CHAPTER 1

INTRODUCTION

The Proteaceae Benth. & Hook. f. is one of the most prominent flowering plants in the southern hemisphere. It is an ancient family made up of two subfamilies (the Proteoideae and Grevilleoideae), which existed before Gondwana began to break up some 140 million years ago. There are about 1,400 species, in more than 60 genera. *Leucospermum (Lsp.)*, *Leucadendron (Lcd.)*, *Banksia* and *Protea* are the genera that are widely used in floriculture. The name *Protea*, given by Linnaeus in 1753, referred to the Greek mythical god, Proteus, who could change his shape at will. It is an apt name due to the diversity of this genus (Rebelo, 1995). The worldwide development of *Protea* has established them as a horticultural crop, with a world sale of approximately 8 million flowering stems per year (Coetzee & Littlejohn, 2001).

The Proteaceae industry in Zimbabwe was founded by a few flower producers in the Eastern Highlands, who began growing proteas in the early 1970's (Archer, 2000). As the industry grew, production areas spread to include Centenary, Chimanimani, Karoi, Makonde, Mvurwi, Norton and Ruwa. In 2001 there was 290 Ha of Proteaceae being grown (Percival, 2002). By 2003, this area had increased to an excess of 350 Ha. There are over 200 growers with plantations ranging from a couple of hundred plants, to 70 hectares in size (Percival, 2004). Between 1997 and 2001 the Proteaceae population in Zimbabwe had doubled to 1,36 million protea plants; of which 42 % was comprised of *Leucadendron*, 39 % *Leucospermum*, 14 % *Protea* and 5 % of other Proteaceae genus, such as *Banksia* and *Grevillea* (Percival, 2002).

Like all floral products, the majority of Proteaceae is exported, and as such, is a major foreign currency earner to Zimbabwe. The main markets include Holland, United Kingdom, South Africa, Germany, Belgium, and Switzerland. During the 1999/2000 season, 18,900 metric tonnes of flowers were exported, of which 10 % were protea (Export Flower Growers Association of Zimbabwe (EFGAZ), 2000). The increases in volumes sold were consistent in 2000 and 2001, at around 30 % per annum (Zimflora, 2003).

The early plantations, or orchards, in Zimbabwe were grown from seed imported from South Africa. Over the last 5 to 10 years, seedling plantations have been gradually replaced

by vegetatively propagated stock of selected species and hybrids. There are very few indigenous Proteaceae in Zimbabwe with commercial characteristics. An example of a successful and widely grown indigenous protea is *Leucospermum saxosum*, chosen for its ability to produce long straight lightweight stems (>40 cm in length) with an attractive flowerhead, good storage and vaselife. It has a long production period (March to September), occurring when market demand is high. *Lsp. saxosum* has a high degree of pest and disease tolerance (Leonhardt, Shingaki & Oka, 2001).

Due to the poor genebank in Zimbabwe, nearly all currently grown commercial protea rootstock has been imported from Australia, New Zealand or South Africa; and all propagation of this stock is done vegetatively. This means that genetic variation in our current rootstock is limited to several 100 plants, representing 20 varieties, brought into the country (Archer, 2000).

Proteas in Zimbabwe are grown in pure stands (monoculture) on commercial plantations. This genetic uniformity within a stand has led to an increasing number of disease problems. Disease is most severe in older orchards; young orchards are relatively clean of disease for the first 2 to 3 years. Many growers have recently experienced huge losses from disease either rendering stems unmarketable, reducing yields, or killing the plant. Plant mortality has been as high as 50 % in some plantations. This amounts to a large loss of revenue from export sales to a grower and the country (Zimflora, 2003).

Many imported protea cultivars, in particular, those from Mediterranean-type regions, have proven not commercially viable in the sub-tropical climatic and soils of Zimbabwe, and are not tolerant to diseases occurring in Southern Africa, such as *Drechslera* spp., *Colletotrichum* spp. or *Fusarium* spp., found in Zimbabwe. Diseases occurring in Mediterranean-type climates are well researched, whereas little attention has been paid to those occurring in summer rainfall areas. Preliminary studies from South Africa suggest that the types and severity of diseases in these areas differ somewhat to those occurring in Mediterranean climates. This was confirmed by the recording of two relatively new diseases in Zimbabwe, *Pestalotiopsis* spp. (Swart, Taylor, Crous & Percival, 1999) and *Sphaceloma protearum* (Teleomorph *Elsinoë* spp.) (Swart, Crous, Kang, Mchau, Pascoe & Palm, 2001).

The pathogen *Sphaceloma protearum*, also know as protea scab, was first isolated by Swart (1998) of the Agricultural Research Council Fynbos Unit, South Africa, from samples

collected from plantations in Marondera, Juliasdale, Norton, Trelawney, Banket and Karoi during a visit to Zimbabwe in March 1998 (Swart, 1998). *Elsinoë,* the teleomorph or sexual state of *Sphaceloma*, causes stem and leaf lesions in young growing tissue. The lesions eventually coalesce, and in severely infected shoots, the tip is killed. This affects production and quality of blooms (Swart, 1998).

A study conducted in Australia on the economic impact of the disease estimated that in 1994 at least 27 % of Proteaceae properties were infected by *Elsinoë* spp. The disease caused a 43 % loss in revenue in affected *Leucadendron* crops, and 80 % in *Leucospermum* crops. This was an average revenue loss of A$20,372 per hectare. Annual costs to the industry were established at A$602,000 (Ziehrl, Pascoe and Porter, 1996).

Extensive surveys to determine the incidence and severity of pests and disease problems affecting protea production were conducted by the Zimbabwe Protea Association (ZPA) in 1997, 1999 and 2001 (Percival, 1997, 2000 & 2002). *Elsinoë* was found to be the most important and devastating disease affecting *Protea* species according to the feedback from 86 % of Zimbabwean protea growers in 1999 (Percival, 2000). Farmers were asked to describe host symptoms and rate the severity of the disease within a plantation. Symptoms of *Elsinoë* spp. infection are distinctive. *Elsinoë* was confirmed to be the causal fungus from isolations and descriptions of infection made by Swart (1998).

Protea compacta x *susannae* cv Pink Ice (*P*. cv Pink Ice) is one cultivar that is very susceptible to *Elsinoë* spp. It was calculated that there were 69,000 *P*. cv Pink Ice planted in Zimbabwe in 1997. This represented 10.1 % of the total Proteaceae cultivated in the country at that time. Since the beginning of 1998, about 37 % of *P*. cv Pink Ice planted were grubbed, because it was uneconomical to maintain the plants due to disease problems. By 2001, this had increased to 77 % of plants due to disease problems caused by *Elsinoë* spp., as well as continual bud damage by *Argyroploce* spp. (black moth) (Percival, 1997, 2000 & 2002). However, removal of plants is not the answer to this problem, as most *Protea* commercial varieties grown in Zimbabwe are susceptible to this disease to some degree. Aversion to planting susceptible genotypes would drastically cut down Zimbabwe's already limited Proteaceae product mix.

Protea cv Pink Ice constituted one of the largest volumes of *Protea* exported to Dutch auction floors from Zimbabwe over the 1995/96 season. Production per plant between 1996-1999 was low (Table 1.1).

Table 1.1 *Protea* cv Pink Ice cut flower production[a] through Zimflora

Season Sept – June	Production (Stems Sold)	No. of Productive Plants [b]	Production [c] Index Per Plant
1995/6	30,898	30,523	1.01
1996/7	35,596	47,301	0.75
1997/8	13,933	41,400	0.34
1998/9	21,936	37,906	0.57
1999/2000	32,447	17,779	1.83
2000/1	32,487	12,400	2.62

[a] Data from Zimflora Private Limited, a flower marketing company who export over 80 % of Proteaceae grown in Zimbabwe

[b] Plants considered productive between 2 and 10 years old

[c] Production index = Production (stems sold) / No. of Productive Plants

Elsinoë had increased in incidence and severity on *Protea* after the 1996/7 and 1997/8 exceptionally wet rainy seasons, and caused dramatic losses in production of *Protea* cultivars. The production index levels fell to its lowest (0.34) in 1997/98 (Zimflora, 2003). Since then, this level has increased primarily due to the drier conditions experienced across the country and sanitation pruning. It is important to understand the epidemiology of *Elsinoë* spp., a disease that is diminishing yields of many normally productive Proteaceae species, cultivars and hybrids.

The disease is difficult to control according to grower reports. However, through information transfer to farmers about the disease and chemical control methods, the disease can be contained. At present there are no chemicals in Zimbabwe registered for use on proteas against any disease or pest. Currently, growers spray with a systemic fungicide, prochloraz manganese chloride complex (Sporgon), once the disease has been observed in new growth.

Elsinoë is being transferred to new plantations via stock that has been produced from cutting material taken from infected motherstock plants. Latent infections can occur on new growth flushes taken for cutting material. This infection is not noticeable on the plant, since no distinct symptoms are visible. The possibility of selecting for greater disease tolerance within species or hybrids from our genebank is limited.

The recent Sanitary and Phytosanitary Agreement ratified by the World Trade Organisation demands a scientific basis for phytosanitary decisions and regulations (Taylor, 2001). It establishes principles that countries are committed to uphold when they work to protect health while trading in plants, animals and their products (World Trade Organisation (WTO), 1994). No other protea producing country has reported infection of *S. protearum* of the kind and severity experienced on *Protea* species and cultivars found in Zimbabwe. The cost to the industry and further spread of the disease threatens the expansion of the industry as a whole and the development of local and export markets. This highlights the need for a study of this pathogen. Scientists visiting from the Fynbos Research Unit, South Africa, believed that it would be disastrous to the *Protea* industry if this destructive fungus, *S. protearum*, established itself in South Africa, especially in the Cape Province region, where *Protea* spp. are cultivated in vast areas. The implications for the trade and exportation of propagated material into other regions could be long lasting.

HYPOTHESIS

1. *Sphaceloma protearum* (Teleomorph *Elsinoë* spp.) infects *Protea* spp.

2. *Elsinoë* spp. are most pathogenic when conditions are extremely wet, and in established orchards.

3. *Elsinoë* can be effectively controlled in established orchards by the use of a combination of fungicides.

OBJECTIVES

1. To characterise the Zimbabwean isolate(s) of the pathogen *Elsinoë*.

2. To study the conditions that are conducive to disease development and survival of the pathogen.

3. To evaluate the efficacy of fungicidal treatments *in vitro* and *in vivo*, and the timing of applications to control the pathogenic stages of *Elsinoë* spp.

CHAPTER 2

LITERATURE REVIEW

2.1 Proteas

A 'protea' is any member of the Proteaceae family. This includes *Banksia*, *Leucadendron*, *Leucospermum* and *Protea,* which are the genera most widely used in floriculture in Zimbabwe (Percival, 2002). Other protea genera grown worldwide are *Aulax*, *Grevillea*, *Isopogon*, *Mimetes*, *Paranomus* and *Serruria* (Coetzee & Littlejohn, 2001). A '*Protea*' is a member of the *Protea* genus.

Protea are only found in sub-Saharan Africa, of which 114 species have been described (Rourke, 1980). The majority of species occur in the Cape Floral Kingdom (or Flora Capensis) (Figure 2.1). They are confined to primarily acidic, nutrient poor soils and have adapted to hot dry summer conditions (Coetzee & Littlejohn, 2001). The Cape Floral Kingdom comprises only 0.04 % of the earth's surface, but due to its remarkable plant species diversity (>8,500 species of flowering plants) and high level of endemism, has been classified as a distinct phytogeographic region (Bond & Goldblatt, 1984). Only 35 *Protea* species occur in tropical Africa (Rebelo, 1995).

All *Protea* are evergreen, woody perennials. Their leaves are sclerophyllous (hard and leathery), which help to prevent water loss and their heavily lignified tissue stops them collapsing when water is scarce (drought tolerant) (Rebelo, 1995). The *Protea* stem grows in spurts, or flushes, during loosely defined growth periods during the year, resulting in clearly defined growth flushes on the stem (Coetzee & Littlejohn, 2001). *Protea* have a dual root system. The primary roots, like other plant genera, reach deep into the ground to anchor the plant and keep it supplied with moisture. These degenerate when flooded with water. For this reason, good drainage is essential. The proteoid roots are highly specialized and unique to Proteaceae. They form at or just below ground surface level and operate in a relatively dry and well-aerated environment. Their function is to absorb and store nutrients from the soil and release them during periods of growth to the plant (Harré, 1995). Regeneration can take place through sprouting from the lignotuber (or rootstock) in some species or by release of achenes. The genus *Protea* is easily recognized by the involucral

bracts which surround the flowerheads, the flowers with 3 completely fused perianth parts and one free, separated part, and their hairy, woody fruits (Rebelo, 1995).

Figure 2.1 Map of Africa, depicting the Cape Floristic Kingdom and the distribution of tropical *Protea* throughout Africa (Coetzee & Littlejohn, 2001)

Almost all cultivated proteas closely resemble those in the wild. This is in sharp contrast to many nursery flowers, for example, roses and chrysanthemums, which have undergone years of hybridisation and improvement over their original species. The majority of commercially used *Protea* flower naturally during the autumn to spring months of the Southern Hemisphere (Coetzee & Littlejohn, 2001).

Protea spp. and cultivars grow best in Mediterranean-type climates with short, wet winters and long, dry summers, such as where the Cape Floral Kingdom is located (Figure 2.1). *Protea* indigenous to this region when translocated to a tropical or sub-tropical

climate, such as in Zimbabwe, are subjected to conditions that are not ideal for plant development. In order to grow protea commercially, a site with excellent drainage and acidic, well-aerated soils is required that is not necessarily rich in nutrients (Coetzee & Littlejohn, 2001). Proteas are therefore planted on sloping ground. Airflow through a plantation is beneficial to the plants, reducing microclimate conditions within the centre of the plants that are conducive to disease development (Harré, 1995).

Improvements in the industry have occurred with such speed that cultural practices have become outdated within a decade. In 1984, proteas were first planted on ridges, like tobacco, instead of flat ground to improve root aeration and soil drainage. The use of irrigation, fertilisers, fungicides and insecticides, once unknown on protea, is now common practise; improving flower quality, yield and return (Archer, 2000). The rapid advances made have been achieved through trial and error of the pioneering protea growers and from some sectors of the agricultural industry. Increasing plant densities has lowered establishment cost per hectare, allowing smaller areas to become more profitable and cost effective. The monoculture of protea on a large scale has resulted in severe disease and insect pest problems. One such disease that is causing major losses is *Elsinoë* scab (Percival, 2002).

2.2 Host Plants of *Elsinoë* spp.

The Loculoascomycete, *Elsinoë*, is responsible for tissue damage of many crops of economic importance in many temperate, subtropical and tropical climates (Fisher, 1969; Gabel & Tiffany, 1987; Munro, Dolan & Williamson, 1988; Ramsey, Vawdrey & Hardy, 1988). Species of *Elsinoë* typically parasitise herbaceous or woody dicots, including raspberry (*Rubus* L.), rough lemon (*Citrus jambhiri* Lush.), sour orange (*C. aurantium* L.) and grapefruit (*C. paradisi* Macf.). The most well known is *E. fawcettii* (citrus scab) which adversely affects the growth of citrus orchards and yields, and also causes economic loss due to poor marketability of crinkled and deformed fruit (Dede & Varma, 1987; Whiteside, 1978 & 1988; Tan, Timmer, Broadbent, Priest & Cain, 1996).

Scab disease, causing severe losses of commercial *Leucospermum* R. Br. plantings, was first observed in South Africa in 1981, and in Australia in 1985 (Benić & Knox-Davies, 1983; Ziehrl *et al*., 1996). The causal agent was identified as a species of *Elsinoë*. It has also been collected from *Banksia* L. f., *Leucadendron* R. Br., *Mimetes* Salisb., *Protea* L. and

Serruria Salisb. in Australia (Ziehrl *et al*., 1996), from *Leucadendron*, *Protea* and *Serruria* in South Africa, and from *Leucospermum* and *Protea* in California and Zimbabwe (Swart *et al*., 2001). There is great variance of susceptibility to *Elsinoë* spp. between Proteaceae cultivars and seedlings (Knox-Davies, Van Wyk & Marasas, 1986). According to a survey of growers in Australia, *Elsinoë* spp. occurred on 27 % of plantations (Ziehrl *et al*., 1996).

2.3 Infection Mechanism and Disease Progression

Elsinoë only infects young growing shoots that are meristematic during the period of exposure or inoculation. This tissue tropism of only immature tissue is thought to be characteristic of all *Elsinoë* spp. regardless of host (Benić & Knox-Davies, 1983; Munro *et al*., 1988; Williamson & McNicol, 1989; Williamson, Hof & McNicol, 1989). It is thought that *Elsinoë veneta* is incapable of penetrating the epidermis of *Rubus* after the cuticle and epicuticular waxes mature, although it has the capacity to invade tissue after infection has been established (Williamson & McNicol, 1989). Older tissue that has been lignified is rarely infected. This could be due to partial restriction of the mycelium to non-suberized cells and polyderm (Gabel & Tiffany, 1987). Munro, Dolan and Williamson (1988) suggested that if only young tissues can be infected, a continuous supply of inoculum with conditions favourable for germination and penetration would be required throughout the season to infect *Rubus* plants over their entire length. The incubation period of *E. veneta* on *Rubus* before symptoms are observed is 3-4 weeks (Munro *et al*., 1988).

Benić & Knox-Davies (1983) suggested that the pathogen, *E. leucospermi*, survives on Proteaceae from season to season in old scab lesions or on host tissue in the form of resting mycelium, stromatic tissue, chlamydospores and microsclerotia. Munro, Dolan and Williamson (1988) reported that early lesions on *Rubus* were initiated by ascospores of *E. veneta* produced in pseudothecia, which are dispersed before new growth on plants commences. Viable conidia of *E. fawcettii* were detected in the stromatic portion of scab pustules on inoculated Valencia orange fruit up to 6-9 months after infection (Whiteside, 1988). Conidia had the potential to cause secondary infection within this period, when conditions were conducive to disease transfer and development on the host. Disease transfer can therefore also occur when plants produced from cutting material are collected from infected stock.

Dissemination of *E. leucospermi* and *E. veneta* chlamydospores and microsclerotia is by rain, irrigation water, wind and spraying operations (Benić & Knox-Davies, 1983; Williamson *et al.*, 1989). Cultural practices will therefore affect scab development. In 1996 and 1997 infection by *Elsinoë* in Zimbabwe was most severe in protea plantations when rainfall was above the average national rainfall (634.9mm); with 700.7mm and 806.7mm rainfall respectively (Dept. of Meteorological Services Harare, 1999). The primary infection develops in wet and humid conditions. It is these conditions that also favour spread of the disease (Munro *et al*., 1988; Wright & Saunderson, 1995). This was confirmed by Ramsey *et al*. (1988) who found that stem and foliage scab (*Sphaceloma batatas*) in sweet potatoes was most severe in mountainous regions where mist, rain and dew were abundant. Dede & Varma (1987) found that severe scab incidence from *E. fawcettii* on rough lemon seedlings was concurrent to areas in Nigeria experiencing high rainfall and humidity. It is interesting to note that in Nigerian savannah regions with low rainfall, some citrus plantations developed scab under conditions of excessive irrigation (Dede & Varma, 1987). Wind dispersal of *E. fawcettii* has been demonstrated by Whiteside (1975). According to Misaki, Tsumuraya & Takaya (1978), *E. leucospila* produces extensive amounts of elsinan (an α-D-glucan) in culture. The glucan is soluble in water to give a highly viscous solution, but at higher concentrations (>2 %), the aqueous solution tends to form a gel. In nature, production of elsinan, under the right conditions, could facilitate adhesion of propagules to the plants from splash or wind dispersal. Insect dissemination is also possible from chewing insects attacking scab lesions (Benić & Knox-Davies, 1983). *E. fawcettii* has been isolated from insect faeces (Jenkins, 1930).

There have been few histological studies, using modern methods such as UV fluorescence microscopy to show autofluorescence of stained plant tissue and the taking of crystat sections, of *Elsinoë* (Benić & Knox-Davies, 1983; Williamson & McNicol, 1989; Gabel & Tiffany, 1987). The disease progression of *E. veneta* on *Rubus* has been described by Williamson and McNicol (1989), and of *E. panici* on *Panicum virgatum* by Gabel and Tiffany (1987). Most *E. panici* infections occurred on the abaxial surface, even though abaxial and adaxial penetration and colonization of the fungus was similar. At infection, the germ tube penetrated the cuticle (Gabel & Tiffany, 1987). Hyphae extensively colonised the primary cortex, developing acervuli containing conidia, and grew along the middle lamella between hypodermal cells and inner parenchyma cells pushing cells upwards. The parenchyma cells, rich in cytoplasm and plastids containing starch, divided 3-4 times within the original mother cell. Increased thickness of diseased leaves was due to the increased

bulk of the stromata. The pseudoparenchymatous cells were compact, thick-walled and darkly pigmented around the periphery of the ascostromata (Williamson & McNicol, 1989; Gabel & Tiffany, 1987). Multiloculate uniascal ascostromata of *E. panici* were observed on *P. virgatum*. Hyphae infected polyderm and non-suberized cells. Phloem fibres, located inside the polyderm, and the middle lamella did not contain hyphae. However, starch was absent from fibre cells adjacent to the mycelium, suggesting starch mobilisation by the fungus. *Elsinoë* penetrated the main vascular tissue by spreading radially between the phloem fibre bundles. Healthy vascular tissue contained extensive starch reserves in plastids, particularly in the ray parenchyma (Williamson & McNicol, 1989; Gabel & Tiffany, 1987). Where thick-walled phloem fibre cells present a complete barrier, *Elsinoë* spp. failed to penetrate vascular tissue. Xylem cells were not penetrated by the fungus (Gabel & Tiffany, 1987). Mature phloem cells were resistant to infection, but if the pathogen reached them before they matured, they became abnormal or were completely suppressed (Williamson & McNicol, 1989).

The yield loss in proteas from *Elsinoë* spp. is due to the killing of shoot tips, and creation of malformed stems and leaves, detracting from the aesthetic value of the bloom, leaving stems unsaleable.

2.4 Host Symptoms, Morphology and Colony Characteristics of *Elsinoë* spp. infecting Proteaceae

Loculoascomycetes are characterised by the presence of a bitunicate ascus with an ascostromatic type ascocarp (Luttrell, 1973). Asci are formed directly in cavities with the stroma or matrix of mycelium and ascocarp (ascostroma). The asci have a characteristic double wall (bitunicate). Other characteristics of this group are haploid mycelium with cross walls, and the production of identifiable types of conidiophores (fruiting bodies). The fungus grows extremely slow in culture on potato dextrose agar. Difficulties in isolation of the fungus occur from contaminants, which out grow *Elsinoë* when grown in culture. Whiteside (1978) recommends swabbing scab pustules with 95 % alcohol to remove some of the contaminating organisms. Colonies grown on potato dextrose agar (PDA) plates were raised, granular and convoluted. *Elsinoë* does not sporulate on PDA (Benić & Knox-Davies, 1983). Production of conidia is sparse and variable. This is thought to be characteristic of *Elsinoë* spp. (Benić & Knox-Davies, 1983; Williamson *et al*., 1989). High

humidity and oxygen levels are thought to be crucial for production of conidia (Williamson *et al.*, 1989).

Based on the internal transcribed spacer (ITS) sequence phylogeny it was found that each *Elsinoë* species has different host ranges, physiological symptoms, and culture characteristics. Molecular tools have become increasingly important in confirming the interpretation of morphological variation; particularly for *Elsinoë* spp. as characterisation of these species is difficult since their teleomorph states are rarely observed and their *Sphaceloma* De Bary anamorphs are morphogically conserved (Tan *et al*., 1996; Swart *et al*., 2001). Random amplified polymorphic DNA (RAPD) markers have proven a quick and useful tool for the identification and differentiation of races and pathotypes of many fungi (Tan *et al*., 1996; Hyun, Timmer, Lee, Yun, Ko & Kim, 2001; Swart *et al.*, 2001).

Swart *et al*. (2001) readily differentiated *Elsinoë* species from Australian, Californian, South African and Zimbabwean biotypes by restriction analysis of the amplified ITS of ribosomal DNA with several endonucleases, by sequencing of the ITS region, and by RAPD analysis. The relatedness between *Elsinoë* species identified on Proteaceae indicates two major clades, *Elsinoë leucospermi* and *Sphaceloma protearum* (Figure 2.2) (Swart *et al*., 2001). Other isolates separated individually. All *S. protearum* isolates used in this study were collected from Zimbabwean plantations (Swart *et al*., 2001).

