that the two fungicides completely protected the rose shrubs against *D. rosae*. These weekly applications are similar to the protective applications, since the a.i. residues are effective on every new generation of conidia formed.

Pyraclostrobin is relatively new in the market. It has been found to control several plant pathogens; it provided excellent control of white rust of spinach caused by *Albugo occidentalis*, on overwintered spinach (Damicone and Trent 2001), and is incorporated into control programmes of several cereal diseases, fruit crops like grapes and apples, and vegetables like beans and potatoes (BASF 2003).

The findings in this study compare very well with the findings of Gold et al (1996) on the effectiveness of kresoxim-methyl (referred to as BAS 490 F in their report), another strobilurin. Their observations revealed that pre-infection treatments 1 d before inoculation with kresoxim-methyl inhibited spore germination and germ tube growth and penetration of the host plant by all tested fungi. Post infection treatment with kresoxim-methyl strongly inhibited mycelial growth and sporulation of *Erysiphe graminis tritici* and *Uncinula necator*, whose mycelium and spores develop on the leaf surface, but had less pronounced effects on the development of the mycelia of *V. inaequalis* and *Uromyces appendiculatus*, whose mycelia develop in the plant. Since the mycelia of most powdery mildew fungi develop exclusively on the leaf surface, Ypema and Gold (1999) argued that this growth characteristic of the powdery mildew fungal mycelia makes all development stages accessible to the kresoxim-methyl. Gold et al (1996) argued that limited effect on the endoparasites may have been due to the limited up take of the a.i., its metabolic breakdown in the leaves and also due to the relative differences in sensitivity between spore germination and mycelial growth. A single eradicative treatment at 6 dpi, a time when the *V. inaequalis* had developed extensive subcuticular mycelium and the first conidia were forming, of apple plants with kresoxim-methyl at its recommended rate of 67 mg AI l^{-1} completely inhibited macroscopically visible apple scab symptoms and almost completely blocked sporulation. These findings agree with those of Knauf-Beiter et al (2000) in which trifloxystrobin was found to greatly reduce the development of *V. inaequalis* such that the disease development was suppressed. The apple scab fungus, *V. inaequalis*, forms only subcuticular mycelia and therefore the inhibition of the macroscopically visible symptoms can be taken to signify the destruction of these

subcuticular mycelia. There was little inhibition of mycelia growth in *U. appendiculatus* whose mycelium develops in the mesophyll layer. The mycelia of *D. rosae* are formed both in the subcuticular and mesophyll layers of the plant tissue, therefore the effectiveness of kresoxim-methyl on the development of *V. inaequalis* whose mycelia develop in the subcuticular layers and of *U. appendiculatus* in the mesophyll layers of their respective hosts, can be compared to that of *D. rosae*. In the case of *D. rosae* the destruction of the subcuticular mycelia by the strobilurins did not stop the fungus from developing further. This is assumed to be because the mycelia in the epidermal and mesophyll layers formed new subcuticular mycelia, leading to further growth and the development of disease symptoms.

In this study a single application of 100 ppm tebuconazole or myclobutanil as soon as the symptoms had developed gave very good control of the disease in that the disease spots did not develop further. However in treatments with myclobutanil the leaves still turned yellow and defoliation followed soon after that. In previous studies Hagan et al (1991) had shown that weekly sprays of tebuconazole at the rates of 20, 40 and 80 ppm gave only some modest reductions in the disease and that the weekly applications of the fungicide at all these rates were equally effective in controlling the blackspot disease. Although the application of tebuconazole at the above mentioned rates gave a season long reduction is disease ratings compared to the untreated control, heavy leaf spotting and some leaf shed was observed on plants treated annually with this fungicide (Hagan et al 1991). In their studies on plants treated with weekly applications of 48 ppm myclobutanil only isolated spots developed. Based on the results obtained in this study tebuconazole (100 ppm) gave better results than myclobutanil (100 ppm), but Hagan et al

(1991) reported myclobutanil (24 ppm) to be more efficacious than tebuconazole (40 ppm).

In summary, the strobilurins provided excellent protective control and only a minimal curative effect in the control of *D. rosae*. This was attributed to the growth pattern of the fungus and the systemic properties of the strobilurins. In the pre-inoculation applications of the DMI fungicides they did not completely inhibit the germination of the conidia but the germinated conidia did not lead to disease development. Their post-inoculation was very effective. Their late application however still led to disease symptom expression but gave significant reductions in disease severity.

Since the strobilurins inhibited conidia germination but were not able to completely stop the fungus once it was inside the leaves, they should be used protectively. The prophylactic treatments should advisably be made as soon as the weather provides the environmental conditions favourable for the development of *D. rosae*. Prophylactic treatments should be on weekly basis where the temperature is above 15 ^{o}C and the leaves are wet. At 10 ^{o}C and below no fungicide applications are required because the fungus does not lead to disease development at these temperatures. Weekly applications on plants already showing the disease symptoms may be effective at inhibiting the germination of each generation of conidia formed and in the long run reduce disease severity. However where the disease symptoms can already be observed it is advisable to use the DMI fungicides because they are more effective at this stage. In advance disease state mixture of a strobilurin and DMI may give good results. Repeated applications of the strobilurins in a single season should be avoided, to reduce the chance of resistance

build up to these single site inhibitors. The heavy chemical residues associated with the applications of myclobutanil are not desirable and therefore the fungicides should not be applied just before harvest especially in the cut roses.

Recommendations of the control of this pathogen should consider the prevailing weather conditions, the disease pressure, and the mode of action of the fungicides. Bowen and Roark (2001) recommended the winter application of fungicides when the temperature roses above -2.2 ^{o}C for 30 days. According to their field studies winter application of the fungicides led to a delay of disease onset in the following spring, to reduced disease severity and defoliation, increased plant vigour and increased flower production in the following season compared to the untreated plants. In this study the fungus did not lead to disease development at 10 ^{o}C even after 20 days of incubation, therefore based on these findings it is not necessary to apply fungicides to control *D. rosae* at temperatures below 10 ^{o}C. However studies should be done to find out if there are isolates of *D. rosae* that infect rose plants at these low temperatures. A fungicide application in late autumn to destroy the inoculum already present on the plants may reduce the overwintering potential of the fungus. Applications in early spring should be determined by the prevailing weather conditions.

Disease management that combines cultural practices and minimal fungicide is more desirable. Therefore cultural practices such as pruning of spent stems, removal of blind shoots to allow good air circulation within the roses bushes and collection of fallen leaves may reduce the disease pressure. Effective non-fungicidal solutions such as sodium biocarbonate (Bowen et al 1995, Horst et al 1992) where disease pressure is low may

serve as alternative to fungicidal sprays. Planting of resistant cultivars where applicable is recommendable. In the control of *D. rosae* fungicides should be used only when necessary. Repeated application of these two classes of fungicides per season should not exceed the manufacturer recommendations because of the risk of resistance development.

Based on the findings of this study, the early treatment of the infected plants has to be emphasized: in late application some fungicides arrested symptom development but defoliation still occurred. This showed that the damage caused by the disease is greater than just the development of the lesions on the infected leaves. Prophylactic treatments need to be applied before the visible symptoms because the pathogen has a latent period of at least 7 days and not all fungicides are able to arrest the development of symptoms when applied after visible symptoms are already present. Much data has been generated from the various studies done on the interaction of *D. rosae* and its host, and the various methods of control. However a disease-forecasting model is still needed for a more economical and optimised use of all the available methods of control. A disease-forecasting model will also optimise the timings of fungicide applications.

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