Figure 2.2 Single most parsimonious tree derived from the alignment of the 5.8S and ITS1 and ITS2 rDNA of 20 *Elsinoë* isolates using parsimony analysis with branch and bound option and 1000 bootstrap replicates. The tree is rooted with the outgroup *Fusarium proliferatum*. The tree length = 239, consistency index = 0.913, retention index = 0.845, rescaled consistency index = 0.772 and homoplasy index = 0.087. The bootstrap values and decay indices are indicated above and below the tree branches respectively (Swart *et al*., 2001)

Five distinct *Elsinoë* Racib. species have been identified on Proteaceae worldwide (Table 2.1) (Swart & Crous, 2001; Swart *et al.*, 2001; Taylor, 2001).

Table 2.1 *Elsinoë* species identified world-wide from hosts *Banksia*, *Leucadendron*, *Leucospermum* and *Serruria*, by country

Species	Australia	South Africa	USA California	USA Hawaii	Zimbabwe
Elsinoë banksiae	B[a]			B	
Sphaceloma spp.	B				
Elsinoë leucospermi	Lcd, Lsp, S	Lcd, Lsp, S	Lsp	Lcd, Lsp	Lcd, Lsp
Elsinoë proteae		P			
Sphaceloma protearum					P
Undetermined species	P		P		

[a]B = *Banksia*, Lcd = *Leucadendron,* Lsp = *Leucospermum*, and S = *Serruria*

***E. banksiae* I. Pascoe et Crous, spp. nov.** (Anamorph. *Sphaceloma* spp.)

Banksia L. f. spp. are the host of this pathogen. *E. banksiae* was identified on *B. serrata* L. f., New South Wales, Australia (Swart *et al.*, 2001).

Lesions 0.5-7 mm in diameter develop as circular to irregular leaf spots. Initially they are small grey specks with a brown margin, encircled by a chlorotic zone. Spots are predominantly epiphyllous on leaves, extending partially through the lamina. Lesions also develop on stems and midribs of leaves, often with large black acervuli appearing as longitudinal ruptures of the epidermis (Swart *et al.*, 2001; Swart & Crous, 2001).

Colonies on MEA are irregular, erumpent and folded with sinuated, smooth margins. Aerial mycelium is sparse. The surface colour of colonies is greenish grey with a grey olivaceous outer red zone. Colonies grew to 8 mm diameter on MEA after 1 month. Attempts of induce sporulation *in vitro* on Fries's medium have been unsuccessful. Cardinal temperatures for growth were minimum 5 ºC, optimum 15 ºC, maximum below 30 ºC (Swart *et al.*, 2001).

***E. leucospermi* L. Swart et Crous, spp. nov.** (Anamorph. *Sphaceloma* spp.)

Hosts of this species include *Leucospermum* R. Br. spp., *Leucadendron* R. Br. spp., *Mimetes* Salisb. spp. and *Serruria* Salisb. spp. (Knox-Davies *et al*., 1986). *E. leucospermi* was identified on the following Proteaceae; *Leucadendron laureolum* (Lam.) Fourc., *Lcd. linifolium* (Jacq.) R. Br., *Lcd. platyspermum* R.Br., *Lcd*. *pubibracteolatum* I. Williams x *Lcd*. *chamelaea* (Lam.) I. Williams, *Lcd*. *thymifolium* (Salisb. ex Knight) I. Williams, *Leucospermum conocarpodendron* (L.) Buek, *Lsp. cordifolium* (Salisb. ex Knight) Fourc., *Lsp*. *cordifolium* cv Vlam, *Lsp. glabrum* E. Phillips x *Lsp. tottum* (L.) Br. cv Scarlet Ribbon, *Lsp. lineare* R. Br., *Lsp. oleaefolium* (Berg.) R. Br., *Lsp. reflexum* Buek. ex Meisn. var. *luteum*, *Lsp.* hybrid (*Lsp. cordifolium* x *Lsp. tottum*) (L.) R. Br. and *Serruria florida* (Thunb.) Salisb. ex Knight (Benić & Knox-Davies, 1983, Knox-Davies, Van Wyk & Marasas, 1986 & 1987, Swart *et al*., 2001). This *Elsinoë* spp. occurs naturally in the south-western Cape in South Africa, from Kogelberg to Elim (Benić & Knox-Davies, 1983). *E. leucospermi* has been reported in Australia, South Africa, California (USA), and Zimbabwe (Swart *et al*., 2001).

E. leucospermi causes a disease called pincushion scab. This disease has been recorded by Benić and Knox-Davies (1983). Small, depressed, elliptical whitish lesions appear on new growth, the surrounding tissue reddens. Lesions augment and coalesce, forming raised reddish-brown areas. These later become corky, roughened and cracked. Leaf lesions are less conspicuous than stem lesions. Heavily infected stems become twisted and deformed. Flowering is reduced, and in extreme cases, infected shoot tips and leaves die (Benić & Knox-Davies, 1983; Von Broembsen, 1989).

Colonies on MEA are irregular, erumpent, folded with sinuate, smooth margins. Aerial mycelium is absent. Colonies are red to coral in colour. Hyaline conidia can be induced using Fries's medium. Cardinal temperatures for growth were minimum above 5 ºC, optimum 15-20 ºC, maximum above 35 ºC (Swart *et al*., 2001).

***E. proteae* P.W. Crous et Swart** (Anamorph. *Sphaceloma* spp.)

E. proteae has been recorded on *Protea cynaroides* (L.) L. and *P. repens* (L.) L. in South Africa (Swart *et al.,* 2001).

Distinctive leaf spots occur on leaves and petioles that are raised, scab-like and white-grey in colour. Black ascomata are visible to the naked eye. Stem lesions can also occur (Swart *et al*., 2001).

On MEA colonies are similar to *E. leucospermi* but with sparse whitish aerial mycelium. The surface colour is rose to red. Older colonies are surrounded by diffuse red pigment. *Sphaceloma* state is induced on Fries's medium. Cardinal temperatures for growth were minimum above 5 ºC, optimum 15-20 ºC, maximum below 30 ºC (Swart *et al*., 2001).

***Sphaceloma protearum* L. Swart et Crous** (Teleomorph *Elsinoë* spp. not observed)

In Zimbabwe, *S. protearum* has been isolated on *Protea compacta* R. Br. x *P. susannae* E. Phillips cv Pink Ice, *P. eximia* (Salisb. ex Knight) Fouc. x *P. susannae* cv Sylvia, *P. neriifolia* R. Br cvs Moonshine and Silvertips, *P. magnifica* Link x *P. susannae* cv Susara and *P. laurifolia* Thunb. cv Regal Mink (Swart *et al*., 2001). *Elsinoë* spp., thought to be *S. protearum* but not formally identified, has been found on *Protea compacta* R. Br. x *P. susannae* E. Phillips cv Pink Ice in Australia (Ziehrl *et al*., 1996).

Symptoms observed on *Protea* occur on growing shoots as leaf and stem lesions. Leaf lesions are red / black in colour. Lesions occur on young stems, which coalesce to form irregular red-black areas. Later the stems become rough and cracked. Where infection is severe, lesions coalesce, causing the whole leaves and shoot tips to die (Swart *et al*., 2001).

Cultures on MES produce red, irregular, erumpent folded colonies with sinuate, smooth margins, with no aerial mycelium. Cardinal temperatures for growth were minimum above 5 ºC, optimum 20-25 ºC, maximum below 30 ºC (Swart *et al*., 2001).

***Sphaceloma* spp.** (undescribed)

Occurs on *Banksia* spp. in Australia. This *Sphaceloma* species was isolated on *Banksia prionotes* Lindl. (Swart *et al*., 2001).

It is distinct from *Elsinoë banksiae*, and is more commonly associated with prominent stem lesions than leaf spots.

Colonies grown on MEA were blood red in colour with a lighter red outer zone, and surrounded by a diffuse red pigment. *Sphaceloma* spp. has optimal growth at 20–25 ºC,

also grows at 30 ºC, appearing more tolerant of higher temperatures than *E. banksiae* (Swart *et al*., 2001).

It is thought that two species of *Elsinoë* are present on Proteaceae in Zimbabwe; *E. leucospermi* and *S. protearum*. The species found on *Protea* spp., namely *S. protearum*, has only been isolated in Zimbabwe. There is the possibility that this pathogen is native to this country on our indigenous proteas. *E. leucospermi* represents the same species infecting *Leucadendron*, *Leucospermum* and *Serruria* in Australia, South Africa, USA and Zimbabwe (Swart *et al*., 2001). It is thought that the disease strains observed on *Leucospermum* were imported into Zimbabwe on infected plant material.

2.5 Control of Scab Disease

Control of latent infections on protea in propagation units can be achieved by dipping cuttings in a fungicide, followed by regular sprays to stop disease development (Benić, 1986). The fungus, *E. veneta*, has an incubation time of about 3-4 weeks before symptoms appear on *Rubus* (Williamson & McNicol, 1989). Mother plants should therefore be sprayed before cuttings are taken (Wright & Saunderson, 1995).

Control measures include use of resistant cultivars, choosing a site with good air-movement, using a drip irrigation system, good sanitation, pruning and strategic spraying of fungicides during growth flushes (Knox-Davies *et al*., 1986). Any cultural practices that reduce relative humidity in the bushes will reduce infection levels. Keeping bushes well pruned and clear of weeds helps to alleviate moist humid conditions, caused from lack of air movement, and reduce infection conditions (Mathews, 1995; Forsberg, 1995). Planting only disease-free material, and in particular, cultivars that have shown resistance, will aid in keeping plantations disease free or at least keep disease occurrence to a minimum. Pruning out and destroying infected material is crucial (Forsberg, 1995). Removal of source material by repeated pruning was shown to reduce 100 % infection early in a season to 0 – 15 %, when combined with routine fungicide sprays (Turnbull, 1995).

At the Tygerhoek Research Farm, Riviersonderend, South Africa, mancozeb (Dithane M45) sprayed at fortnightly applications on infected pincushions obtained good control of *E. leucospermi* (Benić & Knox-Davies, 1983). However Nagata, Hashimoto, DePonte and Ferreira (2002) found that captab (Captan 50% WP) at 2.4 g/L (most effective), followed by

chlorothalonil (Daconil 2787 40% F) at 1.9 g/L, propiconazole (Banner 14.3% EC) at 1.3 ml/L and benomyl (Benlate 50% WP) at 1.2 g/L were better than mancozeb (Dithane F45) 3 ml/L at controlling pincushion scab. Captan is a non-systemic phthalamide, whose mode of action interferes with amino acid production (Agrios, 1969).

In Zimbabwe the incidence of *S. protearum* is higher than that of *E. leucospermi*. Most growers apply mancozeb fortnightly during the rainy season (October to April). Mancozeb is an organic sulphur compound, part of the dithiocarbamates, and is non-systemic. It is fungitoxic, and is able to inactivate the sulphydyl groups in amino acids, inhibiting the production of proteins and enzymes (Agrios, 1969). This fungicide has not successfully controlled protea scab. It may be advisable for plants heavily infected the previous season to be sprayed with a curative chemical before young susceptible shoots emerge. Dichlofluanid controlled cane spot (*E. veneta*) of *Rubus*. The fungicides carbendazim, dicarboximide, iprodione and vinclozolin were ineffective (Munro *et al*., 1988). Only benomyl (a benzimidazole) and chlorothalonil (a benzene compound) significantly reduced the disease 111 days after inoculation with *S. batatas* (Teleomorph *E. batatas*) in sweet potatoes, relative to the control (Ramsey *et al*., 1988). Resistance to carbendazim by *E. veneta* isolate 426 in raspberries (Munro *et al*., 1988) and to benomyl in *E. fawcettii* in citrus plantations (Whiteside, 1980) have been noted in this genus. Benomyl is an organic systemic fungicide, belonging to the benzimidazole group. These compounds interfere with cell nuclear division within fungi by stopping spindle formation, thus preventing mitosis. Translocation of benomyl in woody plants, like protea, is poor as the generated active chemical, methyl benzimidazol-1-yl carbate (MBC), binds with lignin (Agrios, 1969).

Tolylfluanid is used against *E. ampelina* and scab on pome fruit (Bayer, 1997). On account of its mechanism of action, which is directed to multisites on the pathogen, tolylfluanid is suitable for the control of resistant pathogen populations, and also its ability to ‘harden’ the surface of leaves and berries, rendering the establishment and spreading of fungal pathogens more difficult. As a consequence of this crops treated repeatedly with tolylfluanid are characterized by a particularly well developed foliage (Bayer, 1997).

Azoxystrobin is a relatively new product, whose synthesis was inspired by a group of natural products (stobilurin A) with fungicidal activity, that were released by a number of edible mushrooms suppressing fungal growth to help the mushrooms compete for nutrients. Azoxystrobin is active against Ascomycetes, Basidiomycetes, Deuteromycetes and

Oomycetes. It is used to control *E. fawcettii* (citrus scab). The mode of action of azoxystrobin is the prevention of electron transfer between cytochrome b and cytochrome c_1, inhibiting mitochondrial respiration in fungi. This fungicide is a highly potent inhibitor of spore germination and the early stages of fungal development (preventative activity), against post-germination stages of the life cycle in a broad range of fungal species (curative and eradicant activity) and also confers antisporulant action to a wide range of diseases (antisporulant activity) (Zeneca, 1998).

Prochloraz is a system organic fungicide, and falls into the triazole group. Triazole chemicals are able to inhibit ergosterol biosynthesis, disrupting synthesis and function of cell membranes (Agrios, 1969).

CHAPTER 3

IDENTIFICATION AND CHARACTERISATION OF THE PATHOGEN *ELSINOË* SPP. IN ZIMBABWE

3.1 Introduction

Species of *Elsinoë* are responsible for tissue damage of many crops of economic importance (Munro *et al*., 1988; Ramsey *et al*., 1988). *Elsinoë* spp. was first observed on Proteaceae in South Africa in 1981 and was identified as the causal agent of scab by Benić and Knox-Davis (1983). Two species have been identified on *Protea* in southern Africa; *Elsinoë proteae* in South Africa, and *Sphaceloma protearum* in Zimbabwe (Swart *et al*., 2001). *Elsinoë leucospermi*, causing pincushion scab on *Leucospermum*, *Leucadendron* and *Serruria,* is found in both African countries (Swart *et al*., 2001).

In Zimbabwe, the disease known locally as protea scab, caused by *Elsinoë* spp., is the most serious problem encountered on *Protea*. Few *Protea* spp. and cultivars show any sign of tolerance against the pathogen. The disease causes stems and leaves of the host to become distorted and unmarketable resulting in major financial losses of plantations. Entire plantations, made up primarily of *P*. cv Pink Ice, have been destroyed due to high infection incidence and severity. The fungus infects all green tissues of the plant. Red – black leaf and stem lesions develop on young shoot tips, enlarge and coalesce, causing withering and tissue necrosis.

Characterisation of species of *Elsinoë* is difficult since their teleomorph or perfect (sexual) states are rarely observed and their *Sphaceloma* anamorphs are generally morphologically conserved (Swart *et al*., 2001). The objective of this study was to identify the *Elsinoë* spp. causing protea scab in Zimbabwe, characterise the pathogen and determine the best method of inoculation so that Koch's Postulates can be fulfilled.

3.2 Materials and Methods

3.2.1 Identification of pathogen

Samples of diseased plants were collected from 8 protea plantations and 1 indigenous stand in Juliasdale, 6 plantations in Darwendale and 1 in Karoi between January 2000 and June 2002 (Table 3.1 & Figure 3.1).

Table 3.1 Plantations in Juliasdale, Darwendale & Karoi from where plant samples were collected

JULIASDALE	DARWENDALE	KAROI
Aveley Farm	Consolidated Agriculture	Glenellen Estate
Chikomo Estates	Elston Estate	
Froggy Farm	Excelsior Farm	
Gomo Remiti Farm	Howes Farms	
Pine Tree Inn	Mtotwe Farm	
Rodel Orchards	Serui Source Farm	
Rosedale		
Wigmore Investments		
Zorora Trust		

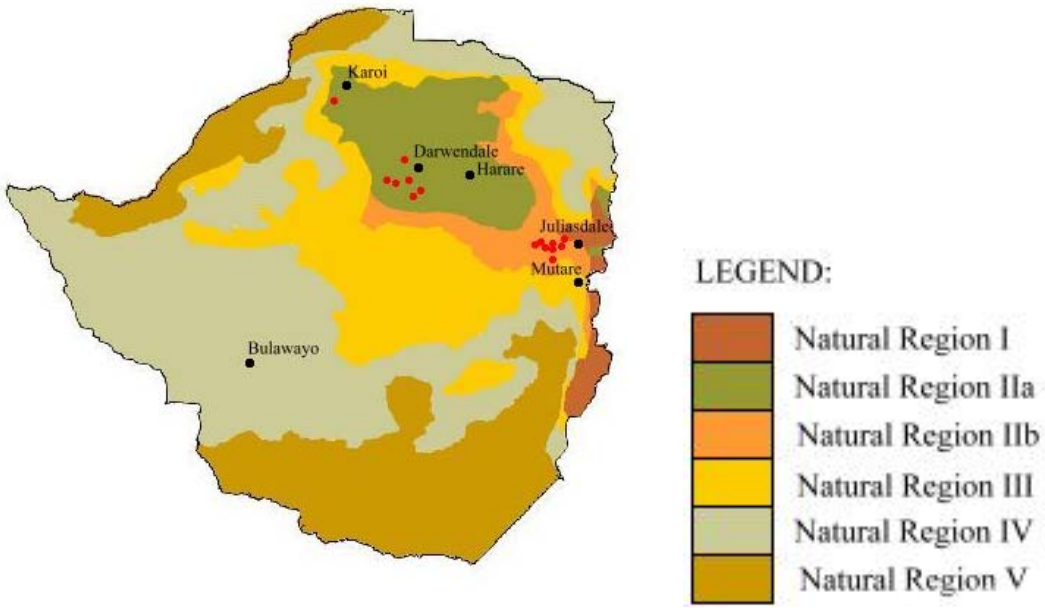

Figure 3.1 Map of Zimbabwe, showing natural regions and locations of plantations where disease samples were collected (1 cm represents 100 km)

Per variety, ten leaf and stem samples showing symptoms of *Elsinoë* infection, were collected from plantations in Julisdale, Darwendale and Karoi regions. A total of 52 sets of samples were collected from 20 *Protea* spp. and cultivars, 2 *Leucospermum* cultivars and 1 *Leucadendron* cultivar. Sample information such as host plant, collection date and source plantation were recorded and an accession number allocated (Table 3.2).

Table 3.2 Accession numbers and data pertaining to suspected *Elsinoë* spp. infected plant samples collected for disease identification

Acc. No	Host[a]	Farm (Area[b])	Collection Date
J01	*P. compacta* x *susannae* cv Pink Ice	Aveley Farm (J)	24 Jan. 2000
J02	*P. neriifolia* cv Moonshine	Aveley Farm (J)	24 Jan. 2000
J03	*P. compacta* x *susannae* cv Pink Ice	Chikomo Estates (J)	24 Jan. 2000
J04	*P. magnifica* x *burchellii* cv Sheila	Chikomo Estates (J)	24 Jan. 2000
J05	*P. eximia* x *susannae* cv Sylvia	Chikomo Estates (J)	24 Jan. 2000
J06	*P. eximia* x *susannae* cv Sylvia	Froggy Farm (J)	24 Jan. 2000
J07	*P. cynaroides*	Froggy Farm (J)	24 Jan. 2000
J08	*P. grandiceps*	Rosedale (J)	24 Jan. 2000
J09	*P. neriifolia*	Rosedale (J)	24 Jan. 2000
J10	*P. repens*	Rosedale (J)	24 Jan. 2000
J11	*P. cynaroides*	Rosedale (J)	24 Jan. 2000
J12	*P. compacta* x *susannae* cv Pink Ice	Rodel Orchards (J)	24 Jan. 2000
J13	*P. neriifolia* cv Moonshine	Rodel Orchards (J)	24 Jan. 2000
J14	*P. eximia* x *susannae* cv Sylvia	Rodel Orchards (J)	24 Jan. 2000
J15	*P. eximia* x *susannae* cv Sylvia	Wigmore Inv. (J)	2 Feb. 2000
J16	*P. laurifolia* cv Regal Mink	Wigmore Inv. (J)	2 Feb. 2000
J17	*P. compacta* x *susannae* cv Pink Ice	Wigmore Inv. (J)	2 Feb. 2000
J18	*P. neriifolia* cv Silvertips	Wigmore Inv. (J)	2 Feb. 2000
J19	*P. neriifolia* cv Moonshine	Wigmore Inv. (J)	2 Feb. 2000
J20	*P. eximia* x *susannae* cv Sylvia	Gomo Remiti Farm (J)	2 Feb. 2000
J21	*P. cynaroides* cv Tsitsikamma	Gomo Remiti Farm (J)	2 Feb. 2000
J22	*P. grandiceps* x *eximia* cv Rosie	Gomo Remiti Farm (J)	2 Feb. 2000
J23	*P. compacta* x *burchellii* cv Brenda	Gomo Remiti Farm (J)	2 Feb. 2000
J24	*P. longifolia* cv Satin Pink	Gomo Remiti Farm (J)	2 Feb. 2000

Acc. No	Host[a]	Farm (Area[b])	Collection Date
J25	*P. compacta* x *obtusifolia* cv Red Baron	Gomo Remiti Farm (J)	2 Feb. 2000
J26	*P. neriifolia*	Gomo Remiti Farm (J)	2 Feb. 2000
J27	*P. repens* cv Guerna	Gomo Remiti Farm (J)	2 Feb. 2000
J28	*P. magnifica* x *susannae* cv Susara	Gomo Remiti Farm (J)	2 Feb. 2000
J29	*P. neriifolia* cv Moonshine	Gomo Remiti Farm (J)	2 Feb. 2000
J30	*P. laurifolia* cv Regal Mink	Gomo Remiti Farm (J)	2 Feb. 2000
J31	*P. laurifolia* cv Regal Mink	Zorora Trust (J)	14 Feb. 2000
J32	*P. neriifolia* cv Silvertips	Zorora Trust (J)	14 Feb. 2000
J33	*P. compacta* x *susannae* cv Pink Ice	Zorora Trust (J)	14 Feb. 2000
J35	*P. eximia* x *susannae* cv Sylvia	Zorora Trust (J)	14 Feb. 2000
J36	*P.* F2 hybrid cv Niobe	Gomo Remiti Farm (J)	3 Mar. 2000
J37	*Lcd. salignum* x *laureolum* cv Safari Sunset	Gomo Remiti Farm (J)	3 Mar. 2000
J38	*P. magnifica* x *susannae* cv Susara	Gomo Remiti Farm (J)	22 Apr. 2001
J39	*P. neriifolia* cv Moonshine	Gomo Remiti Farm (J)	22 Apr. 2001
J40	*P.* F2 hybrid cv Niobe	Gomo Remiti Farm (J)	22 Apr. 2001
J41	*P. neriifolia*	Gomo Remiti Farm (J)	22 Apr. 2001
J42	*P. neriifolia* cv Silvertips	Gomo Remiti Farm (J)	22 Apr. 2001
J43	*P. eximia* cv Fiery Duchess	Gomo Remiti Farm (J)	22 Apr. 2001
J44	*P. grandiceps* x *eximia* cv Rosie	Gomo Remiti Farm (J)	22 Apr. 2001
J45	*P. gaguedi*	Pine Tree Inn (J)	2 Jun. 2002
D01	*P. compacta* x *susannae* cv Pink Ice	Mtotwe Farm (D)	30 May 2000
D02	*P. cynaroides* cv Tsitsikamma	Consolidated Agriculture (D)	30 May 2000
D03	*P. compacta* x *susannae* cv Pink Ice	Excelsior Farm (D)	30 May 2000
D04	*P. eximia* x *susannae* cv Sylvia	Excelsior Farm (D)	30 May 2000
D05	*Lsp. cordifolium* cv Yellow Bird x *patersonii* cv High Gold	Howes Farms (D)	15 Aug. 2000
D06	*Lsp. cordifolium* cv Yellow Bird x *patersonii* cv High Gold	Excelsior Farm (D)	15 Aug. 2000
D07	*P. compacta* x *susannae* cv Pink Ice	Elston Estate (D)	6 Nov. 2000

Acc. No	Host[a]	Farm (Area[b])	Collection Date
D08	*P. compacta* x *susannae* cv Pink Ice	Serui Source (D)	6 Nov. 2000
K01	*P. compacta* x *susannae* cv Pink Ice	Glenellen Estate (K)	1 Jun. 2000

[a] *P. = Protea*; *Lcd. = Leucadendron*; *Lsp. = Leucospermum*

[b] J = Juliasdale; D = Darwendale; K = Karoi

Stem and leaf lesions were swabbed with 95 % alcohol to remove contaminating micro-organisms. Thin sections were cut with a scalpel from infected plant tissue and deposited in a sterile Petri dish. The surface sterilised sections of stem and leaf infected tissue were then transferred onto semi-selective potato dextrose agar (sPDA) (Appendix 1) for 4 weeks at 25 ºC.

Arising fungal colonies from sPDA plates were sub-cultured on malt extract agar (MEA) (Appendix 2). Isolates were observed under a dissecting microscope (Wild M3 Heerbrugg, Switzerland) (x 40) for colonies resembling *Elsinoë* spp. A mycelial plug of *Elsinoë* spp. was inoculated into healthy *P. compacta* R. Br. x *Protea susannae* E. Phillips cv Pink Ice stem tissue. After 3 weeks, the pathogen was re-isolated from the resultant stem lesions. Pure cultures of *Elsinoë* isolates were maintained on MEA slants at 4 ºC for 6 months.

Three cultures of *Sphaceloma protearum* were received from the Agricultural Research Council (ARC) Fynbos Unit, South Africa as positive controls. These cultures were isolated from samples collected in Zimbabwe (Table 3.3) and were determined to be *S. protearum* through its morphology, host symptomatology and ITS sequence phylogeny. The cultures were used to compare with collected isolates for positive identification of *S. protearum.* The colonies are distinct to that of other fungal genus.

Table 3.3 *Sphaceloma protearum* cultures received from the Agricultural Research Council, South Africa

Isolate Number	Host	Geographic Origin
86	*Protea compacta x susannae* cv Pink Ice	Zorora, Juliasdale
87	*P. eximia* x *susannae* cv Sylvia	Wigmore Inv., Juliasdale
88	*P. compacta x susannae* cv Pink Ice	Serui Source, Darwendale

3.2.2 Morphological Characterisation of pathogen

Finely cut free-hand sections of diseased *Protea* leaves were placed on a microscope slide with two drops of a solution of cotton blue in lactophenol. These were observed under a light microscope (Leitz Laborlux K, Germany) (eye piece lens x 10, objective lens x 40). Sexual structures were observed and measured (average of 25 measurements).

Whiteside's (1975) method was used to induce sporulation of three selected *S. protearum* isolates. The isolates J24, J35 and D01 were chosen as they were isolated from different *Protea* cultivars, with a minimum of one isolate from Juliasdale and Darwendale regions (Table 3.2). Sections of mycelial growth were taken from the middle of colonies cultured on MEA and fragmented in a Petri dish into which 10 ml of modified Fries' medium (Appendix 3) was poured. The solution was stirred vigorously to release hyphal fragments. After 2-4 days at 20 ºC colonies attached themselves to the bottom of the Petri dish, which was then flushed with sterile distilled water three times. Colonies were scraped from the dish with a scalpel and transferred to drops of distilled water on microscope slides. Slides were kept at 25 ºC for 2-3 hours at near-ultraviolet light. Morphological characteristics were observed under a light microscope (Leitz Laborlux K, Germany) (eye piece lens x 10, objective lens x 40) and the colour, shape and distinguishing features described, and dimensions measured (average derived from 25 measurements).

3.2.3 Inoculation of Host Plants

A randomised complete block design was implemented in this experiment, using a 4 x 4 factorial arrangement, with 4 replications. The factors were four inoculum (including control) and four inoculation treatments.

Conidia production *in vitro,* using Fries' medium, was sporadic and harvesting of the conidia was poor. Therefore, a mycelium solution was used to inoculate plants. A 1-1.5 cm diameter mycelium plug from 3 isolates (J24, J35 and D04) grown on MEA at 25 ºC for 4 weeks was collected. The isolates were chosen for their quick growth in culture, with a minimum of one isolate from Juliasdale and Darwendale regions. A mashed mycelium suspension was made by crushing the mycelium plug in 50 ml distilled water containing 0.01 % Tween 80. The control contained no isolate.

Protea cv Pink Ice cuttings were vegetatively propagated in pine bark and quarry dust / river sand, using material collected from a mother plant that had no previous *Elsinoë* spp.

infection. The medium was steam sterilized and the rooted cuttings were sprayed fortnightly alternating mancozeb, captan, chlorothalonil and prochloraz to ensure that no *Elsinoë* spp. was present. All fungicide spray routines were ceased 4 weeks before inoculation date. One to one and a half year old plants were inoculated in February 2003.

Inoculations were made shortly before sunset to avoid rapid drying (Whiteside, 1988). Inoculations of the three *Elsinoë* isolates were applied to the first expanding leaf of the growing shoots, and the internode beneath the first expanded leaf tagged to identify tissue exposed to inoculation. A minimum of 4 shoots on 4 plants per treatment were inoculated using the following methods:-

a) injecting the isolate suspension into the young soft stem tissue of the plants,

b) wrapping a 15 x 5 cm strip of absorbent cotton around 2 young leaves and a soft stem, dipping the wrapped section in water and then pouring 5 ml of the solution onto the leaves and stem just above the cotton wrapping,

c) inserting a mycelium plug beneath a small flap of young soft stem tissue and wrapping the wound with a parafilm,

d) spraying a mashed mycelial suspension onto the youngest matured leaves, both unwounded and wounded (by gently rubbing leaves with fine sand),

Distilled water and 0.01 % Tween 80 (method a, b and d), and sterilised agar (method c), were used as a control.

The inoculated tips were covered with a polyethylene bag to maintain high humidity during incubation. The bags were slit to increase ventilation after 24 h and were removed after a week (Williamson & McNicol, 1989; Munro *et al*., 1988; Williamson *et al*., 1989). The cotton wrap was removed after 1 day. Plants were kept in a propagation unit and separated from each other by sheets of plastic to prevent cross-contamination. Humidity was kept high by overhead sprinklers that were turned on 2-4 times a day.

Plants were assessed twice weekly after inoculation to follow disease development. An inoculation efficiency was determined for each treatment, using the formula:

$$\text{Inoculation efficiency} = (x / n) \times 100\ \%$$

whereby x = number of stems that developed lesions per plant

n = total number of stems inoculated per plant

The pathogen was re-isolated from infected *P.* cv Pink Ice tissue that had been inoculated with isolate J35, J24 and D04. The isolation method described in 3.2.1 was used. Cultures were compared with the original inoculates.

3.3 Results

3.3.1 Identification of Pathogen

A brief description of disease symptoms from plants where samples were taken is presented in Appendix 4.

Initial leaf symptoms on *Protea* were small (1-3 mm diam), circular to elliptical, red-brown lesions accompanied by reddening of adjacent tissue, partially extending through lamina, on abaxial and / or adaxial sides of young shoots (Plate 3.1.1). Lesions were primarily located on leaf margins or midrib, however leaf interveinal regions were also infected. Lesions were covered with erumpent conidiomata, which enlarged (3-15 mm), coalesced, becoming irregular, red-black and extended through the lamina (Plate 3.1.2-4). Leaf twisting and deformation occurred (Plate 3.1.5-6, Plate 3.2.1), leading to necrosis, withering and death of shoot tips (Plate 3.2.2-4).

Stem lesions were less conspicuous than leaf lesions. Lesions were whitish to grey, elliptical to irregular, sometimes with reddening of surrounding tissue (Plate 3.3.1-2). Lesions enlarged (2-15 mm diam) and coalesced to form raised, irregular areas, which became roughened and cracked (Plate 3.3.3-5). In extreme cases, shoot tips, leaves and flowers on infected shoots died (Plate 3.3.6).

Scab lesion on leaves and stems are typically seen in clusters, separated by discrete intervals of uninfected tissue.

Clean cultures were initially difficult to isolate. Many contaminants or secondary fungi were isolated from diseased material. It was easy for a culture to become over grown by other organisms present in the lesions. Subsequent isolations were made by vigorously scrubbing plant lesions with 95 % alcohol, and plating out a minute piece of host tissue onto MEA, with added chlorothalonil (see Chapter 5). This fungicide actively controlled contaminants without affecting growth of *Elsinoë* spp. However, cultures did become non-viable after the third successive isolation onto this medium. The third isolation therefore was inoculated onto plain MEA to get continued isolate viability.

The isolates grew extremely slowly in culture. After 12-14 days fungal colonies 2-5 mm in diameter had developed, and after 4 weeks were 10-14 mm in diameter. Sub-cultures of isolates were similar to the colony morphology of *S. protearum* cultures from the ARC. Isolates typically formed very slow-growing colonies. On MEA plates the colonies were irregular, raised and deeply fissured, with smooth margins, light brown to reddish brown in colour, which darkened to red-dark brown as the colony aged (Plate 3.4). A diffuse red pigment was released by the colony into the agar. Occasionally white aerial mycelium was observed.

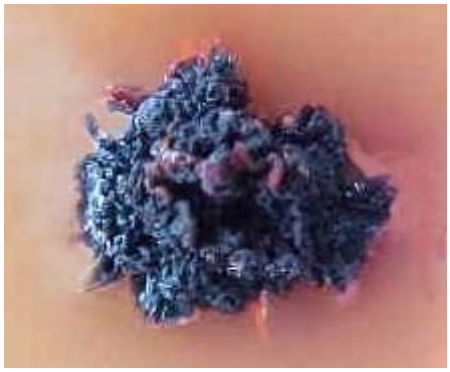

Plate 3.4 Culture of protea scab fungus grown on MEA at 22-25 ºC for 4 weeks

The inoculated *Protea* cv Pink Ice plant developed stem red-brown lesions at the inoculation sites after 3 weeks. Fungal colonies arising from thin sections of these stem lesions when plated onto sPDA were characteristic of the initial inoculum colony.

Sphaceloma protearum was isolated from eleven *Protea* spp. and cultivars (Table 3.4).

Table 3.4 Proteaceae cultivars from which *Sphaceloma protearum* was isolated and positively identified as the causal agent

Host	Isolate
Protea compacta x susannae cv Pink Ice	J01, J33, D01, D07
P. eximia x susannae cv Sylvia	J14, J20, J30, J35, D04
P. grandiceps x eximia cv Rosie	J22
P. compacta x burchellii cv Brenda	J23
P. longifolia cv Satin Pink	J24
P. compacta x obtusifolia cv Red Baron	J25
P. repens cv Guerna	J27
P. laurifolia cv Regal Mink	J31
P. neriifolia cv Silvertips	J32, J44
P. magnifica x susannae cv Susara	J38
P. F2 hybrid cv Niobe	J40

Initial symptoms on *Leucospermum* and *Leucadendron* occur as elliptical white stem lesions (1-5 mm diam) with reddening of the surrounding tissue. Lesions become raised. They enlarge and coalesce forming large areas that are roughened, cracked and corky in appearance (Plate 3.5.1). Heavily infected stems become twisted and deformed.

Leaf lesions are less prominent than stem lesions. They are circular to elliptical in shape (1-3 mm diam), white to tan coloured, developing into rough, raised, scab-like lesions with age (Plate 3.5.2). Lesions extended through the leaf lamina.

The fungus grew slowly in culture. Colonies grown on MEA were raised, convoluted, beige to light brown in colour, which darkened with age. Colonies were 8-15 mm in diameter after 4 weeks.

Two *Elsinoë leucospermi* isolates (D05, D06) were isolated from *Lsp.* cv High Gold and positively identified as the causal agent of the disease, pincushion scab.

3.3.2 Morphological Characterisation of pathogen

The most common fungal structures on diseased host tissue were branched, septate, hyaline to brown acervular stromata, 2.5-4 µm. Mycelium was present internally, and ruptured the leaf cuticle erratically as it developed. Conidiophores were light brown, cylindrical, 0-1 septate, 5.5-6 x 12-19 µm. No conidia were observed on host material. Hyaline, aseptate conidia, (4-)5.5(-7) x (1.5-)2.5 µm, developed in Fries' medium. Conidia were elliptic with a slightly flattened base (Figure 3.2).

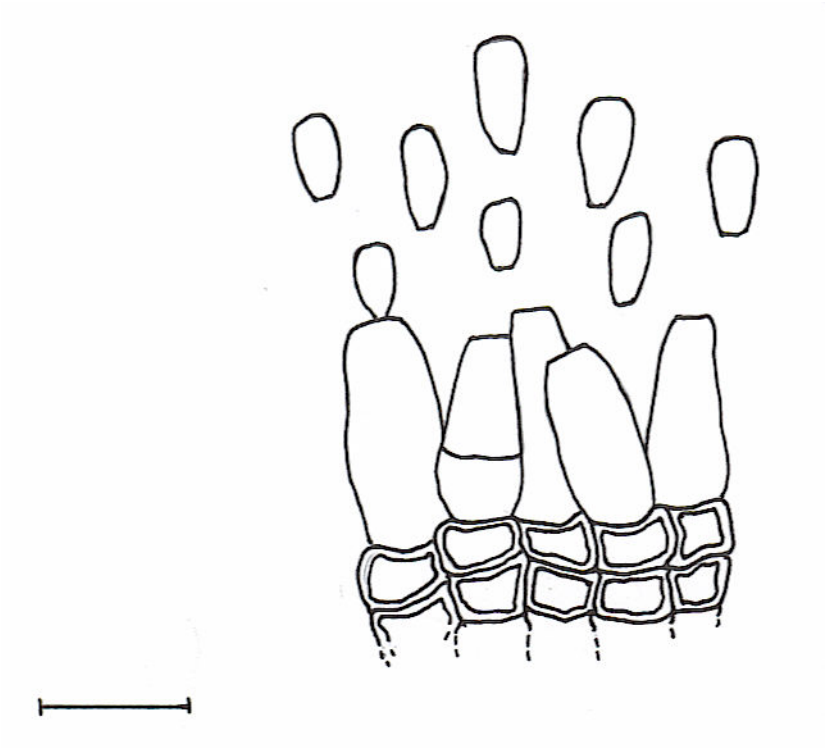

Figure 3.2 Condiophores and conidia of *S. protearum*. Bar = 10 µm

3.3.3 Inoculation of Host Plants

The first lesions occurred in week 3 on at least one shoot of all *P*. cv Pink Ice plants that were inoculated with a mycelium plug into the stem (inoculation method c in materials and methods) (Appendix 5). The inoculation efficiency was 35.4 % in week 3, and 56.3 % in week 4. The lesions appeared as a single, small translucent brown–red blemish, accompanied by reddened surrounding host tissue. Lesions continued to develop over the next 3 to 4 weeks, and were characteristic of *S. protearum* host symptoms as described in section 3.3.1. Isolate J35 had a lower inoculation efficiency at week 4 to J24 and D04; 43.75 % compared to 62.5 % and 62.5 % respectively. The type of isolate used to inoculate had no discernable difference to the timing of the appearance of the first small lesion, or its development.

Only one plant inoculated with isolate J35 through injection of a mycelium suspension (inoculation method a in materials and methods) developed two stem lesions, between week 3 and 3.5. The inoculation efficiency was 2.1 % in week 3, and 4.2 % in week 4.

None of the other methods of inoculation gave positive results. No lesions were observed at the sites of inoculation, and where treatments were representative of the controls.

Samples taken from stem lesions of *P.* cv Pink Ice inoculated with *S. protearum* when grown on MEA were red-brown, irregular, raised and convoluted. These were comparable to original cultures used to inoculate.

3.4 Discussion

The level of genetic variability of *Elsinoë* spp. has allowed distinct species to be classified, especially since host species specificity, symptomatology and morphology differences can be minor (Tan *et al*., 1996; Hyun *et al*., 2001). Swart *et al*. (2001) readily differentiated five scab organisms infecting the Proteaceae. The host *Protea* L. is susceptible to two species of *Elsinoë*; *E. proteae* and *Sphaceloma protearum* (Swart *et al*., 2001).

The *Protea* fungal isolates obtained in this study agree with previously described *Elsinoë* spp. and *Sphaceloma* spp. in respect of very slow growth in culture (Hyun *et al*., 2001; Benić & Knox-Davies, 1983; Whiteside, 1975), production of colonies that are pulvinate (convex) and deeply fissured (Hyun *et al*., 2001; Swart *et al*., 2001), the presence of long thin vegetative hyphae (Benić & Knox-Davies, 1983) and the ability to produce a red pigment (Benić & Knox-Davies, 1983; Swart *et al*., 2001).

The hyaline to brown acervuli and erumpent conidophores from samples of host tissue were similar to those described for *Sphaceloma* (Benić & Knox-Davies, 1983; Swart *et al*., 2001). Conidiophores and conidia were seldom observed, but many species of *Elsinoë* rarely produce abundant conidia *in vitro* (Ramsey *et al*., 1998; Williamson *et al*., 1989; Benić & Knox-Davies, 1983). *E. veneta* and *E. leucospermi* have hyaline elliptical conidia (Williamson *et al*., 1989; Benić & Knox-Davies, 1983). The teleomorph state was not observed, which is not uncommon for *Sphaceloma* spp. (Ramsay *et al*., 1989). Gabel and Tiffany (1987) suggested that sexual structures of *E. panici* may not be easily visible by light microscope techniques.

The fungus caused lesions on both the stem and leaf of *Protea* L. spp. Leaf lesions were typical of that caused by *Sphaceloma protearum*, being small, circular, red-brown in colour, eventually coalescing and killing the entire leaf (Swart *et al*., 2001).

When healthy plants were inoculated with a pure culture of the causal organism, they consistently reproduced disease symptoms, which when re-isolated was identical to the fungus originally isolated. Thus, with Koch's postulates fulfilled, it was concluded that the fungus causing the disease on *Protea* spp. was *Sphaceloma protearum*.

The inoculation method with the highest efficacy was the insertion of a mycelium plug of *S. protearum* into immature stem tissue of the host *P*. cv Pink Ice. This method has also been used to successfully inoculate *E. leucospermi* into *Leucospermum* spp. (Benić & Knox-Davies, 1983). Development of disease symptoms from injection of a *S. protearum* mycelium suspension into host tissue was not consistent. No disease symptoms developed from immersion or sprays of a *S. protearum* mycelium suspension as is recommended for inoculation of *S. batatas* on sweet potatoes (Ramsey *et al*., 1988), *E. fawcetti* on *Citrus* spp. (Whiteside, 1978) and *E. veneta* on *Rubus* spp. (Williamson *et al*., 1989). The success of these inoculation methods is likely to be more dependant on maintaining high relative humidity and temperature levels within a propagation unit, which is difficult to regulate in a large unit as that used in the experiment. Similarly to all inoculation methods using *Elsinoë* spp., disease symptoms only developed on immature tissue (Whiteside, 1978; Benić & Knox-Davies, 1983; Ramsey *et al*., 1988; Williamson *et al*., 1989).

Protea scab has been the most important disease of *Protea* spp. in Zimbabwe. Eleven *Protea* species and cultivars were identified to be susceptible to infection by *Sphaceloma protearum*; eight of which have not been previously documented. These include *P. grandiceps* Tratt. *x eximia* (Salisb. ex Knight) Fourc. cv Rosie, *P. compacta* R. Br. *x burchellii* Stapf cv Brenda, *P. longifolia* Andrews cv Satin Pink, *P. compacta* R. Br. *x obtusifolia* H. Buek ex Meisn. cv Red Baron, *P. repens* (L.) L. cv Guerna, *P. laurifolia* Thunb. cv Regal Mink, *P. neriifolia* R. Br. cv Silvertips and *P*. F2 hybrid cv Niobe. The parents of these cultivars are all indigenous to Mediterranean type climates found in the Cape Floral Kingdom. No tolerant cultivars were observed. Severe infection of stems caused large yield losses, when deep penetrating lesions damage the vascular tissues and cause distortion or death of lateral shoots. *S. protearum* was identified from 4 of the 9 protea plantations in Juliasdale, and 3 of the 6 plantations in Darwendale.

It is suspected from the host symptoms that *P. cynaroides* cv Tsitsikamma (J07 and J21) could have been infected with *E. proteae.* *E. proteae* has been isolated from *P. cynaroides* (L.) L. before by Crous in South Africa (Swart *et al*., 2001). This *Elsinoë* spp. has not been identified in Zimbabwe. No viable cultures were obtained from plant samples J07 and J21. Disease lesions were found on the leaves and petioles of last season's growth. Symptoms were similar to that described by Swart *et al*. (2001), although lesions were more grey-tan than grey-white, and were also found on the stem. The non-viability of the fungus could be due to fungicide sprays that had been applied routinely during the 1999/2000 rainy

seasons. No recent infection symptoms have since been observed at these plantations. The optimum growth temperature for *E. proteae* is between 15-20 ºC (Swart et al., 2001). It may be that the conditions for survival of this species require a more moderate climate.

It was concluded that the pathogen isolated from *Leucospermum cordifolium* cv Yellow Gold x *patersonii* cv High Gold was *Elsinoë leucospermi*. This is the only known *Elsinoë spp.* affecting *Leucospermum* spp., *Leucadendron* spp. and *Serruria florida* (Thumb.) Salisb. (Swart *et al*., 2001). The identification of this fungus agrees with the previously described *E. leucospermi* with respect to host symptoms and colony morphology (Benić and Knox-Davies, 1983; Swart *et al*., 2001).

Sphaceloma protearum causes protea scab on *Protea* spp. and cultivars in Zimbabwe. Insertion a mycelium plug into immature host tissue was the most efficient inoculation method of *S. protearum*. Pincushion scab, caused by *Elsinoë leucospermi,* was found infecting *Leucospermum* spp.

CHAPTER 4

STUDY OF THE CONDITIONS THAT ARE CONDUCIVE TO DISEASE DEVELOPMENT AND SURVIVAL OF THE PATHOGEN, *ELSINOË* SPP.

4.1 Introduction

Elsinoë spp. infects young, actively growing tissue (Ziehrl *et al*., 1996; Whiteside, 1978). Older tissue is rarely infected. Disease symptoms are seen in discrete sections along a stem, indicating the need for particular conditions to be present for infection to occur during the growing season (Ziehrl *et al*., 1996; Munro *et al*., 1988).

Moisture favours infection and disease development, with scab tending to be most severe in regions where rain, mist and dews are abundant. *Sphaceloma batatas* (Teleomorph *Elsinoë batatas*) was found to sporulate on the surface of lesions during wet weather (Ramsey *et al*., 1988). Benić & Knox-Davies (1983) noted that shaded *Leucospermum* plants, and the shaded areas within plants, were noticeably more subject to pincushion scab, caused by *E. leucospermi*, than those plants, or portions of plants, in exposed sites. The optimal conditions of humidity and temperature or period of leaf / stem wetness are unknown (Benić & Knox-Davies, 1983; Ziehrl *et al*., 1996).

Sphaceloma protearum causes severe disease symptoms on *Protea* spp. when climatic conditions are hot and wet. Usually scab disease is first noticed only when symptoms have become severe, since earlier and more subtle symptoms are liable to be missed initially. Fungicide treatments if implemented late are not effective at controlling the pathogen. Late treatment resulted in a loss of revenue from the sale of poor quality disease damaged stems. In this study, it was decided to elucidate the climatic conditions that cause re-initiation of fungal development and host disease symptoms.

4.2 Materials and Methods

4.2.1 Optimum Growth Conditions of the Pathogen *In vitro*

A randomised complete block design was implemented in this experiment, using a 3 x 5 x 5 factorial arrangement, with 4 replications. The factors were: three isolates of *S. protearum*, five relative humidity levels and five temperature levels.

Mycelium plugs (2 mm squared), were taken from the actively growing edge of colonies from three isolates on MEA, and inoculated centrally into MEA plates. There were 4 replicate plates per treatment for each isolate (J35, J40 & D04). The isolates were chosen for their quick growth in culture, with at least one isolate originating from Juliasdale and Darwendale.

Relative humidity was obtained using five saturated solutions of salts (Commonwealth Mycological Institute, 1968) (Table 4.1). Salts were added to 50 ml distilled water until no further salt could be dissolved. An A5 piece of newsprint paper was placed in each saturated salt solution. The soaked paper was folded in half and placed around a set of isolate plates, both of which were then inserted into a plastic bag and sealed.

Table 4.1 Humidity control by saturated solutions of salts

Salt, saturated solution	RH per cent At 20 ºC
$CaCl_2.6H_2O$	32
$NaNO_2$	66
NH_4Cl	79
$ZnSO_4.7H_20$	90
$Na_2SO_3.7H_2O$	98

Sets of humidity treatment plates were kept in a dark incubator at 5 ºC intervals between 15 ºC and 35 ºC. After 4 weeks, colonies were scraped free of the agar, and dried in a kiln at 45 ºC. Dry weight measurements of the fungus were taken. ANOVA (SAS) was used to analyse data (SAS, 1995).

An experiment to deduce the minimum, optimum and maximum temperatures, or cardinal temperatures, required for conidia germination was not possible due to the poor and inconsistent sporulation of *S. protearum* on Fries' medium.

4.2.2 Survival of Pathogen in the Field

The experiment was conducted at Rodel Orchards, Juliasdale (Natural Region I) (18º43'S, altitude 1820 m), where there was a known recurrence of *S. protearum* on *Protea* cv Pink Ice. Plants were grown on ridges 1 m apart and 1.8 m between rows. Cultural practises, such as watering via a micro-jet system, weeding and spraying for insect pests, were implemented as and when required.

Spore traps were set up in three sites diagonally spaced within a block of *P.* cv Pink Ice, and at two heights, giving a total of six traps (Jenkyn, 1974). The spore traps consisted of a microscope slide clipped vertically on to a block that was drilled into a stake (Plate 4.1.1-3). A Perspex rain shield (12 cm diameter) was attached to the stake, above the block. The sticky surface consisted of a piece of single sided cellotape 14 mm wide and 50mm long, coated with vaseline over a length of 30 mm on the smooth side and secured to a glass microscope slide (200 mm x 750 mm). The extra length of cellotape was labelled with the date and height position. Each trap was set up with the stake facing south and with two microscope slides per stake, one sited with the sticky surface 500 mm above the ground (at half the height of the *P.* cv Pink Ice) and the other 1500 mm above the ground (above the *P.* cv Pink Ice). The traps were sited in the plant row to be out of the way of the sprayer. The labelled microscope slides were changed weekly and kept separate so that date and position could be identified. The number of spores, across the width of three random sections of the cellotape on each spore trap, were counted. The counts were converted to a mean number of conidia/cm, using ANOVA (SAS, 1995).

Climatic records of the plantation's daily maximum and minimum temperature (ºC), relative humidity (%), soil moisture levels (centibars), soil temperature (ºC) and rainfall (mm) were taken. The maximum and minimum, and wet and dry bulb thermometers were kept in a Stevenson screen stationed adjacent to the plantation. The rain gauge was placed in an open area at the edge of the plantation. Tensiometers (at 30 cm and 60 cm depth) and the soil thermometer were placed in one of the ridges between two *P*. cv Pink Ice, located in the middle of the plantation.

Samples of lesioned and symptomless tissue were collected from known host plants, weeds, other protea or crops in the surrounding area. Samples were plated on MEA for 4 weeks at 25ºC. The cultures and original host samples were observed under a light microscope (Leitz Laborlux K, Germany) (eye piece lens x 10, objective lens x 10, x 40) to determine the presence and structural stage of *Elsinoë* spp.

Disease assessments were done monthly on 25 % of the plants (200 plants), rating the first 5 pairs of leaves on 5 stems per plant, throughout the 2000/1 and 2001/2 wet seasons. Stems were selected randomly, 3 on the north facing side of the plant and 2 on the south. These assessments were rated on a 1-6 scale (Ramsey *et al*., 1988). Whereby:

1 = shoot tips free of disease (Plate 4.2.1),

2 = less than 5 scab lesions on leaves, may be slight leaf distortion, no stem lesions (Plate 4.2.2),

3 = 5-50 scab lesions on leaves, lesions on leaf veins causing slight leaf distortion, stem lesions (Plate 4.2.3),

4 = more than 50 scab lesions on leaves, leaves distorted, stem lesions (Plate 4.2.4)

5 = more than 50 scab lesions on leaves, scab lesions confluent over large areas of the stem, severe leaf and terminal distortion (Plate 4.2.5), and

6 = more than 50 scab lesions on leaves, leaves dead, severe leaf and terminal distortion, terminal meristem killed (Plate 4.2.6).

Disease progress curves (DPC) were constructed from disease incidence data (Strange, 1993) and analysed using SAS (1995).

4.3 Results

4.3.1 Optimum Growth Conditions of the Pathogen *In vitro*

Because of the irregular growth of the fungi (occurring in 3 dimensions), the growth of the fungus could not be measured in 2 directions at right angles as originally planned. It was therefore decided to use the dry weight of the fungi as a measurement of growth. The average dry weight of three isolates grown at five temperatures and five relative humidity levels are shown in Appendix 6. The dry weight is representative of the growth of isolates under various conditions. Isolate, temperature and relative humidity all significantly affected growth at a 99.9 % confidence level (Appendix 7). There were significant reactions between isolate x temperature, temperature x relative humidity, relative humidity x isolate, and isolate x temperature x relative humidity.

The three-way interaction between isolate, temperature and relative humidity is shown in figure 4.1. The maximum temperature for survival of the *S. protearum* is below 30 ºC. No growth of any isolate occurred at 30 and 35 ºC at any level of relative humidity. There was no comparable difference in growth of isolate J35 at 15 ºC regardless of relative humidity. However at 20 ºC, at 90-98 % growth increased from 27 mg to 46 mg in weight. There was little change in dry weight of J35 at other relative humidity levels at this temperature. Dry weight was greatest at 25 ºC for relative humidity levels 79 % (69 mg), 32 % (62 mg), 66 % (56 mg) and 90 % (52 mg), except for 98 % relative humidity, which decreased slightly from the weight at 20 ºC (46 to 41 mg). For isolate J40, the dry weight at 90 % relative humidity at 15 ºC was considerably higher than all other relative humidity levels (31 mg compared to 1-17 mg). At 20 ºC the heaviest dry weight obtained was at 66 % relative humidity (53 mg). All other relative humidity levels peaked on the growth curve at 25 ºC, with the most growth at 32 % (62mg), 79 % (56 mg) and 90 % (53 mg) relative humidity. Dry weight values of isolate D04 at 15 ºC were considerably heavier than for other isolates. At 20 ºC, only 79 % relative humidity resulted in an increase in dry weight of D04. There was a decrease in dry weight for all other relative humidity levels. Growth was highest at 25 ºC for relative humidity levels of 66 % (84 mg), 79 % (79 mg) and 32 % (73 mg).

Conidia plate inoculations could not be performed due to the poor and inconsistent sporulation obtained using Whiteside's method. The spores that did form tended to stick to the surface of the glass microscope slides and could not be transferred into a solution.

4.3.2 Survival of Pathogen in the Field 2000/1

Old protea scab stem lesions were present on plants from infection during the previous rainy season. Initial symptoms developed on young, meristematic tissues of the leaf and stem; either from a shoot arising after pruning within the plant, or from a new growth flush of a stem at the top of the plant. After heavy rains new leaf lesions could be seen developing immediately below an old protea scab lesion (Plate 4.3).

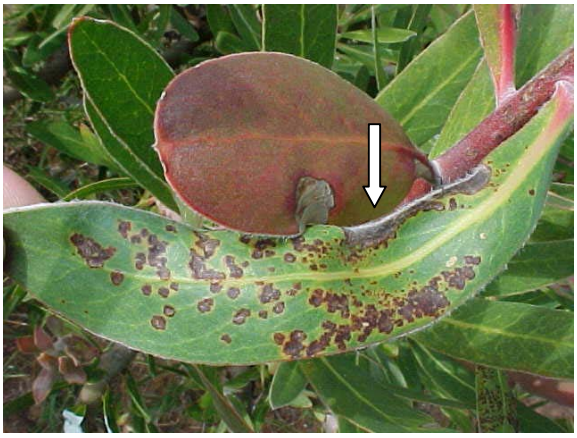

Plate 4.3 New protea scab lesions infection caused by water dispersal

Conidia and ascospores were caught in the spore traps. There were hyaline conidia present from other pathogens of similar shape, but only those within the range, (4-)5.5(-7) x (1.5-)2.5 µm, for *S. protearum,* were counted. Ascospores were seldom observed on spore traps. Ascospores were hyaline, ellipsoid, 4 transversely septate, and 0-1 vertical septa, 11-13(-16) x 4-5 µm (Figure 4.2).

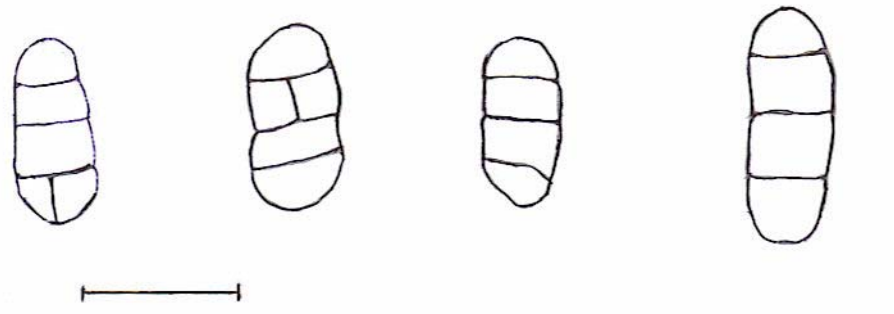

Figure 4.2 *Elsinoë* spp. Ascospores. Bar = 10 µm

The teleomorph state has not been previously documented for *S. protearum*. Morphological characteristics were similar to that described for *E. proteae* and *E. leucospermi* (Swart *et al*., 2001), and therefore included in spore counts. Sightings tended to occur early on in the season, with an initial increase in conidia production during December to February.

The timing and increase in spore counts correlated to periods of host infection during the 2000/1 season (vertical lines), and, three weeks later, increased incidence and severity of disease symptoms of host tissue as shown by increased disease scores (arrows) (Figure 4.3). There was no significant difference between spore counts at mid-plant height (from lower spore traps) to that above plant height (from upper spore traps) ($P > 0.05$).

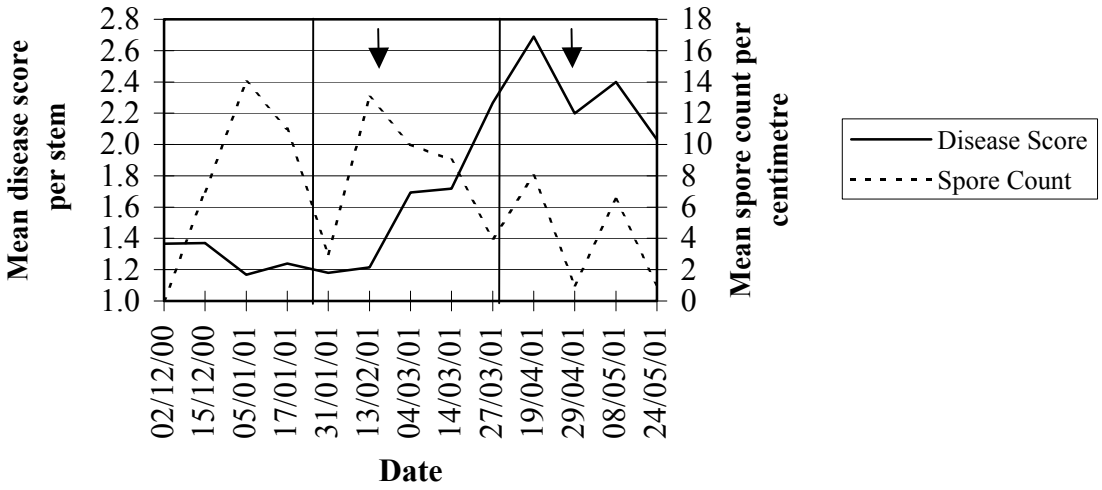

Figure 4.3 Weekly totals of spore counts and disease development in *Protea* cv Pink Ice during the fungicide trial conducted in 2000/1

Climatic data shown relates to the period of protea scab infection and disease development. The arrows highlighting dates 13/01/01 and 19/04/01 on climatic graphs for the 2000/1 season (Figures 4.4-6) relate to the period where protea scab symptoms on *P*. cv Pink Ice were first observed to increase i.e. a rise in the mean score per plant (Figure 4.3). It had already been determined that the period from *S. protearum* infection to visible host symptoms on *P.* cv Pink Ice was 3 weeks (Chapter 3). Vertical lines transect figures at the date of suspected infection for ease of reference. The maximum temperatures at 21 days before 13/01/01 and 19/04/01 were in the range of 22-27 ºC, and minimum temperatures (10-)12-14 ºC (Figure 4.4).

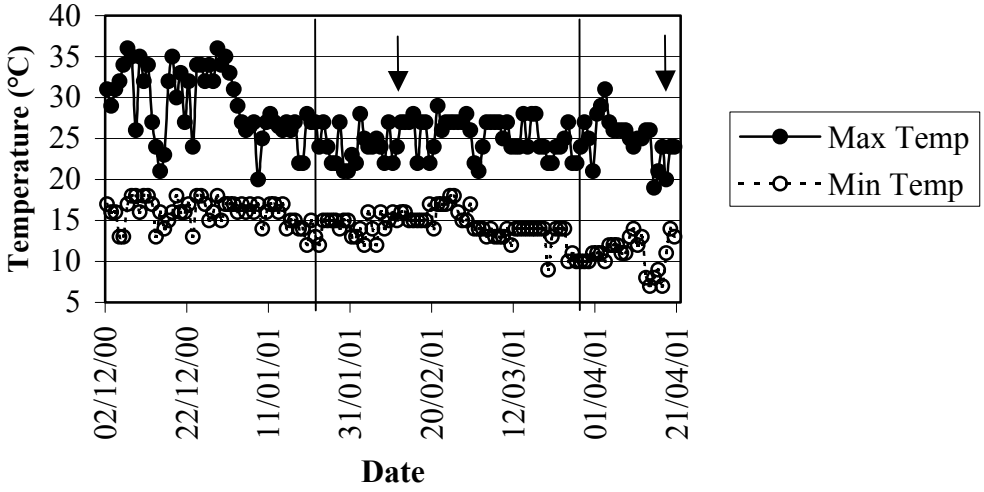

Figure 4.4 Maximum and minimum temperatures at Rodel Orchards, 2000/1

Relative humidity was 65-90 % for first infection period and 45-90 % for the second (Figure 4.5). Rainfall had occurred within the prior week.

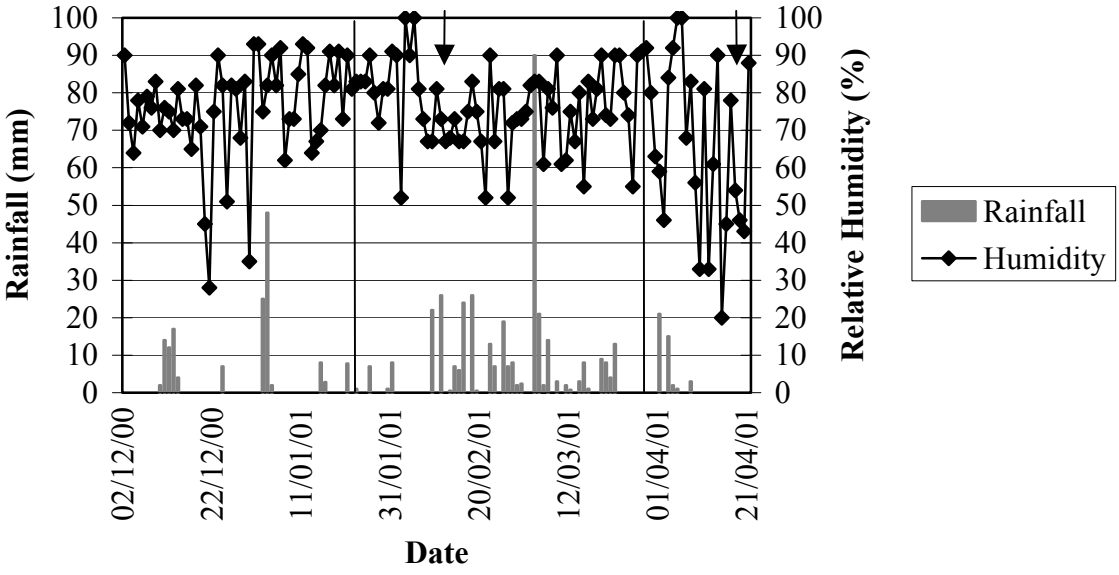

Figure 4.5 Rainfall and relative humidity at Rodel Orchards, 2000/1

Soil moisture is of particular significance, coinciding immediately before periods of increased mean disease scores (Figure 4.6). The water pump had broken down during both periods of infection, as seen by the dry soil conditions (peaking at 78 and 59 cb, respectively). This would have caused severe water stress on the plants.

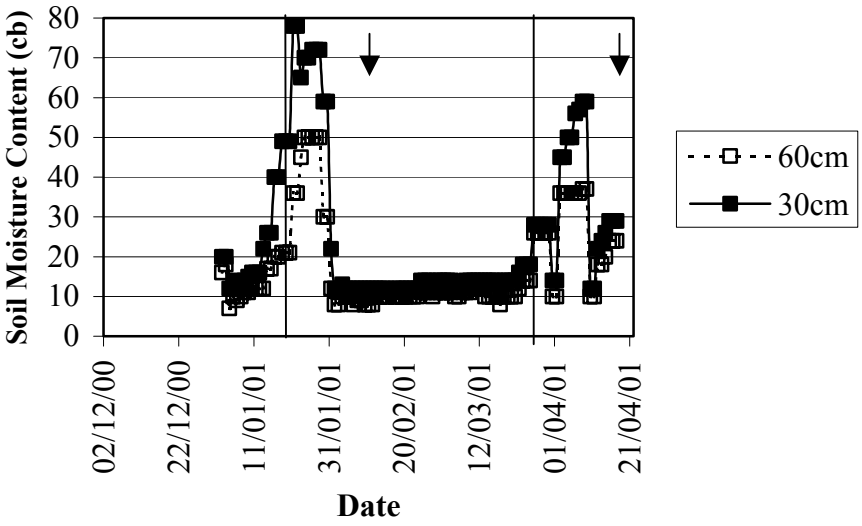

Figure 4.6 Soil moisture readings at 30 and 60 cm depth on Rodel Orchards, 2000/1

Soil temperature during this period ranged from 17-20 ºC.

Conidia levels did increase from the first day that disease scores were taken (Figure 4.3), but high temperatures (25-36 ºC) experienced limited the pathogens ability to survive in host tissue (Figure 4.4). Host infection only took place when conditions were between 22-27 ºC, 65-90 % and when plants were more susceptible to infection were due to water stress (Figure 4.4-6).

4.3.3 Survival of Pathogen in the Field 2001/2

There was a positive correlation between cycles of spore counts and disease infection (Figure 4.7). An increase in the mean number of spores found on the spore traps correlated to an increase in disease scoring, or disease incidence and severity, three to four weeks later.

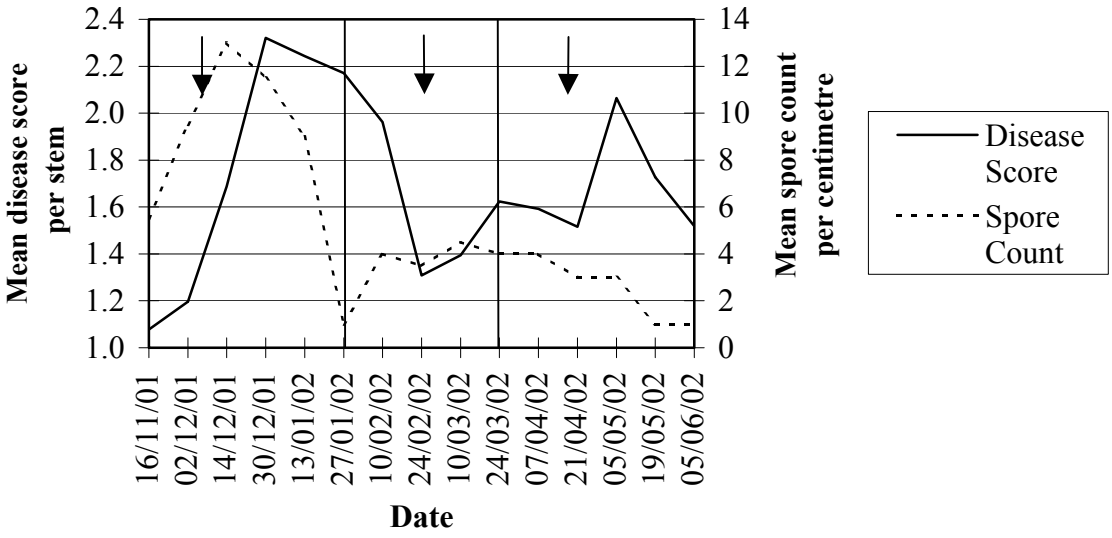

Figure 4.7 Spore count weekly totals and disease development in *Protea* cv Pink Ice during the fungicide trial conducted in 2001/2

Mean disease score per stem rose on dates 2/12/01, 24/02/02 and 21/04/02 for the 2001/2 season (Figure 4.7). The maximum temperatures 21 days before these dates were in the range 24-29 ºC (Figure 4.8). Minimum temperatures were 17-19 ºC for day 17, and 6-10 ºC for days 100 and 156.

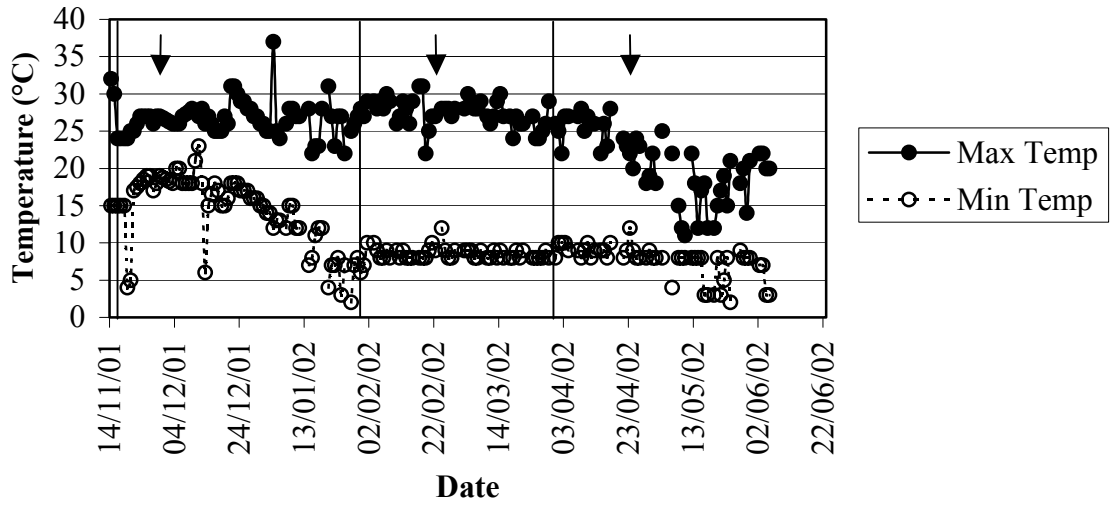

Figure 4.8 Maximum and minimum temperatures at Rodel Orchards, 2001/2

Relative humidity was 45-100 %, with rainfall above 30 mm, during periods of disease development (Figure 4.9).

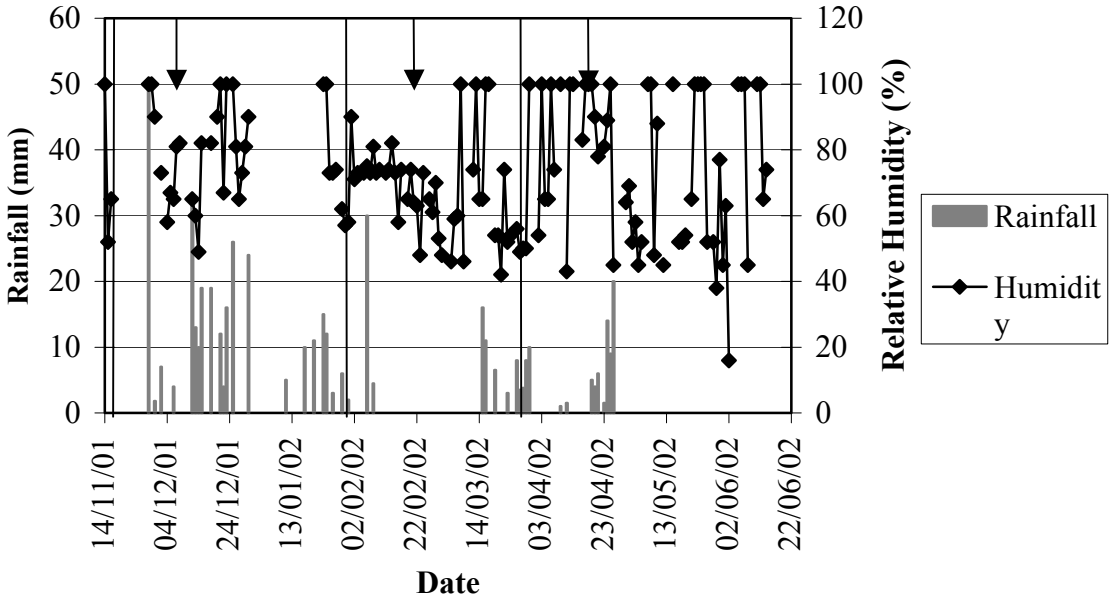

Figure 4.9 Rainfall and relative humidity at Rodel Orchards, 2001/2

Extreme soil water shortage again coincided with the start of disease cycles on *P.* cv Pink Ice (dates 2/12/01 and 24/02/02 only) (Figure 4.10).

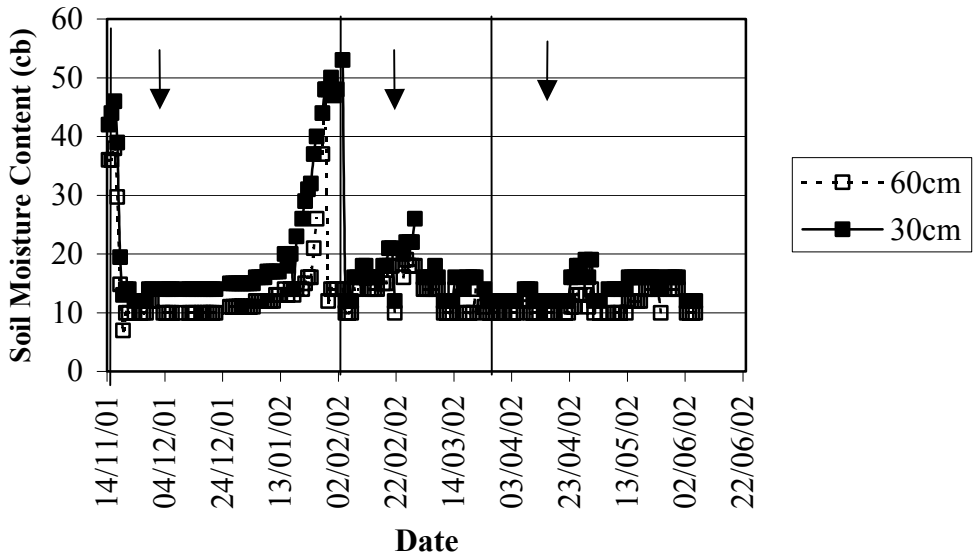

Figure 4.10 Soil moisture readings at 30 and 60 cm depth on Rodel Orchards, 2001/2

Soil temperatures were consistently between 16-20 ºC between 14/11/01 and 22/06/02.

S. protearum was consistently isolated from samples collected from *P.* cv Pink Ice, and *P. eximia* x *susannae* cv Sylvia, situated with the sample field block during 2000/1 and

2001/2. Observations of plant samples revealed that conidia from mycelial conidiomata as the main source of infection. No asci or ascospores were observed in host tissue. However, some ascospores were occasionally present on the leaf surface and were of similar shape and size to that observed on spore traps.

4.4 Discussion

The lack of success of conidia plate inoculations could be due to the secretion of substance, such as elsinan, which is produced by *E. leucospila* to aid adhesion of propagules to a surface (Misaki *et al*., 1978). Further research is required.

Cardinal temperatures were optimum at 25 ºC (humidity levels 90 % and below) and 20ºC (98 % humidity), and maximum below 30 ºC. Temperature results correlated with data from Swart *et al*. (2001) for *S. protearum*. *E. proteae* had optimal growth at 15-20 ºC, enabling temperature to be a factor in confirming species identification of *Elsinoë* spp. infecting *Protea* spp. (Swart *et al*., 2001). This was not the case for many *Elsinoë* spp. infecting other hosts, whose morphological and cardinal characteristics differences are minor (Tan *et al*., 1996; Hyun *at al*., 2001).

The dry weight of isolate D04 (Darwendale, Natural Region IIa) was consistently heavier than isolates originating from Juliasdale, Natural Region I. Infection of susceptible hosts with D04, under the right climatic conditions, resulted in tissue damage occurring at a quicker rate.

The incubation period of 3-4 weeks before the first appearance of lesions agreed with observations of *Elsinoë* spp. (Swart & Crous, 2001), *E. leucospermi* (Benić & Knox-Davies, 1983; Ziehrl *et al*., 1996) and *E. veneta* (Munro *et al*., 1988; Williamson *et al*., 1989). Disease symptoms always occurred on *Protea* shoots that were meristematic at the time of infection. This is thought to be characteristic of all *Elsinoë* spp. regardless of host (Benić & Knox-Davies, 1983; Williamson & McNicol, 1989; Munro *et al*., 1988). Older tissue was rarely infected by *S. protearum*, only occurring when infection had already been established. Similar observations were made by Williamson & McNicol (1989) of *E. veneta* on *Rubus,* and by Gabel & Tiffany (1987) of *E. panici* on *Panicum*.

Links explored by correlating the morphological over-wintering stage of *Elsinoë* spp. with the timing of pathogenic infection symptoms on the plants revealed that initial infection by ascospores or resting mycelium, in old scab lesions. The characteristics of ascospores have not been documented for *S. protearum*. Ascospores observed were hyaline and ellipsoid, like *E. banksiae*, *E. leucospermi* and *E. proteae* (Swart *et al*., 2001), but differed in size and number of transverse and vertical septa. Double walled asci would have formed within an ascostromatic type ascocarp, each containing eight ascospores as is

characteristic for all Loculoascomycetes (Luttrell, 1973). Asci would release ascospores from old scab lesions as they degraded. Munro, Dolan & Williamson (1988) reported that *E. veneta* overwintered on *Rubus* in a similar state, as did *Elsinoë* spp. in *Leucospermum* (Benić & Knox-Davies, 1983). No chlamydospores and microsclerotia, sometimes associated with *Elsinoë* spp., were observed in *Protea* host tissue. However, after initial infection, conidia production was the prevalent source of infection. *S. protearum* over-wintered in the trial plants, *P.* cv Pink Ice, as well as other susceptible *Protea* spp. nearby. Disease onset and progress were influenced by the physiological age of tissue maturity. If overwintering stems are heavily infected it may be justified to destroy the inoculum before susceptible shoots or fruit emerge (Munro *et al*., 1988; Whiteside, 1988). Sanitation pruning in October alleviated disease pressure. The disappearance of scab from previously infected *Citrus* plantations has been attributed to the loss of inoculum-producing ability of the pustules (after 10-12 months) on the previous year's fruit before the next crop became exposed to infection (Whiteside, 1988). The length of time that the *S. protearum* can survive as a viable propagule is an area that requires future research.

There was no significant difference between spore counts at mid-plant height to that above plant height. Wind dispersal of *E. fawcetti* was demonstrated by Whiteside (1975). The fact that conidia and ascospores were found on spore traps above plant height and that disease symptoms were observed at a distance of 20-40 cm above old lesions suggests that wind dispersal of *S. protearum* is possible. Dissemination of *Elsinoë* spp. can also be by water (Benić & Knox-Davies, 1983, Williamson *et al*., 1989). Rainfall and heavy dew dispersed propagules over shorter distances through gravity to infect *Protea* tissue lower down on the plant. Fall out from drops splashing upwards would allow new infection just above old lesions. Water dispersal of propagules up the plant over a longer distance would be possible from sprays of chemical applications and overhead irrigation.

Much of the work on the influence of water potential on fungal germination and growth has been determined using osmotically controlled systems using salts, sugars or glycerol (Magan, 1988). Whiteside (1975) found that conidia production was highest at 24 ºC (optimal between 21-27 ºC) on *E. fawcettii*. Microcolonies that were dried and then exposed to 50-60 % relative humidity for 2 days at 24 ºC retained their ability to produce conidia. However relative humidity was required to be above 95 % for conidiophores on host tissue to continue their growth as hyphae, or if wetted, produce hyaline conidia (Whiteside, 1975). Environmental conditions observed in the field concurred with *in vitro*

results. Initial infection occurred during periods of hot, humid weather, at the start of the rainy season. Infection of *Protea* host material by *S. protearum* was likely when environmental conditions were 20-27 ºC with 45-90 % relative humidity, particularly when plant immunity was low during periods of water stress / drought.

If some factor of the environment is unfavourable to the host, various physiological processes, including disease resistance are commonly impaired. The effect of stress generally is not on initial establishment of the pathogen in the host, but rather on development of established infections (Cook & Papendick, 1972). This was highlighted by the regular increase in disease scoring after infection during both the 2000/1 and 2001/2 seasons during periods of water restriction. Water management should be considered not only in terms of satisfying the plantations water requirement, but also its effect on disease. Plants may be more or less susceptible to adverse effects of water stress depending on the stage of growth. Cook and Papendick (1972) reported that the most critical period for moisture in the growth of spring wheat was the jointing stage. Stress at flowering was less detrimental to yield.

Initial infection of *Protea* spp. by *S. protearum* is through wind or water dispersal of ascospores or resting mycelium. The fungus infects actively growing host tissue during periods of hot (20-27 ºC) and humid (45-90 % relative humidity) weather. Disease symptoms are observed 3-4 weeks after infection. Plants under stress, such as lack of water, are particularly susceptible to infection.

CHAPTER 5

EVALUATION OF FUNGICIDE TREATMENTS *IN VITRO* AND *IN VIVO* TO CONTROL THE PATHOGENIC STAGE OF *SPHACELOMA PROTEARUM* INFECTION

5.1 Introduction

Sphaceloma protearum infects *Protea* spp., causing heavy losses in yield and income. To date very little work has been carried out on the chemical control of this pathogen. Cultural control has focused on the removal and destruction of diseased material from plantations, as *Elsinoë* spp. survive for long periods in infected tissue (Forsberg, 1995; Ramsey *et al*., 1988). Keeping bushes well pruned by thinning out non-saleable stems, and cleared of weeds improves air movement through the plants, alleviating conditions such as high humidity within the plants, which aid disease development (Mathews, 1995).

No fungicide trials have been completed on *S. protearum*. Chemical control has been based on fungicide trial results of *E. leucospermi*. Experimental work by Nagata *et al.* (2002) concluded that captan was most effective at controlling pincushion scab. This was followed by chlorothalonil, propiconazole, benomyl, and lastly, mancozeb. However, results of chemical control of *S. protearum* in protea plantations using these fungicides have been disjointed and not conclusive.

Elsinoë leucospermi and *S. protearum* have different morphology, host specificity and geographic distribution, which may translate to different tolerances to fungicides. This experiment was therefore carried out to determine the efficacy of fungicide treatments on *S. protearum* and deduce an effective spray program regime.

5.2 Materials and Methods

5.2.1 *In vitro* fungicide trial

A randomised complete block design was implemented in this experiment, using a 4 x 4 x 8 factorial with three replications. The factors were: four isolates of *S. protearum*, four fungicide treatments and eight fungicide concentrations. Malt extract agar (MEA) was amended with fungicides to obtain concentrations of 0, 1, 5, 10, 50, 100, 500 and 1000 µg a.i./ml at 50 °C after autoclaving for 25 minutes (Appendix 8). Aliquots of 7 ml were conferred into 10 cm Petri dishes. Initial fungicides tested were chlorothalonil (Bravo 50 % f.w.), mancozeb (Dithane M45 80 % w.p.), prochloraz manganese chloride complex (Sporgon 50 % w.p.) and azoxystrobin (Ortiva 25 % s.c.) (fungicide experiment 1). Azoxystrobin plates at 500 and 1000 µg a.i./ml were not tested, as these concentrations were greater than the commercial recommended field concentrations.

The trial was repeated using 1000 µg a.i./ml chlorothalonil in addition to the fungicide treatments. Chlorothalonil was used to control contaminants, as it had no effect on the growth of *S. protearum*. A randomised complete block design was implemented in this experiment, using a 3 x 5 x 8 factorial with three replications. The factors were: three isolates of *S. protearum*, five fungicide treatments and eight fungicide concentrations. Fungicide experiment 2 treatments were captan (Captan 50 % w.p.), tolylfluanid (Eurapen 50 % w.p.), mancozeb (Dithane M45 80 % w.p.), prochloraz manganese chloride complex (Sporgon 50 % w.p.) and azoxystrobin (Ortiva 25 % s.c.). Concentrations were as for fungicide experiment 1.

Four *S. protearum* isolates (J24, J31, D01 and D04) were used to inoculate fungicide plates (fungicide experiment 1). Isolates were chosen for their relatively quicker growth rates compared to other isolates, and the fact that they were isolated from different *Protea* host cultivars originating from Juliasdale and Darwendale regions. Three mycelium plugs (2 mm squared) of each isolate, cut with a sterilised scalpel from the actively growing edge of a 4-6 week old colony on MEA, were inoculated centrally onto a fungicide plate. Three replicates were made of each fungicide treatment. The control plates were MEA without the addition of fungicide. Plates were sealed in a plastic bag and incubated at room temperature (22-25 ºC) for 6 weeks. The plates were weighed (plate wet weight) at 0 weeks (t_0), and every 2 weeks thereafter, until 6 weeks (t_6). After this time, colonies were scraped free of agar, dried in a kiln (48 °C for 5 days) and reweighed (dry weight). Only dry weight

measurements of isolates J24, D01 and D04 were taken after 6 weeks incubation at room temperature (25 ºC) for fungicide experiment 2. Isolate J31 was not used as it had lost its viability from extended use on selective MEA with added chlorothalonil.

A separate *in vitro* fungicide trial (fungicide experiment 3) was conducted on an *E. leucospermi* isolate (D06), a different *Elsinoë* species affecting *Leucospermum* and *Leucadendron* cultivars in Zimbabwe. This was done to ascertain the effectiveness of chemical control against *E. leucospermi* and compare it to the results obtained against *S. protearum*. Isolate D06 was obtained from *Lsp. cordifolium* cv Yellow Bird x *patersonii* cv High Gold, Darwendale. A complete randomised block experimental design was used, with a 4 x 8 factorial and three replications. The factors were: four fungicide treatments and eight fungicide concentrations. Dry weight measurements (mg) were obtained using the method described above for the second *in vitro* trial. Chlorothalonil (Bravo 50 % f.w.), 1000 µg a.i./ml, was added in addition to fungicides tested: tolylfluanid (Eurapen 50 % w.p.), mancozeb (Dithane M45 80 % w.p.), prochloraz manganese chloride complex (Sporgon 50 % W.P.) and azoxystrobin (Ortiva 25 % s.c.). Concentrations were as for fungicide experiment 1. Dry weight measurements were taken after 6 weeks incubation at room temperature (25 ºC).

Results were analysed using SAS, General Linear Model procedures (SAS, 1995). The concentration of active ingredient that causes a 50 % reduction in colony growth (EC_{50}) was determined (Koomen, Cross & Berrie, 1993).

5.2.2 *In vivo* fungicide spray trial

Site. Rodel Orchards, Juliasdale (Natural Region I), latitude 18º43' S, altitude 1820 m. The field trial was carried out within a commercial plantation in Juliasdale, where there was a known recurrence of *S. protearum* on mature *Protea* cv Pink Ice, planted in 1994. The plants were grown on ridges 1 m apart and 1.8 m between rows. The plantation was irrigated during the dry season using micro-jets. A minimum of 40 *Protea* cv Pink Ice plants were used per fungicide treatment of which there were five treatments, with 4 replicate plots. Three rows of untreated plants were left between treatment plots to reduce the effect of fungicide drift. Cultural practises such as weeding, irrigation by microjet and harvesting of marketable stems were done as and when required.

Fungicide Treatments. Since only young, immature tissue is infected by *S. protearum*, the fungicide treatments began with the first rains, when conditions were conducive to disease development, namely December 2000 to May 2001, and November 2001 to June 2002. Tolylfluanid, due to its poor control of *S. protearum in vitro,* was not tested in the field. Five fungicide treatments were applied weekly through a 20 litre Hatsuta AM-351M mist-blower (Appendix 9). The spray treatments were:

a) Farmer's practice: weekly sprays alternating mancozeb (Dithane M45 80 % w.p) with captan (Captan 50 % w.p.); when disease symptoms were observed, 2 sprays of prochloraz manganese chloride complex (Sporgon 50 % w.p.) were applied 5 days apart in-between routine spraying (positive control),

b) weekly sprays of captan (Captan 50 % w.p.) plus benomyl (Benlate 50 % w.p.),

c) weekly sprays of prochloraz manganese chloride complex (Sporgon 50 % w.p.),

d) weekly sprays of mancozeb (Dithane M45 80 % w.p.),

e) weekly sprays of azoxystrobin (Ortiva 25 % s.c.).

The fungicide concentrations used in the spray tanks are listed in Table 5.1. A volume of 6.7 m^3/Ha was sprayed. Care was taken to spray on calm days to keep spray drift to a minimum. Border rows were not sprayed. The spray tank was thoroughly cleaned and rinsed before applying the next fungicide treatment.

Table 5.1 Spray concentration of fungicides (as recommended by the manufacturers)

Active Ingredient (a.i.)	% a.i.	Rate a.i. (mg / l)
benomyl	50	500
captan	50	2000
captafol	80	3200
mancozeb	80	1600
prochloraz	50	500
azoxystrobin	25	250

Disease assessments were done monthly on 25 % of the plants (10 plants) per plot, as described in 4.2.2.

Disease progress curves (DPC) were plotted (Strange, 1993). To deduce the effectiveness of the various fungicides, the area under the disease progress curve (AUDPC) was calculated:

$$AUDPC = \sum_{i}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i)$$

(Campbell & Madden, 1990), using the trapezoidal integration function in Sigma Plot 2000. Standardised AUDPC was calculated to compare the AUDPC for seasons 2000/1 and 2001/2, where the epidemics did not have the same length of time. A standardised AUDPC is calculated by total duration ($t_n - t_1$) resulting in a scale 0-1, with proportions (Campbell & Madden, 1990). Results were subjected to an analysis of variance using SAS, General Linear Model System (SAS, 1995).

5.3 Results

5.3.1 *In vitro S. protearum* fungicide trial

The mean plate wet weight results of mycelial inoculations of *S. protearum* isolate J24 for weeks 0 (t_0), 2 (t_2), 4 (t_4) and 6 (t_6) weeks per treatment are seen in Appendix 10. Fungicide plates missing a reading at 6 weeks (t_6) were heavily contaminated, and the weight therefore disregarded. As can be seen from the data there was a negative growth rate, for example, of the azoxystrobin control plates from 27.957 g in t_0 to 26.483 g at t_6. Data for isolates J31, D01 and D04 is not shown. The difference in weight between plates for all isolates at week 2 ($t_2 - t_0$), 4 ($t_4 - t_0$) and 6 ($t_6 - t_0$) with week 0 (t_0), are tabulated in Appendix 11 & 12. All results gave a negative growth rate to that being observed on the plates.

A new method to indicate fungal growth was required. Therefore, dry weight measurements were taken of isolate colonies at week 6 (t_6) (fungicide experiment 1) (Table 5.2). A dry weight measurement is the sum weight of the three colonies inoculated per treatment plate. There was a strong correlation of decreasing dry weight of isolates to increasing fungicide concentration for all treatments, except with chlorothalonil, where the reverse was true.

Table 5.2 Mean dry weight of *Elsinoë* isolates after 6 weeks (t_6) on fungicide plates of varying concentrations (fungicide experiment 1)

Fungicide	Concentration	Mean dry weight of isolate at 6 weeks (mg)			
	(µg a.i./ml)	J24	J31	D01	D02
azoxystrobin	0	239	114	149	39
	1	25	43	7	19
	5	32	31	24	32
	10	37	94	75	54
	50	6	22	70	16
	100	2	70	29	36
chlorothalonil	0	193	114	149	39
	1	189	106	80	104
	5	166	148	109	101
	10	138	107	112	158
	50	58	111	115	76
	100	197	137	189	116
	500	210	133	119	132
	1000	233	198	161	136
Mancozeb	0	4	75	2	2
	1	2	10	9	48
	5	4	3	18	8
	10	101	<1	11	<1
	50	18	<1	16	5
	100	39	<1	<1	<1
	500	<1	<1	3	<1
	1000	<1	<1	<1	<1
Prochloraz	0	4	75	2	2
	1	61	63	56	48
	5	<1	<1	<1	8
	10	4	<1	<1	<1
	50	4	<1	<1	5
	100	<1	<1	<1	<1
	500	<1	<1	<1	<1
	1000	<1	<1	<1	<1

Analysis of variance (SAS, 1995) of dry weight results of fungicide experiment 2 revealed that all fungicide, isolate and fungicide concentration interactions were significant to at least a 99 % confidence level, except [isolate x concentration], which was not significant (Appendix 13). Azoxystrobin had the greatest effect on *Sphaceloma* growth, which had mean dry weight measurements of 7.3 mg, followed by captan (20.4 mg), prochloraz (32 mg), mancozeb (41.8 mg) and lastly, tolylfluanid (136.1 mg). Isolates collected from Darwendale (D01 & D04) were significantly more tolerant to fungicides than isolate J24, from Juliasdale (Figure 5.1).

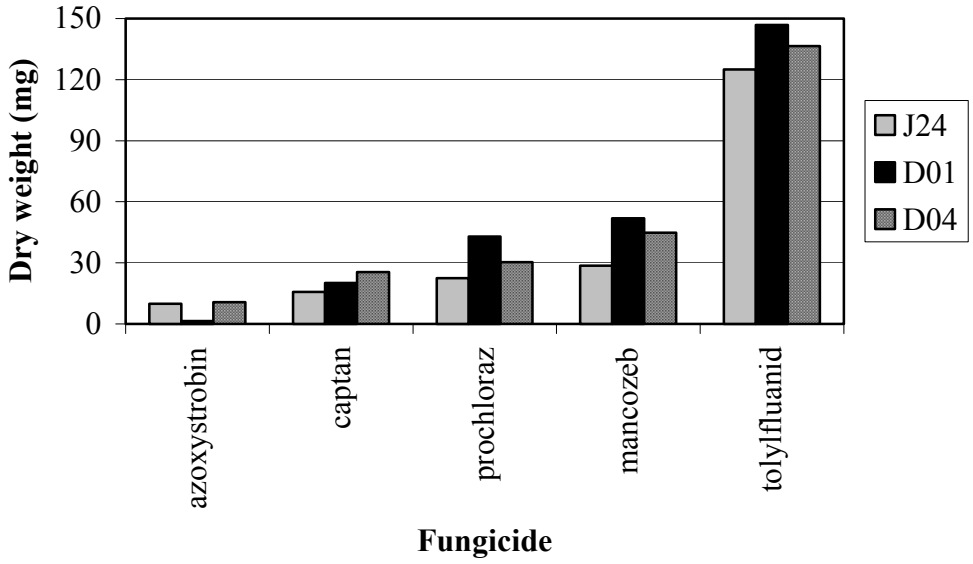

Figure 5.1 The mean dry weight showing the relationship between fungicide and *Elsinoë* isolates (SE = 9.1 g)

An increase in active ingredient, or fungicide concentration, caused colony dry weight to decrease, as fungal growth was restricted (Figure 5.2). However, 10 µg a.i./ml resulted in slightly heavier colonies than 5 µg a.i./ml, but the difference was non-significant.

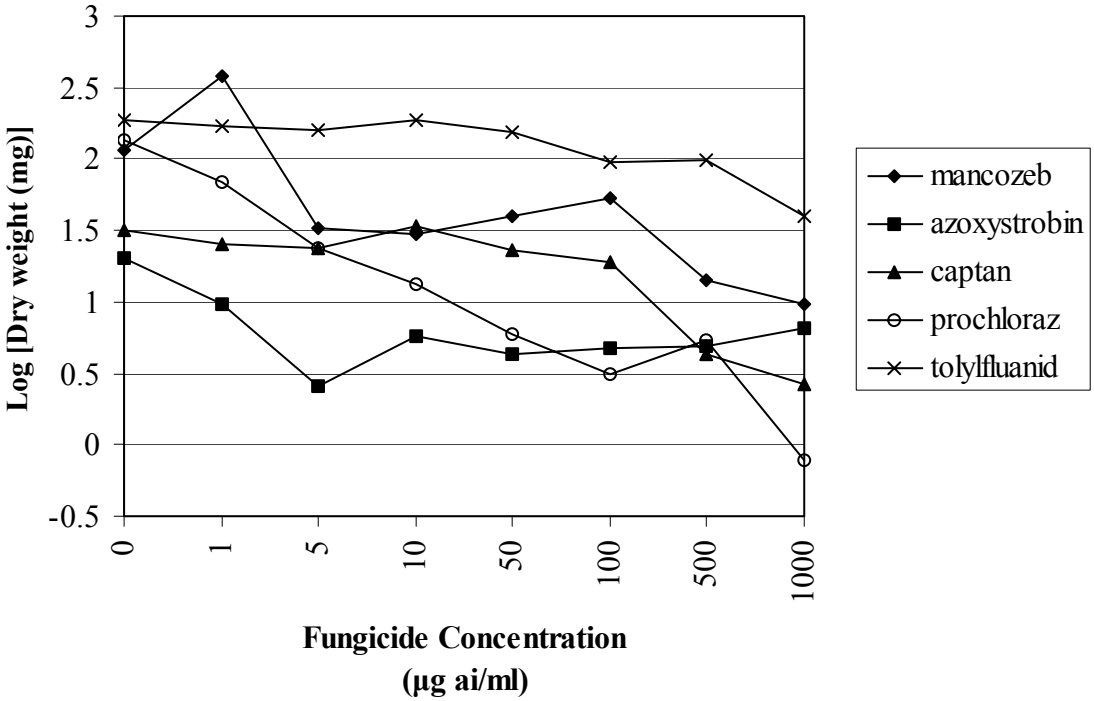

Figure 5.2 The mean dry weight of isolates showing the relationship between fungicide and concentration [SE = log(6.1) = 0.78]

Results showing the three-way interaction between fungicide, concentration and isolate are summarised on Figure 5.3.

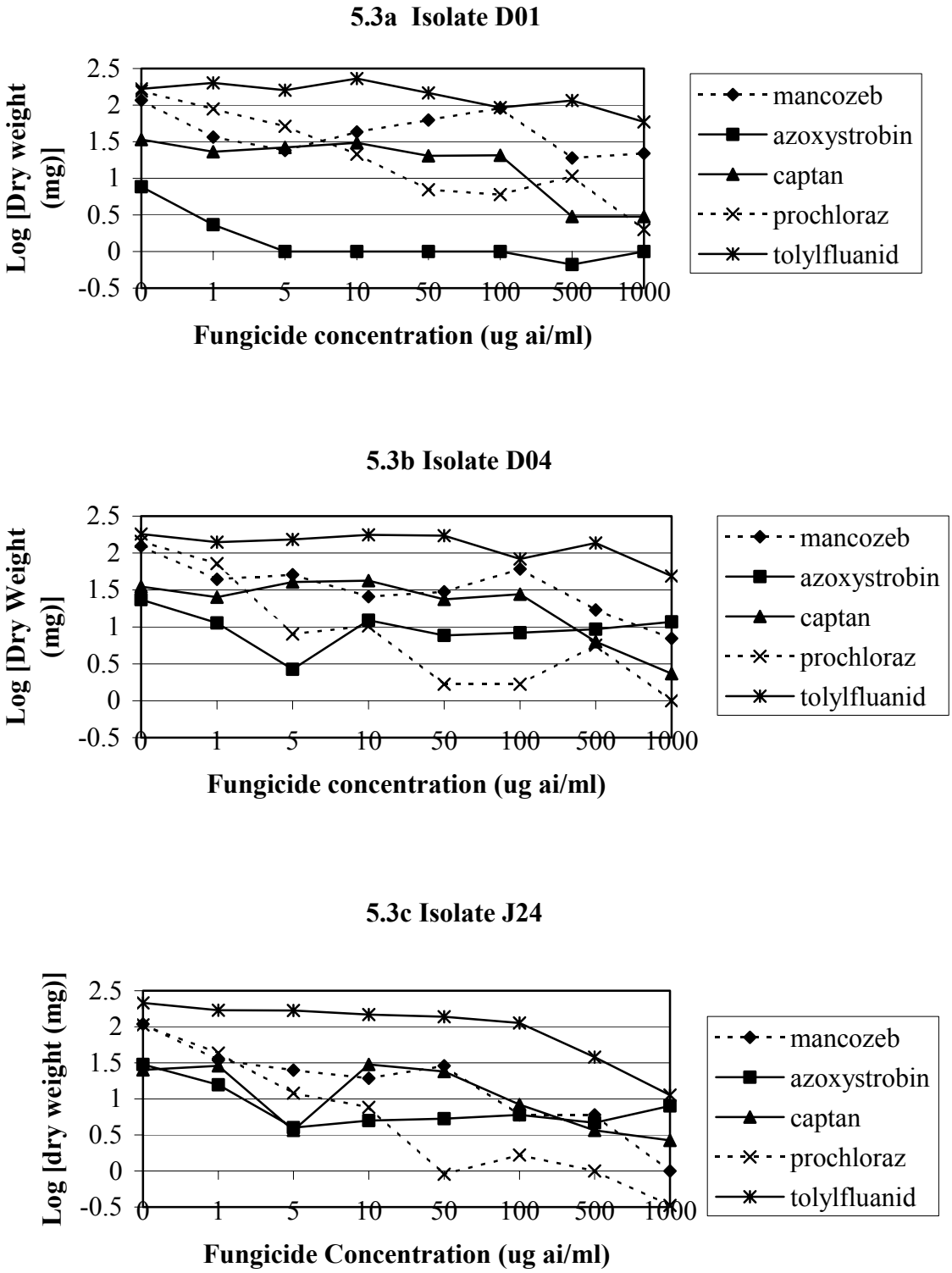

Figure 5.3 The mean log of dry weight of each isolate, showing the relationship between fungicide and concentration [SE = log (4.2) = 0.63]

The EC_{50} for mycelial growth of fungicide experiment 2 was below the recommended field concentration for captan and prochloraz in all isolates, mancozeb in isolate J24, and azoxystrobin in isolate D01 (Table 5.3). None of the isolates were sensitive to tolylfluanid. Comparison of the EC_{50} for the three isolates indicates that J24, isolated from Juliasdale, is more sensitive to mancozeb than isolates collected from Darwendale.

Table 5.3 The EC_{50} (mg a.i./ml) and % inhibition at the recommended field concentration (IRC) for fungicides used to control *S. protearum* (fungicide experiment 2)

Active Ingredient	Recommended Concentration (mg a.i./ml)	J24		D01		D04	
		EC_{50}	IRC	EC_{50}	IRC	EC_{50}	IRC
Azoxystrobin	250	>1000 [a]	14	<50	100	>1000	3
Captan	2000	<1000	100	<1000	100	<1000	100
Mancozeb	1600	<500	100	>1000	9	>1000	19
Prochloraz	500	<50	100	<50	100	<50	100
Tolylfluanid	2500	>1000	19	>1000	9	>1000	8

[a] < or > indicates that the EC_{50} is between this concentration and the previous or following concentrations tested

5.3.2 *In vitro E. leucospermi* fungicide trial

Prochloraz had the greatest effect on *E. leucospermi* mycelial growth (0.3 mg) (fungicide experiment 3), followed by tolylfluanid (7.1 mg), azoxystrobin (7.9 mg) and mancozeb (63.5 mg), whose result was significantly different to the others (Figure 5.4 & 5.5).

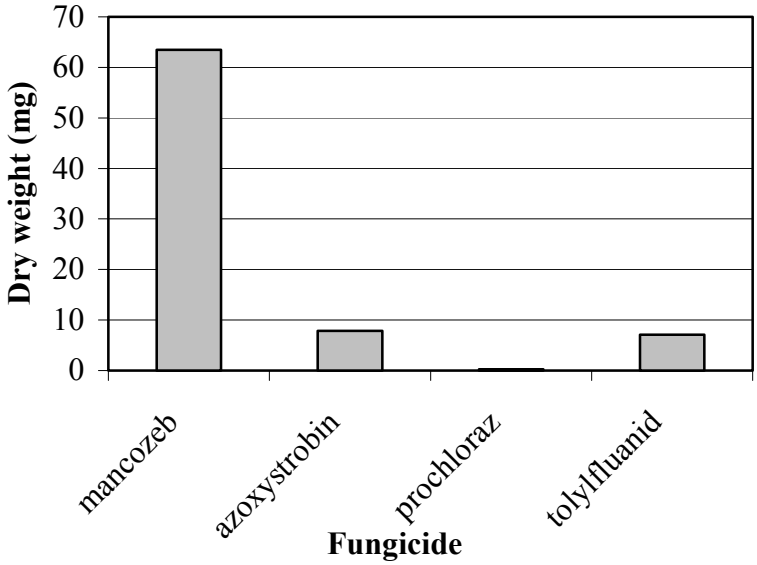

Figure 5.4 Effectiveness of fungicides on growth of *E. leucospermi* (Isolate D06). [LSD = 10.1, at P = 0.05]

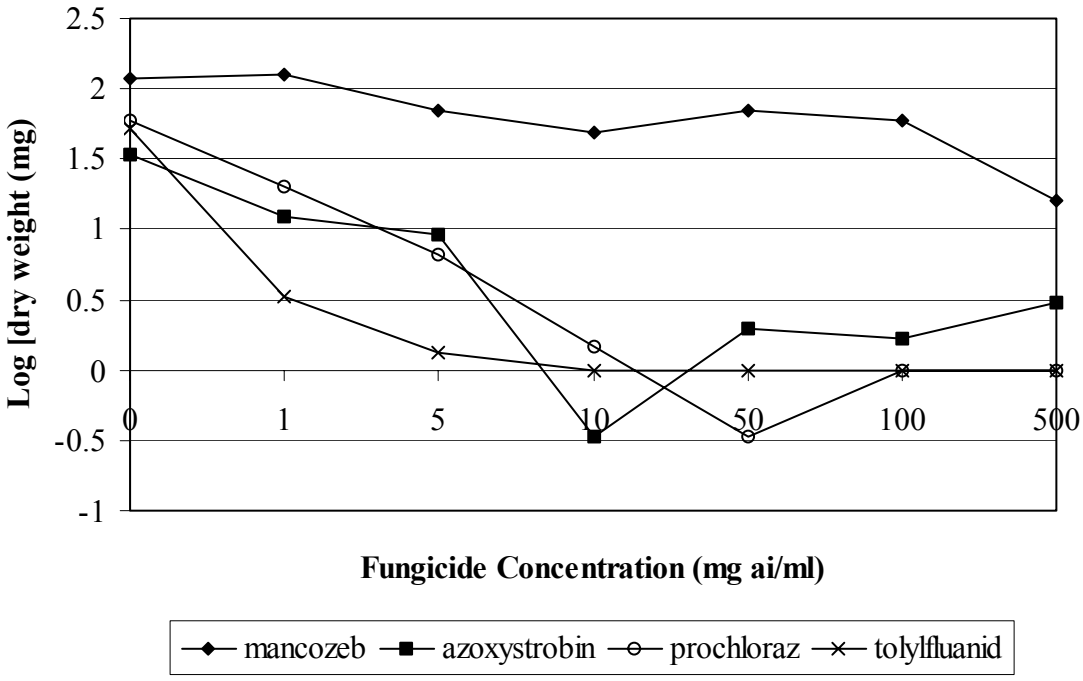

Figure 5.5 The mean dry weight of *E. leucospermi* (Isolate D06) showing the relationship between fungicide and concentration. [SE = log(8.1) = 0.91]

Prochloraz, tolylfluanid and azoxystrobin all offered 100 % inhibition of the fungus at the recommended field concentration of the fungicides (Table 5.4).

Table 5.4 The EC_{50} (mg a.i./ml) and % inhibition at the recommended field concentration (IRC) for fungicides used to control *E. leucospermi*

Active Ingredient	Recommended Concentration (mg a.i./ml)	D06	
		EC_{50}	IRC
Azoxystrobin	250	<50 [a]	100
Mancozeb	1600	>1000	17
Prochloraz	500	<10	100
Tolylfluanid	2500	<5	100

[a] < or > indicates that the EC50 is between this concentration and the previous or following concentrations tested

5.3.3 *In vivo* fungicide trial 2000/1

Residual infection was observed on *Protea* cv Pink Ice plants from the beginning of the trial period (Figure 5.6 & 5.7). Plants prior to the trial period had been sprayed fortnightly with mancozeb. Severity and incidence of infection symptoms observed on the plants increased from day 74 (February 13th 2001) to day 139 (April 19th 2001) for all treatments. The mean score per stem went from under 1.35 at day 74 to range from 2.03 (prochloraz) to 3.38 (captan + benomyl) at day 139. After this time there was a general lowering of the mean score per stem, as disease incidence and therefore symptoms lessened, except for a brief period between days 149 and 158.

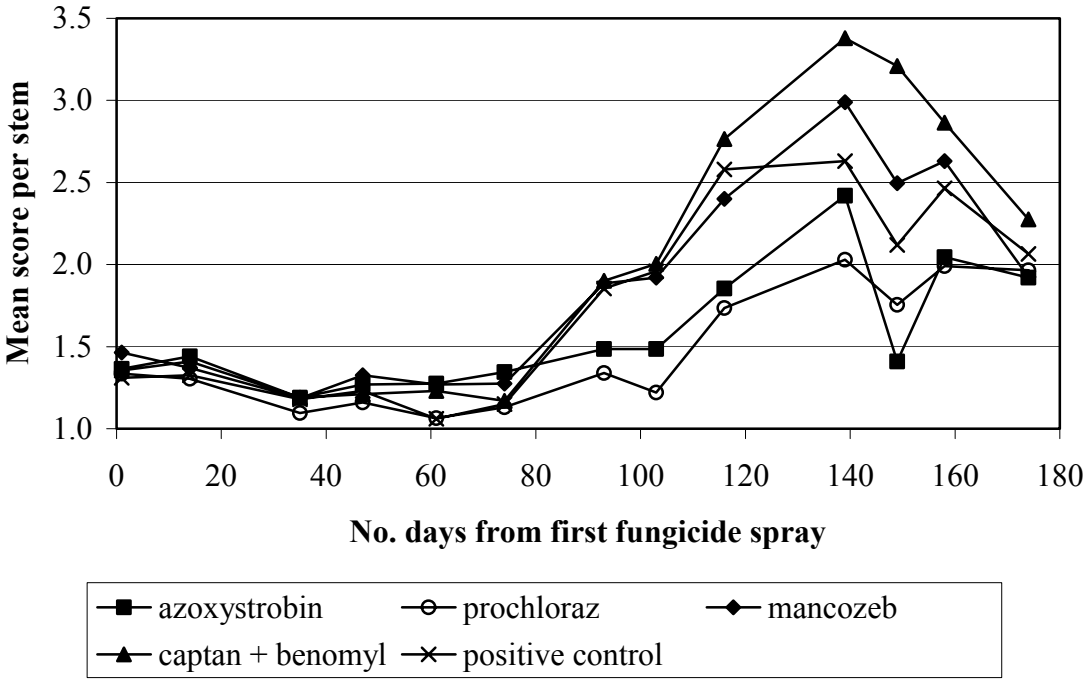

Figure 5.6 Disease progress curves of mean score per stem of field fungicide treatments for 2000/1

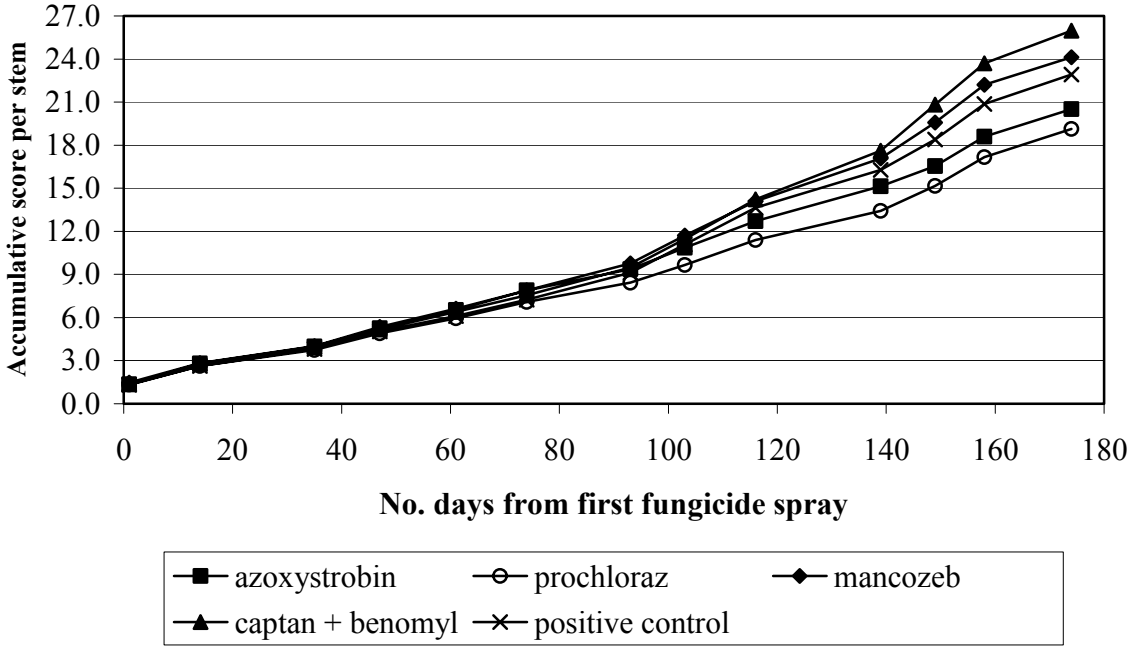

Figure 5.7 Disease progress curves of accumulative mean score per stem of field fungicide treatments for 2000/1

Prochloraz and azoxystrobin treatments gave the best results of the *in vivo* fungicide trial for the 2000/1 season, with a mean AUDPC of 252 and 274 respectively, which were not significantly different from each other (Table 5.5). The positive control had a mean AUDPC of 306, followed by mancozeb (321) and (captan + benomyl) (344).

Table 5.5 Mean area under a disease progress curve (AUDPC) to deduce effectiveness of fungicides against *Elsinoë* infection (2000/1)

Fungicide Treatment	Mean AUDPC (N=4)	Significant Difference[1] (LSD=34.57)
captan + benomyl	344.12	A
mancozeb	321.06	AB
positive control	305.96	BC
azoxystrobin	274.48	CD
prochloraz	251.92	D

[1]Means followed by the same letter do not differ significantly ($p<0.05$)

5.3.4 *In vivo* fungicide trial 2001/2

The trial period for season 2001/2 was extended due to symptom development still occurring at day 170 (Figure 5.8 & 5.9). Initial disease incidence was lower than in the previous year (the mean score per stem was below 1.1 in 2001/2 compared to 1.47 in 2000/1). This could have been due to the more intensive spray programs used on farm during the first fungicide trial. During this season there were at least two distinct disease infection cycles (Figure 5.8). From day 17 (December 2nd 2001) the mean score per stem rose from below 1.325 to peak between 1.52 (azoxystrobin) and 3 (mancozeb) by day 72 (January 27th 2002). The second disease cycle began from day 100 (February 24th 2002) to day 170 (May 5th 2002) with mean scores rising to between 1.5 (azoxystrobin) and 2.41 (captan + benomyl). Within this second cycle, the score of symptoms did reduce slightly between day 128 and 156, before rising again.

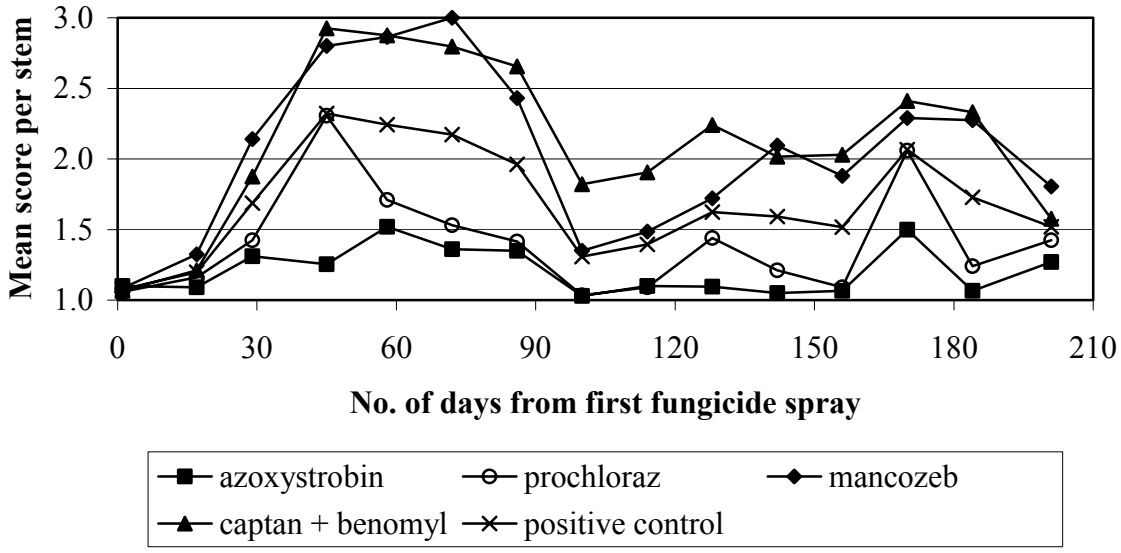

Figure 5.8 Disease progress curves of mean score per stem of field fungicide treatments for 2001/2

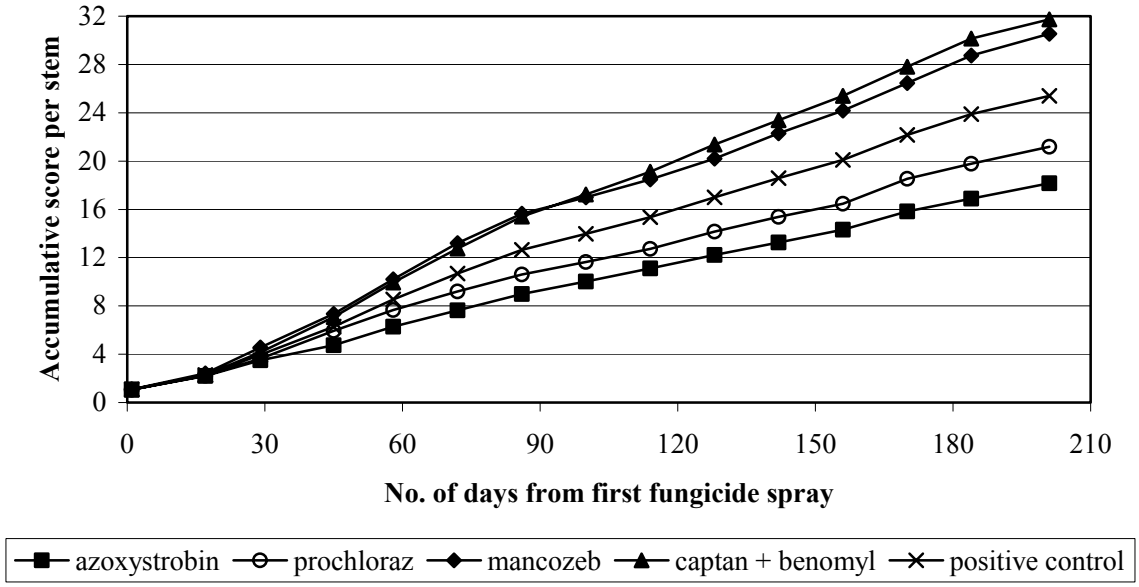

Figure 5.9 Disease progress curves of accumulative mean score per stem of field fungicide treatments for 2001/2

Azoxystrobin was the most effective fungicide used (mean score per stem below 1.5), followed by prochloraz, the positive control, mancozeb and (captan + benomyl), with a mean AUDPC of 242, 285, 389, 415 and 433 respectively (Table 5.6).

Table 5.6 Mean area under a disease progress curve (AUDPC) to deduce effectiveness of fungicides against *Elsinoë* infection (2001/2)

Fungicide Treatment	Mean AUDPC (N=4)	Significant Difference[1] (LSD=20.68)
captan + benomyl	432.62	A
mancozeb	414.57	A
positive control	389.21	B
prochloraz	284.69	C
azoxystrobin	242.12	D

[1]Means followed by the same letter do not differ significantly ($p<0.05$)

The relationship between [season x fungicide] was significant between 2000/1 and 2001/2 seasons (Appendix 13). Good control was achieved using azoxystrobin (1.39 mean AUDPC) and prochloraz (1.43), followed by the positive control (1.85), mancozeb (1.96) and then (captan + benlate) (2.06) (Table 5.7).

Table 5.7 Standardised area under a disease progress curve (AUDPC) to deduce effectiveness of fungicides against *Elsinoë* spp. infection (2000/1 and 2001/2)

Fungicide Treatment	Standardised AUDPC (N=8)	Significant Difference[1] (LSD=0.1132)
captan + benomyl	2.0638	A
mancozeb	1.9550	AB
positive control	1.8475	B
prochloraz	1.4325	C
azoxystrobin	1.3900	C

[1]Means followed by the same letter do not differ significantly ($p<0.05$)

Azoxystrobin was more effective at controlling *S. protearum* infection in the 2001/2 season, when disease incidence was lower than at the start of the fungicide trial in 2000/1 (Figure 5.10). Prochloraz for both seasons had a standardized AUDPC of 1.42. Fungicide treatments of the positive control, mancozeb, and captan + benlate produced better control in the 2000/1 season than 2001/2; and in both seasons were the least effective chemicals.

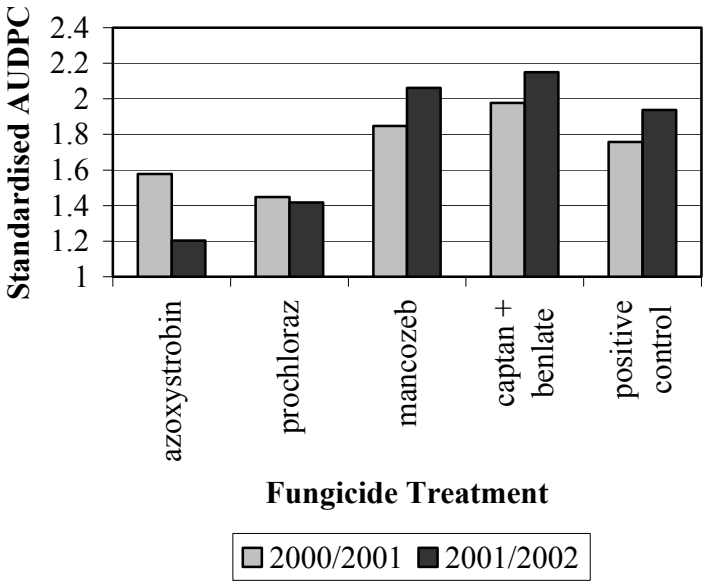

Figure 5.10 Standardised AUDPC's for both seasons and fungicide treatments [SE = 0.024]

The mean standardised AUDPC for season 2000/1 was 1.722, and for 2001/2 was 1.754 (LSD = 0.0716 at P = 0.05).

5.4 Discussion

Sphaceloma protearum grew very slowly on agar. By week 6 (t_6), many of the plates were over run with fungal contaminants; in particular, control plates. Observations of chlorothalonil plates (Bravo 50 % f.w.) revealed that this fungicide did not retard growth of *S. protearum*, even at 1000 µg a.i./ml, but did markedly reduce growth of contaminants. Colonies grown at the higher chlorothalonil concentrations had heavier dry weight results than those at lower concentrations due to reduced inhibition of colony growth through control of contaminants. It was concluded from this initial experiment that 1000 µg a.i./ml chlorothalonil be applied to MEA and used as a selective media to control fungal contaminants. Addition of streptomycin sulphate and tetracycline hydrochloride as suggested by Whiteside (1978) gave little control of fungal contamination, and was only useful against bacterial contaminates, which seldom occurred.

The wet weight measurements taken of plates were not representative of fungal growth from initial inoculations, as seen by the negative growth rate of *S. protearum* with increasing fungicide concentration. Weight measurements, in fact, decreased irregularly with time, primarily due to water loss from the agar, consumption of nutrients by *Elsinoë* isolates and contaminants.

The new method of taking dry weight measurements of colonies revealed a graduated response to the effect of fungicide concentration for all fungicides trialled, except chlorothalonil, by which fungicide efficacy could be determined.

Isolates collected from the Darwendale region were more tolerant to fungicides tested than that from Juliasdale, with the exception of azoxystrobin, which is a relatively new chemical on the market. This inherent tolerance of fungicides could be due to the intensive spray regimes implemented by growers in Darwendale compared to that applied by growers in Juliasdale. This results in the survival and proliferation of more fungicide tolerant isolates. Although all isolates collected from *Protea* plantations within Zimbabwe belong to the same species, *S. protearum*, there are genetic differences between isolates collected from Juliasdale and Darwendale sites (Figure 2.2). Isolates 2035, 2037 and 2038 were from Juliasdale plantations and isolates 2034 and 2036 from Darwendale (Swart *et al*., 2001). It is possible that these genetic differences allow for different fungicide tolerances of isolates between the areas in which they are found.

Initial control methods and spray programs against protea scab were based on the extensive studies on citrus scab (Benić & Knox-Davies, 1983). Benomyl gave good control of *S. batatas* (sweet potato) (Ramsey *et al*., 1988). Munro *et al*. (1988) found that dichlofluanid (Elvaron) controlled cane spot (*E. veneta*) of raspberry, but carbendazim (Bavistin), vinclozolin (Ronilan) and iprodione (Rovral) were ineffective. Chlorothalonil (2.5 ml/l) was reported to give excellent control on potted *Leucospermum* against *E. leucospermi*, at maximum recommended rates, although phytotoxicity symptoms were observed on host plants (Nagata *et al*., 2002; Turnbull, 1995), and against *S. batas* on sweet potato (Ramsey *et al*., 1988), which is in contrast to what was observed in this trial. Chlorothalonil had no effect on growth of *E. leucospermi* or *S. protearum,* and was added to MEA to be a selective media for both species. It is apparent that more fungicide efficacy studies are required per species, rather than to a genus level.

Azoxystrobin had the greatest growth inhibitory effect *in vitro* against *S. protearum*, followed by captan, prochloraz, mancozeb and then tolylfluanid. However, prochloraz and captan had a higher overall efficacy (EC_{50}) over azoxystrobin, with 100 % IRC. Azoxystrobin only had 100 % IRC of isolate D01. Growth of colonies grown at 0 mg a.i./ml was low for both captan and azoxystrobin (below l.5 compared to between 2 and 2.25 for other fungicides). This lower dry weight did considerably affect the slope of the line of best fit from which EC_{50} is deduced. Had these weights for D04 and J24 at 0 mg a.i./ml azoxystrobin been higher, IRC would be close to 100 %. Nagata *et al*. (2002) concluded that captan (Captan 50% WP) at 2.4 g/l was most effective at controlling pincushion scab. Good control was also achieved against protea scab with this chemical *in vitro*. Mancozeb is used on sweet potatoes to control *S. batatas* at the early stage of disease development (Ramsey et al., 1988). *In vitro* control of pincushion and protea scab using mancozeb was poor, confirming field trial results by Nagata *et al*. (2002), who rated mancozeb above iprodione, the least effective of six fungicides trialled against *E. leucospermi*. Mancozeb did offer 100 % IRC for isolate J24, but only 9 and 19 % IRC for D01 and D04. Mancozeb is commonly used as a preventative spray against both pincushion and protea scab (Percival, 2002; Benić & Knox-Davies, 1983). In Darwendale, spray regimes are routinely applied using mancozeb, and have resulted in mancozeb tolerant strains. This highlights the lack of knowledge by growers on disease control, and the reasons for the high disease incidence and severity experienced in Zimbabwe. Tolylfluanid offered little or no protection at all against *S. protearum*, but was effective against *E.*

leucospermi. Prochloraz had the greatest inhibitory effect on *E. leucospermi*, followed by tolylfluanid and azoxystrobin, but all offered 100 % IRC.

Field trials confirmed *in vitro* results, in particular, the effect of fungicide on fungal growth. Azoxystrobin was the most effective chemical against *S. protearum*, closely followed by prochloraz, then the positive control, mancozeb and lastly, captan + benlate. Azoxystrobin and prochloraz offered greater protection during the 2001/2 season, than for 2000/1. Lower residual disease levels at the start of the 2001/2 season could be the reason for this lower infection severity. Azoxystrobin is steadily taken up into the leaf after application, ensuring that some active ingredient remains on the leaf surface to combat infection from incoming fungal spores. This uptake can be enhanced if the foliar surface is re-wetted, for example, following dew or rain (Zeneca, 1998), when infection of *S. protearum* propagules is highest. Excellent control of protea scab in the field was achieved by spraying at the recommended rate (0.25 g/L). The positive control was third most effective fungicide. The spraying of octave when new disease symptoms were observed in the host plants (positive control treatment) would have been responsible for the slightly greater efficacy against *S. protearum* compared to mancozeb and captan + benomyl. Captan + benomyl gave the poorest protection of plants against protea scab in the field, which is in contrast to *in vitro* results for captan (100 % IRC).

Infection occurred on *Rubus* canes in the region which had been meristematic during the period of exposure. This is a general characteristic of the genus *Elsinoë* (Munro *et al*., 1988; Benić & Know-Davies, 1983; Swart & Crous, 2001; Swart *et al*., 2001), and was observed of *S. protearum* on *P*. cv Pink Ice. Plants should be sprayed with a preventative fungicide after flowering or heavy pruning when young growth is expected. The importance of good, directional spray coverage and the use of correct spray nozzles and air volume outputs to penetrate the dense inner foliage of Proteaceae cannot be over emphasised.

It is necessary to implement certain cultural practices in order to optimise chemical control of *Elsinoë* spp. Sprays with phosphoric acid (Fosject) and chlorothalonil (Daconil) effectively reduced 100 % infection to 15 % and 0 %, respectively, when done in conjunction with removal of source disease material by heavy pruning. If diseased material was not removed, infection levels increased rapidly, resulting in plant death (Turnbull; 1995). Diseased material must be removed from the plantation and destroyed, as the fungus

survives for long periods in infected tissue (Forsberg, 1995; Ramsey *et al*., 1988). Keeping bushes well-pruned, thinning out non-saleable stems, and cleared of weeds improved air movement through the plants, alleviating conditions such as high humidity with in the plants, which caused disease development (Mathews, 1995). This is particularly important for plantations that are irrigated overhead (Agricura, 1996). Overhead irrigation will also facilitate splash dispersal of conidia (Ziehrl *et al*., 1996, Ramsey *et al*., 1988). Preventative spraying is necessary when wet weather and growth flushes coincide. Other cultural practices include planting disease tolerant or disease free cultivars, after a sufficient quarantine period (Ramsey *et al*., 1988; Swart & Crous, 2001).

Greatest control of *S. protearum in vitro* (100 % IRC) was achieved through use of prochloraz and captan (all isolates), azoxystrobin (D01 only) and mancozeb (J24 only), and of *E. leucospermi* by prochloraz, azoxystrobin and tolyfluanid. In the field, azoxystrobin and prochloraz offered the greatest protection, followed by the positive control, mancozeb and then captan + benlate.

CHAPTER 6

OVERALL DISCUSSION

Cultivation of *Protea* in areas outside their natural habitat has increased dramatically (Coetzee & Littlejohn, 2001). Extensive monoculture of crops with a limited genetic base is an open invitation to plant disease on a grand scale (Strange, 1993). *S. protearum* is a relatively new discovered disease of *Protea* spp. (Swart *et al*., 2001). Similarly to that observed by Swart & Crous (2001), few *Protea* spp. or cultivars examined showed any resistance or tolerance to *S. protearum*. There was one species, *P. cynaroides,* which was noticeably devoid of the typical host symptoms. This species is known to be susceptible to the other *Elsinoë* spp. infecting *Protea*, *E. proteae* (Swart *et al*., 2001). Several species of *Protea* are indigenous to Zimbabwe. Too few indigenous stands were examined to determine the extent the disease occurs in the wild. Isolates collected from Darwendale were more tolerant of fungicides than those from Juliasdale, which suggests some genetic difference. This genetic difference was confirmed from work completed by Swart *et al*. (2001).

During this study, it was confirmed that the fungus causing scab of proteas is *Sphaceloma protearum*. Ascospores were rarely seen, except on the surface of host leaf samples and on spore traps. Ascocarps and asci were not observed at all. The ascospores were characteristic of the Loculoascomycete *Elsinoë* (Benić & Knox-Davies, 1983; Swart *et al*., 2001; Luttrell, 1973). No additional spore forms, such as chlamydospores or microsclerotia, as described by Benić & Knox-Davies (1983) on *Lsp. cordifolium* were observed. The hyaline to brown mycelium and erumpent conidophores from samples of host tissue were similar to those described for *Sphaceloma* (Benić & Knox-Davies, 1983; Swart *et al*., 2001) and *E. panici* (Gabel & Tiffany, 1987). Conidiophores and conidia were seldom observed in culture, but many species rarely produce abundant conidia *in vitro* (Ramsey *et al*., 1988; Williamson *et al*., 1989; Benić & Knox-Davies, 1983). The hyaline conidia produced in Fries' medium were similar to that described by Benić & Knox-Davies (1983) for *Elsinoë* spp. and Whiteside (1975) for *E. fawcettii*. Results concerning the identification of the pathogen causing Protea scab confirm the existence of *Sphaceloma protearum*, with an *Elsinoë* spp. teleomorph, infecting *Protea* L. spp. in Zimbabwe.

Fungal isolates agreed with previously described *Elsinoë* spp. and *Sphaceloma* spp. in respect of very slow growth in culture (Hyun *et al*., 2001; Benić & Knox-Davies, 1983; Whiteside, 1975), production of colonies that are pulvinate (convex) and deeply fissured (Hyun *et al*., 2001; Swart *et al*., 2001), the presence of long thin vegetative hyphae (Benić & Knox-Davies, 1983) and the ability to produce a red pigment (Benić & Knox-Davies, 1983; Swart *et al*., 2001). In contrast to that noted by Swart *et al*. (2001), aerial mycelium was occasionally observed on colonies. This study has shown that *S. protearum* is insensitive to the fungicide, chlorothalonil. As such, chlorothalonil was added to MEA to create a selective medium, to control fungal contamination.

Young actively growing flushes were most susceptible to infection, correlating with information collected, that *Elsinoë* spp. typically infected immature leaf and stem tissue that is meristematic (Ramsey *et al*., 1988; Williamson *et al*., 1989; Ziehrl *et al*., 1996). *Protea* grow vigorously in early spring to summer and are therefore most susceptible to Protea scab during this period. Infection was also prevalent on the first flushes of stems arising from the prune of a harvestable stem (late summer to autumn). The incubation period of 3 weeks before the first appearance of lesions agrees with observations of *Elsinoë* spp. (Swart & Crous, 2001), *E. leucospermi* (Benić & Knox-Davies, 1983; Ziehrl *et al*., 1996) and *E. veneta* (Munro *et al*., 1988; Williamson *et al*., 1989).

Cardinal temperatures of the fungus grown *in vitro* were optimum at 25 ºC (humidity levels 32-90 %), and 20ºC (98 % humidity), maximum below 30 ºC. Temperature results correlated with data from Swart *et al*. (2001) for *S. protearum*. It is likely that conidia production and germination, like *E. fawcettii,* would be optimal between 20-25 ºC, with a marked decline at above 30 ºC (Whiteside, 1975). The upper temperature limit would have little epidemiological significance in Zimbabwe because when shoots are wetted by dew or rain, the temperature would usually be below 30 ºC.

Moisture is an important factor contributing to the disease development and severity caused by *Elsinoë* spp. (Benić & Knox-Davies, 1983; Ramsey *et al*., 1988; Ziehrl *et al*., 1996). Wetting of leaves, even for a few hours, can promote abundant conidial development, thereby increasing inoculum potential (Whiteside, 1975). The tendency of infection to occur at the leaf margin or midrib is probably because of the longer retention of water at these locations. Infection periods were linked to weather patterns, and were particularly high when wet weather, growth flushes and plant stress coincided. If a factor of

the environment is unfavourable to the host, such as lack of water, various physiological processes, including disease resistance, can commonly be impaired (Cook & Papendick, 1972). The effect of stress generally is not on initial establishment of the pathogen in the host, but rather on development of established infections (Cook & Papendick, 1972).

Like other *Elsinoë* spp., *S. protearum* infection is thought to be possible from old scab lesions during periods of canopy wetting by dew, rain, overhead irrigation or non-fungicidal sprays (Whiteside, 1975; Benić & Knox-Davies, 1983; Dede & Varma, 1987; Ziehrl *et al*., 1996). Hyaline conidia of *E. fawcettii* required liquid water for their production, dispersal, survival and germination, and a minimum wetting period of 2.5 to 3.5 hours to cause infection (Whiteside, 1975). This relatively short period for conidia formation and germination is of epidemiological importance. Coloured conidia, also produced by *E. fawcettii*, were disseminated from conidiophores by both wind and water, which were still viable after a temporary desiccation over a 24-hour period. The distribution of protea scab suggested that splash dispersal is more significant epidemiologically than dry, airborne dispersal, similar to that observed by Whiteside (1975 & 1978) and Munro *et al*. (1988). Dissemination by insects commonly occurring in or attacking scab lesion is a possibility (Benić & Knox-Davies, 1983), but not observed in this study.

The length of time that *S. protearum* can survive in old scab lesions has not been identified. Viable mycelium and conidiophores of *E. fawcettii* on *Citrus* were still detected for the first 6-9 months after infection, but few were detected after 10-12 months (Whiteside, 1988). Improvement of previously infected *Protea* plantations has been observed in 2002/3. This improvement is thought to have occurred primarily due to drier conditions prevailing during the rainy season, which could be attributable to a loss of inoculum-producing ability of pustules before conditions were conducive for sporulation, transmission and germination.

Chemical control of *Elsinoë,* even within those species that infect only Proteaceae, is species specific. Plantations with known *S. protearum* infection should begin fungicide spraying when conditions first become favourable for pathogen sporulation and germination, after winter. Preventative sprays of mancozeb (Benić & Knox-Davies, 1983) and chlorothalonil (Nagata *et al*., 2002; Ramsey *et al*., 1988; Turnbull, 1995), recommended for *E. leucospermi* and *S. batatas,* previously applied by growers in Zimbabwe, offered little or no protection against *S. protearum*. Azoxystrobin had the greatest inhibitory effect *in*

vitro on growth of *S. protearum*, followed by captan, prochloraz, mancozeb and tolylfluanid. Tolylfluanid did not control *S. protearum*, but was effective against *E. leucospermi*. Field trials confirmed *in vitro* results, except for captan. Sprays of captan + benlate were the least effective fungicides trials *in vivo*. Azoxystrobin and prochloraz, like for control of *E. leucsopermi* (Mathews, 1995; Nagata *et al*., 2002), offered the greatest protection. It would be advisable for growers to use these two curative chemicals alternatively against *S. protearum*. Azoxystrobin (250mg/l) and prochloraz (500 mg/l) would be sprayed twice, five days apart when symptoms are first observed. In addition to this, weekly sprays of mancozeb (1600mg/l) should be applied in Juliasdale, and captan (2000mg/l) in Darwendale. Further screening of other fungicides is necessary so that growers can alternate usage of chemical groups so that the possibility of inducing disease resistance is limited.

Due to the extreme susceptibility of *Protea* spp. to *S. protearum*, implementation of cultural methods in addition to application of fungicides is necessary for effective control. These include quarantining new plants so disease is not introduced in planting material (Dede & Varma; 1987; Ramsey *et al*., 1988; Swart & Crous, 2001), applying a pre-planting fungicide treatment to rooted cuttings (Ramsey *et al*., 1988) and planting in drier regions, such as Karoi, which had an absence of the disease. Although even in such areas, scab could proliferate under excessive irrigation from overhead sprinklers (Dede & Varma, 1987; Whiteside, 1975). Keeping bushes thinned out and clear of weeds would alleviate moist conditions and lack of air movement favouring disease development (Mathews, 1995). Sanitation pruning of diseased material, which harbours the fungus, is crucial to reduce disease inoculum, and pressure (Ramsey *et al*., 1988; Whiteside, 1988; Turnbull, 1995; Ziehrl *et al*., 1996, Swart & Crous, 2001). This should be done throughout the year and infected material destroyed, in particular, before young shoots emerge (Munro *et al*., 1988). Pruning tools should be sterilised following contact with infected plant material (Ziehrl *et al*., 1996). Cuttings should be taken from plants with no previous history of Protea scab infection, and should be quarantined to ensure that no latent infection is present (Benić & Knox-Davies, 1983; Swart & Crous, 2001). The avoidance of susceptible cultivars to restrict disease spread, such as use of resistant *Citrus* spp. used to restrict *E. fawcettii* (Dede & Varma; 1987) or *Leucospermum* cultivars of *E. leucospermi* (Littlejohn, van den Berg & Lubbe, 2003), is not possible due to the lack of tolerance of all *Protea* spp. and cultivars.

CHAPTER 7

CONCLUSION

The severity of symptoms and devastating effect of *S. protearum* on *Protea* plantations in Zimbabwe is of great concern to other *Protea* producing countries if transmission were to occur, particularly into areas with a sub-tropical climate. Strict phytosanitary measures have been imposed on *Protea* plant material exported from Zimbabwe to South Africa to prevent the spread of the disease. Further sampling of indigenous stands of *Protea* spp. is required to determine the extent the disease occurs naturally. *S. protearum* can now be identified from other *Elsinoë* spp. through molecular analysis (Swart *et al*., 2001), host symptoms, colony morphology and characteristics. From the research undertaken, no longer will lack of knowledge about protea scab and its control be a major factor in the proliferation of this disease.

Host symptoms caused by *S. protearum* have been comprehensively documented in this study. Growers will be able to easily identify the initial leaf and stem lesion symptoms as they first appear on the host. Conditions conducive to disease development have been elaborated, and will allow greater understanding of the epidemiology of the disease. Curative fungicides can be applied where *S. protearum* has been known to occur when periods of hot humid weather (20-27 ˚C and 45-90 % humidity) and growth flushes of host plants coincide. A grower who utilizes this information will be able to timeously apply effective fungicides with in the 3-week period between infection and appearance of host symptoms. Good fungicide control will be achieved, with minimal damage caused to host tissue by the pathogen.

Chemical control of *Elsinoë* tends to be species specific. Weekly preventative sprays of mancozeb (1600mg/l) should be applied in Juliasdale, and captan (2000mg/l) in Darwendale during the rainy season. Azoxystrobin (250mg/l) and prochloraz (500 mg/l) should be used alternatively, after an application of two sprays, five days apart, from when symptoms are first observed or during periods that are conducive to disease development. Growers should apply prochloraz (500 mg/l), azoxystrobin (250mg/l) and tolylfluanid (2500mg/l) to control *E. leucospermi*. Tolylfluanid is a chemical that has not been commonly used on Proteaceae in Zimbabwe. The testing of a wider range of fungicides will allow growers to alternate more chemical groups in a spray regime, preventing pathogen

fungicide-resistance. Resistance to fungicides, such as the Darwendale isolates to mancozeb, is already occurring.

Cultural methods also provide a means of managing protea scab. As nearly all *Protea* spp. are susceptible to *S. protearum*, new plants must be quarantined so that the disease is not introduced into clean land. Removal and destruction of diseased host tissue before new shoots emerge will relieve disease pressure, and alleviate humid conditions within the plant that would cause disease development. The length of time that *S. protearum* can survive as a viable propagule in old scab lesions is an area that requires future research. If factors of the environment are unfavourable to the host, various physiological processes, including disease resistance are commonly impaired. Plant management should be constantly monitored to alleviate stress conditions, such as lack of water or fertiliser. Irrigating with drip line if preferential, as overhead irrigation will facilitate spread of the disease.

Implementing the fungicide treatments along side cultural control methods will decrease disease incidence and severity that has crippled the Zimbabwe *Protea* spp. industry over the past 5 years. It will now be in the grower's means to develop a *Protea* crop through effective disease management, increasing their returns from improved quality and yield of marketable stems.

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APPENDICIES

Appendix 1 Potato Dextrose Agar (PDA)

Basal medium (g) in 1 L water:	Potato extract	4.0
	Glucose	20.0
	Agar	15.0

pH 5.6 ± 0.2

Suspend 39 g basal medium in 1 L of distilled water. Sterilise by autoclaving at 121ºC for 15 minutes.

PDA is a carbohydrate rich medium. Although PDA is useful for the isolation of *Elsinoë* spp. from plant materials many saprophytic fungi and bacteria also grow on PDA, and may inhibit the recovery of *Elsinoë*. Antibiotics are added to PDA (sPDA), enabling isolation of *Elsinoë* species, whilst inhibiting growth of other fungi and bacteria. The autoclaved medium is cooled to 50°C before adding:

Streptomycin sulphate[1]	100 mg
Tetracycline hydrochloride[2]	100 mg

(Whiteside, 1988)

The prepared plates should be allowed to cool before use. Characterisation of most species of *Elsinoë* spp. is difficult since their teleomorph states are rarely observed, and their *Sphaceloma* anamorphs are generally morphologically conserved. Sporulation is poor.

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[1] Streptomycin is effective against Gram negative bacteria
[2] Tetracycline is broad spectrum antibiotic

Appendix 2 Malt Extract Agar (MEA)

Basal medium (g) in 1 L water:	Malt extract	30.0
	Mycological peptone	5.0
	Agar	15.0
		pH 5.4 ± 0.2

Suspend 50 g basal medium in 1 L of distilled water. Sterilise by autoclaving at 115ºC for 10 minutes.

Fungicides and antibiotics are added to MEA (sMEA), enabling the selective isolation of *Elsinoë* species. They are inhibitory to most fungi and bacteria but allow growth of *Elsinoë*, which form small colonies of up to 20mm diameter after 4 to 6 weeks. The autoclaved medium is cooled to 50°C before adding:

Streptomycin sulphate[1]	100 mg
Tetracycline hydrochloride[2]	100 mg
Chlorothalonil (Bravo)[3]	1000 µg

The prepared plates should be allowed to cool before use. Characterisation of most species of *Elsinoë* spp. is difficult since their teleomorph states are rarely observed, and their *Sphaceloma* anamorphs are generally morphologically conserved. Sporulation is poor. *Elsinoë* colonies should be maintained on MEA alone because addition of fungicide results in cultures becoming non-viable.

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[1] Streptomycin is effective against Gram negative bacteria
[2] Tetracycline is broad spectrum antibiotic
[3] Chlorothalonil is a broad-spectrum fungicide

Appendix 3 Fries' medium

Basal medium (g) in 1 L water:	Sucrose	20.0
	$(NH_4)_2C_4H_4O_6$ (ammonium tartrate)	5.0
	NH_4NO_3	1.0
	K_2HPO_4	1.0
	$MgSO_4.7H_2O$	0.5
	$CaCl_2$	0.1
	NaCl	0.1

Appendix 4 Description of infected host samples collected

Host[a] Isolate[a]	Symptom Description
***P. compacta* x *burchellii* cv Brenda**	
J23	Oval leaf lesions only. Resulting in leaf distortion.
***P. compacta* x *obtusifolia* cv Red Baron**	
J25	Extensive small lesions on leaves, which eventually coalesce causing leaf distortion. Most lesions occur on leaf margin and leaf tips. Few stem lesions present. No reddening of surrounding tissue.
***P. compacta* x *susannae* cv Pink Ice**	
J01	Leaf and stem lesions occur on young shoot tips. Leaf lesions oval, red to black in colour, leaf tissue becomes necrotic; with red surrounding tissue, (1-)2-5 x 1.5-5 mm diam. Lesions found primarily on leaf margin, but also interveinal areas and midrib. Necrotic areas enlarge, withering leaf. Leaf growth distorts leaf. Black - grey stem lesions, oval to irregular in shape, with reddened surrounding tissue. Stem lesions occur in same general section of plant as leaf lesions.
J03	Severely infected stems and leaves (+/- 25 % leaf area). Younger tissue has more lesions, but they are smaller in size than that on more mature leaves. Older stem lesions have coalesced.
J12	New stem lesions brown, oval. The woodier the stem, the more defined the lesions were. Older stem lesions were cracked.
J17	Severe leaf lesions on young shoot tips. Stem lesions were brown and oval in shape.
J33	Severe leaf and stem infection. Shoot tips distorted and necrotic. Black conidiomata visible on withered leaves. Lesions were 3-7 mm diam.
D01	Round to oval, red-black leaf lesions, 2-4 mm diam, on young shoot tips. One oval red raised stem lesion.
D03	Withered young shoots. Numerous dark brown oval leaf lesions, 2-4 mm diam, and irregular stem lesions present. Black conidiomata visible.
D07	Old infection. Twisted, deformed stem growth from stem lesions. Infected leaves with numerous red-black lesions, 3-6 mm diam. Newer growth above infected area was red, a sign of plant stress.

***P. compacta* x *susannae* cv Pink Ice (cont.)**

D08 Large dark brown leaf lesions on maturing growth, 5-7 mm diam. Lesions occurring on leaf margin, giving leaves are twisted appearance. Stem lesions irregular in shape, generally oriented vertically on plant stem.

K01 Two round sunken black leaf lesions, 6-8 mm diam, on leaf margin. No stem lesions present.

P. cynaroides

J11 Red, raised stem lesions, with grey centre. Scabs were woody looking and occurred around the base of leaf nodes.

P. cynaroides cv Tsitsikamma

J07 Small stem and leaf petiole lesions. Stem lesions were black with tan centre, became cracked. Older lesions looked watersoaked. Petiole lesions sometimes extended onto midrib. These were tan – grey in colour, with lightly reddened surrounding tissue.

J21 Raised stem and petiole lesions. Striated scabs. Old lesions were grey, woody looking and sunken.

D02 Black round leaf lesion. Tissue necrosis apparent around leaf spot. No stem lesions present. Not likely to be *Elsinoë* spp. infection.

P. eximia cv Fiery Duchess

J43 Small leaf lesions, 0.5-1 mm diam, boarded by 1.5-2 mm of red surrounding tissue. One or two possible stem lesions (could be old scale damage).

***P. eximia* x *susannae* cv Sylvia**

J05 Round to oval, black – grey lesions, 1.5-2 mm diam, on shoot tips.

J06 Old diseased tissue mainly on lower mature leaves. *Coleroa* spp. and *Colletotrichum gloeosporioides* present. (Symptoms not consistent with *Elsinoë* infection.)

J14 Infection on growing shoot tips at leaf tips and midrib. Oval-circular lesions on either adaxial or abaxial sides of leaf. Lesions were raised, red-brown in colour and were not translaminae. Corresponding leaf surface to lesion was pink / red. Red-black sporodochia present in centre of lesion, as leaf tissue becomes necrotic, lesions coalesce and leaf withers and dies. Relatively few stem lesions to leaf lesions. Lesions first occur as brown – tan oval spots. Stems lesions irregular in shape, become black in colour as they enlarge and crack. Tissue around infected area turns red.

***P. eximia* x *susannae* cv Sylvia (cont.)**

J15 Leaf had variable sized lesions; from new infection (0.5–1 mm diameter) to large, older necrotic lesions (2-5 mm diameter). Leaves withered. Grey–brown, irregularly shaped stem lesions.

J20 Severe infection symptoms on leaf and stem. Small to large leaf spots (0.5–5 mm diam), turn grey–black as leaf tissue becomes necrotic, with red surrounding tissue.

J35 Large oval leaf lesions, red-black, 5-9 mm diam. Secondary infection present just below one large leaf lesion. Over 20 red-brown, raised lesions, 0.5-2 mm diam, possibly due to splash dispersal of conidia. Irregular to oval, red-black stem lesions.

D04 Large (6-7 mm diam) oval red-black leaf lesions, with red surrounding tissue. Irregular to oval, red-black stem lesions.

***P.* F2 hybrid cv Niobe**

J36 Oval dark brown leaf lesions, 2-4 mm diam. Grey-tan, raised stem lesions were striated, as if split by the growth of the stem.

J40 Leaf lesions oval, 1-3 mm diam; surrounding tissue speckled red - brown. Irregular stem lesions.

P. gaguedi

J45 Only indigenous species of *Protea* seen with *Elsinoë*-like symptoms. 2-3 leaf lesions, 2-3 mm diam, black, slight reddening of surrounding tissue. One oval, tan coloured, raised stem lesion, 2 mm diam.

P. grandiceps

J08 No leaf spots. Small, thin, vertical to irregular shaped brown stem lesions; usually occurring just above leaf node. (Symptoms not consistent with *Elsinoë* infection.)

***P. grandiceps* x *eximia* cv Rosie**

J22 No leaf lesions. Possible tan lesions below leaf nodes.

J44 As above. Cultivars exhibits greater tolerance to *Elsinoë* infection.

***P. laurifolia* cv Regal Mink**

J16 Sample had few leaf lesions (one lesion only on 2 leaves), lesions were large.

J30 Few leaf lesions that extend through leaf laminae, lesions are large. Tissue immediately surrounding lesion turns red (1-2 mm diam); lesion turns necrotic causing leaf to distort.

Stem lesions are black.

***P. laurifolia* cv Regal Mink (cont.)**

J31 Red-black, circular leaf lesions, 3-5 mm diam. Irregular raised red stems lesions, with black erumpent conidiomata.

***P. longifolia* cv Satin Pink**

J24 Extensive small leaf lesions along midrib and leaf margins. Occasionally occur on one side of leaf, causing curling and necrosis. Stem lesions are small, becoming cracked as tissue matured.

***P. magnifica* x *susannae* cv Susara**

J28 Numerous leaf lesions on young growth, with reddening of surrounding tissue. Leaves wither, and curl. Irregular stem lesions.

J38 Numerous irregular stem lesions. Initially are brown in colour, become raised with red surrounding tissue. Leaf lesions are similar – with black sporodochia in middle of lesions (2-3mm diam). Lesions coalesce, leaf tissue dies.

P. neriifolia

J09 Small stem lesions, mainly found below leaf nodes.

J26 Severe old stem scab lesions on last growth flush. New oval stem lesions (2 – 4 mm diameter) on youngest flush. Few leaf lesions present.

J41 Old black coloured leaf lesions. Leaves distorted.

P. neriifolia cv Moonshine

J02 Few *Elsinoë* symptoms observed. Leaf lesions oval. Grey stem lesions. Tip die-back of smaller leaves at internodes. Plants were not in healthy condition. *Coleroa* spp. present on leaves.

J13 Small grey – brown lesions on stem. Tip die-back of leaves at internodes. Leaf spots observed were caused from damage by insects.

J19 Oval necrotic leaf lesions, causing leaf curl and distortion. Small brown stem lesions present.

J29 Oval to irregular shaped stem lesions, grey in colour and became cracked on older tissue. Round to oval raided leaf spots, with red surrounding tissue.

J39 As above. Large black lesions, extending from leaf margins to midrib, were also present (*Botryosphaeria* spp.).

***P. neriifolia* cv Silvertips**

J18 Severe infection on young tissue. Leaf lesions are oval to irregular in shape, causing leaf distortion. One leaf lesion present in older tissue. Irregular black stem lesions.

J32 Old infection. Large leaf lesions, 5-7 mm diam, red-black conidiomata, some tissue necrosis. Stem lesions tan-black, becoming cracked.

J42 Raised red-brown oval leaf lesions (1-4 mm diam), which coalesce as disease progression occurs. Surround tissue is yellow – red. In other lesions the sporodochia turn a brown – black colour. Stem lesions were elliptical and raised. Severe infection.

P. magnifica x *burchellii* cv Sheila

J04 Lesions on leaves extending through leaf lamina; leaf spots oval to circular, reddish-purple, covered with erumpent conidiomata, appearing as reddish - black sporodochia on the necrotic tissue, leading to blackening, withering and death of shoot tips. Stem lesions were irregular in shape.

P. repens

J10 No leaf lesions. Small black stem lesions.

J27 Striated tan coloured scab lesions on stems**.** No reddening of tissue surrounding lesions.

Leucadendron salignum x *laureolum* cv Safari Sunset

J37 Prominent stem lesions. Tan coloured, raised, scab-like, with red surrounding tissue. Stems were severely twisted and distorted. Rough small, raised, whitish leaf lesions, 1-2 mm diam. Likely *Elsinoë leucospermi* infection.

***Leucospermum cordifolium* cv Yellow Bird x *patersonii* cv High Gold**

D05 Severe infection up length of stem. Newest lesions small, 3 mm diam, oval, whitish on young growth, becoming raised tan coloured, with red surrounding tissue. Lesions of older infection had coalesced, giving stem a corky appearance. Small rough raised leaf lesions. Infection by *Elsinoë leucospermi*.

D06 New shoots growing were twisted and deformed from coalesced stem lesions. Stem lesions were raised, scab-like, tan coloured. Few small raised leaf lesions visible. Infection by *Elsinoë leucospermi.*

[a] Host & Isolate number in **bold** were positively identified to be infected with *Elsinoë* spp.

Appendix 5 Efficiency of inoculation treatments for isolates J24, J35 and D04 at week 3 and 4

Inoculation Isolate	Inoculation Treatment	Week 3 % Efficiency per plant					Week 4 % Efficiency per plant				
		1	2	3	4	Mean	1	2	3	4	Mean
J24	A	-	-	-	-	0.00	-	-	-	-	0.00
	B	-	-	-	-	0.00	-	-	-	-	0.00
	C	25	25	75	25	37.50	50	75	75	50	62.50
	D	-	-	-	-	0.00	-	-	-	-	0.00
J35	A	-	-	25	-	6.25	-	-	50	-	12.50
	B	-	-	-	-	0.00	-	-	-	-	0.00
	C	50	25	25	50	37.50	50	25	50	50	43.75
	D	-	-	-	-	0.00	-	-	-	-	0.00
D04	A	-	-	-	-	0.00	-	-	-	-	0.00
	B	-	-	-	-	0.00	-	-	-	-	0.00
	C	25	25	50	25	31.25	75	50	50	75	62.50
	D	-	-	-	-	0.00	-	-	-	-	0.00
Control	Control A	-	-	-	-	0.00	-	-	-	-	0.00
	Control B	-	-	-	-	0.00	-	-	-	-	0.00
	Control C	-	-	-	-	0.00	-	-	-	-	0.00
	Control D	-	-	-	-	0.00	-	-	-	-	0.00

Appendix 6 Mean dry weight of isolates J35, J40 and D04 at five different humidity levels and temperatures

		Mean Dry Weight (mg)				
Isolate	Humidity	Temperature (ºC)				
	Level (%)	15	20	25	30	35
J35	32	27.25	29.25	62.25	<1.00	<1.00
	66	26.25	23.5	56.00	1.25	<1.00
	79	27.75	25.00	69.00	<1.00	<1.00
	90	27.25	46.50	52.50	<1.00	<1.00
	98	30.00	46.75	41.25	<1.00	<1.00
J40	32	5.50	35.75	62.50	<1.00	<1.00
	66	1.00	52.75	41.75	<1.00	<1.00
	79	17.00	7.75	56.00	<1.00	<1.00
	90	31.75	47.00	53.00	<1.00	<1.00
	98	1.00	17.25	32.75	<1.00	<1.00
D04	32	54.75	43.75	73.00	<1.00	<1.00
	66	31.00	10.75	83.75	<1.00	<1.00
	79	51.00	70.75	78.75	<1.00	<1.00
	90	51.50	49.00	51.25	<1.00	<1.00
	98	42.75	10.25	43.50	<1.00	<1.00

Appendix 7 Relationship between isolate, humidity and temperature with regard to mean dry weight of *Elsinoë* spp.

Source	DF	Type III SS	Mean Square	F Value[1]	Pr>F[2]
Temperature	4	135142.047	33785.512	203.68	0.0001 *
Humidity	4	3984.080	996.020	6.00	0.0001 *
Isolate	2	6338.667	3169.333	19.11	0.0001 *
Temperature x Humidity	16	7834.220	489.639	2.95	0.0002 *
Temperature x Isolate	8	8947.633	1118.454	6.74	0.0001 *
Humidity x Isolate	8	4271.800	533.975	3.22	0.0017 *
Temperature x Humidity x Isolate	32	15810.400	494.075	2.98	0.0001 *

[1]F Value = the ratio of variance associated with the particular effect measured to the natural variance

[2]Pr>F = the probability (or confidence level) of the effects concerning

* = significant at 99 % confidence level

Appendix 8 Fungicide solutions for resistance tests

Two stock solutions (solution A and B) were made for each fungicide treatment. Flasks labelled A and B contained 500 ml and 100 ml sterile distilled water, respectively. The amount of fungicide added in each flask depended on its percentage active ingredient.

Active Ingredient (a.i.)	Percentage a.i.	Solution A (g/500ml)	Solution B (g/100ml)
chlorothalonil	50 %	1 [a]	5 [a]
mancozeb	80 %	0.625	3.125
prochloraz	50 %	1	5
azoxystrobin	25 %	2	10
captan	50 %	1	5
tolylfluanid	50 %	1	5

[a] Chlorothalonil was measured in ml/500ml or 100ml, not grams

i.e. Prochloraz solution A = 1 g prochloraz was dissolved in 500 ml sterile distilled water
Prochloraz solution B = 5 g prochloraz was dissolved in 100 ml sterile distilled water

The appropriate quantity of solution was added to 250 ml of MEA before pouring to give the required concentration in each plate.

Concentration	Volume of Solution to add to 250 ml MEA
0	0 ml
1	0.25 ml solution A
5	1.75 ml solution A
10	2.5 ml solution A
50	0.5 ml solution B
100	1 ml solution B
500	5 ml solution B
1000	10 ml solution B

i.e. 1 ppm prochloraz = 0.25 ml prochloraz solution A in 250 ml MEA

Appendix 9 Hatsuta Mist Blower

Model	AM-351 M
Application	Mist Blower
Dimensions (L x W x H)mm	460 x 390 x 630
Weight (dry)	9.0Kg
Capacity of Tank	20 litres
Discharge volume (Max.)	3.5 litres/minute
Engine:	
Type	Air-cooled 2-cycle engine
Displacement	43.1cc
Fuel	25 Gasoline : 1 Lubricant oil
Lubricant oil	Special oil for 2-cycle engine
Capacity of fuel tank	1.0 litre
Ignition system	Electronic ignition system
Starter	Recoil starter
Nozzle:	
Type & Use	Medium range for general use
Misting volume (litres/minute)	0 – 3.3
Width (meter)	
At 0.1 meter from nozzle	0.7
At 1.0 meter from nozzle	4.2
Distance (meter)	8 – 10
Engine Revolution (rpm)	7,000 (full)

Appendix 10 Mean wet weight of Isolate J24 fungicide plates at 0, 1, 5, 10, 50, 100, 500 and 1000 µg a.i./ml at week 0, 2, 4, and 6

Fungicide Concentration (µg a.i./ml)	Week	Mean wet weight of isolate J24 fungicide plates (g) Fungicide treatment: azoxystrobin	chlorothalonil	prochloraz	mancozeb
0	0	27.957	27.957	26.469	26.469
	2	27.158	27.158	26.336	26.336
	4	26.909	26.909	26.339	26.339
	6	26.483	26.483		
1	0	23.870	28.363	25.199	25.857
	2	22.878	27.492	25.077	25.752
	4	22.591	27.291	24.890	25.484
	6	22.242	26.957	24.773	
5	0	26.513	31.091	27.335	26.011
	2	25.547	30.178	27.179	25.878
	4	25.259	29.877	27.006	25.674
	6	24.973	29.326	26.945	
10	0	27.430	30.190	28.445	28.478
	2	27.335	29.199	28.321	28.370
	4	26.995	28.913	28.129	28.206
	6	26.650	28.375	28.059	28.111
50	0	24.707	24.776	25.872	26.423
	2	24.494	24.530	25.717	26.303
	4	24.186	24.175	25.565	26.082
	6	23.800	23.704	25.554	
100	0	28.534	28.215	25.379	23.466
	2	28.388	28.021	25.227	23.363
	4	27.447	27.676	25.030	23.247
	6	27.061	27.376	24.975	23.155
500	0		31.845	27.045	26.608
	2		30.776	26.895	26.494
	4		30.414	26.725	26.336
	6		29.895	26.554	26.260
1000	0		32.812	28.992	29.590
	2		31.656	28.876	29.456
	4		31.197	28.730	29.350
	6		30.827	28.523	29.236

Appendix 11 The difference in wet weight of fungicide plates between week 2 (t_2), week 4 (t_4) and week 6 (t_6) with week 0 (t_0) of isolates J24 and J31

Fungicide	Concentration	δ wet weight (g) of Isolate J24			δ wet weight (g) of Isolate J31		
	(μg a.i./ml)	$t_2 - t_0$	$t_4 - t_0$	$t_6 - t_0$	$t_2 - t_0$	$t_4 - t_0$	$t_6 - t_0$
azoxystrobin	0	-0.799	-1.048	-1.474	-0.557	-0.766	-0.929
	1	-0.991	-1.278	-1.628	-1.001	-1.207	-1.505
	5	-0.966	-1.254	-1.540	-0.757	-0.905	-1.164
	10	-0.095	-0.436	-0.780	-0.075	-0.350	-0.705
	50	-0.213	-0.521	-0.907	-0.065	-0.268	-0.502
	100	-0.146	-1.087	-1.474	-0.097	-0.506	-0.724
chlorothalonil	0	-0.799	-1.048	-1.474	-0.557	-0.766	-0.929
	1	-0.871	-1.072	-1.406	-0.725	-0.865	-1.039
	5	-0.913	-1.214	-1.765	-0.731	-0.916	-1.150
	10	-0.991	-1.278	-1.815	-0.945	-1.131	-1.501
	50	-0.246	-0.601	-1.072	-0.111	-0.394	-0.688
	100	-0.194	-0.539	-0.840	-0.109	-0.346	-0.535
	500	-1.069	-1.432	-1.951	-0.979	-1.202	-1.411
	1000	-1.156	-1.615	-1.985	-0.880	-1.067	-1.230
mancozeb	0	-0.134	-0.130		-0.340	-0.521	
	1	-0.105	-0.373		-0.424	-0.657	
	5	-0.133	-0.337		-0.204	-0.420	
	10	-0.108	-0.271	-0.367	-0.154	-0.335	
	50	-0.120	-0.342		-0.169	-0.416	
	100	-0.103	-0.219	-0.311	-0.151	-0.279	-0.476
	500	-0.114	-0.272	-0.348	-0.214	-0.457	-0.608
	1000	-0.134	-0.240	-0.354	-0.273	-0.420	-0.642
prochloraz	0	-0.134	-0.130		-0.340	-0.521	
	1	-0.122	-0.308	-0.426	-0.220	-0.482	-0.672
	5	-0.156	-0.330	-0.391	-0.208	-0.401	-0.560
	10	-0.124	-0.316	-0.386	-0.234	-0.435	-0.882
	50	-0.155	-0.307	-0.318	-0.141	-0.309	-0.312
	100	-0.151	-0.348	-0.404	-0.143	-0.356	-0.530
	500	-0.151	-0.321	-0.491	-0.251	-0.446	-0.723
	1000	-0.116	-0.262	-0.468	-0.151	-0.346	-0.592

Appendix 12 The difference in wet weight of fungicide plates week 2 (t_2), week 4 (t_4) and week 6 (t_6) with week 0 (t_0) of isolates D01and D04

Fungicide	Concentration	δ wet weight (g) of Isolate D01			δ wet weight (g) of Isolate D04		
	(µg a.i./ml)	$t_2 - t_0$	$t_4 - t_0$	$t_6 - t_0$	$t_2 - t_0$	$t_4 - t_0$	$t_6 - t_0$
azoxystrobin	0	-0.441	-0.633	-0.836	-0.751	-0.982	-1.186
	1	-1.031	-1.480	-1.838	-0.902	-1.086	-1.244
	5	-0.655	-0.793	-1.076	-0.760	-0.947	-1.147
	10	-0.080	-0.296	-0.582	-0.091	-0.352	-0.586
	50	-0.053	-0.246	-0.450	-0.071	-0.274	-0.480
	100	-0.105	-0.301	-0.560	-0.072	-0.992	-1.211
chlorothalonil	0	-0.441	-0.633	-0.836	-0.751	-0.982	-1.186
	1	-0.597	-0.746	-0.910	-0.832	-0.981	-1.182
	5	-0.594	-0.751	-0.925	-0.807	-1.027	-0.654
	10	-0.932	-1.179	-1.452	-0.953	-1.149	-1.534
	50	-0.164	-0.371	-0.528	-0.171	-0.417	-0.574
	100	-0.101	-0.358	-0.544	-0.101	-0.342	-0.540
	500	-0.568	-0.843	-1.002	-0.970	-1.229	-1.537
	1000	-0.837	-0.937	-1.102	-0.961	-1.207	-1.372
mancozeb	0	-0.296	-0.561		-0.248	-0.421	
	1	-0.246	-0.601		-0.169	-0.424	
	5	-0.244	-0.642		-0.165	-0.461	
	10	-0.326	-0.646		-0.154	-0.453	
	50	-0.256	-0.633		-0.114	-0.332	
	100	-0.341	-0.566	-0.577	-0.106	-0.279	-0.346
	500	-0.247	-0.476	-0.483	-0.112	-0.266	-0.328
	1000	-0.331	-0.659	-0.696	-0.134	-0.269	-0.346
prochloraz	0	-0.296	-0.561		-0.248	-0.428	
	1	-0.235	-0.554	-0.649	-0.148	-0.335	-0.409
	5	-0.292	-0.615	-0.626	-0.218	-0.460	-0.488
	10	-0.286	-0.686	-0.393	-0.174	-0.448	-0.384
	50	-0.310	-0.772	-0.367	-0.211	-0.480	-0.389
	100	-0.334	-0.653	-0.761	-0.210	-0.392	-0.461
	500	-0.340	-0.698	-0.727	-0.198	-0.405	-0.577
	1000	-0.393	-0.693	-0.780	-0.190	-0.375	-0.665

Appendix 13 Relationship between isolate, fungicide and fungicide concentration with regards to the dry weight measurement of *Sphaceloma* spp.

Source	DF	Type III SS	Mean Square	F Value[1]	Pr>F[2]
Fungicide	4	0.75396507	0.18849127	458.69	0.0001 **
Isolate	2	0.00981141	0.00490570	11.94	0.0001 **
Concentration	7	0.21402564	0.03057509	74.40	0.0001 **
Fungicide x Isolate	8	0.01016224	0.00127042	3.09	0.0024 *
Isolate x Concentration	14	0.00525771	0.00037555	0.91	0.5444 NS
Fungicide x Concentration	28	0.18234688	0.00651239	15.85	0.0001 **
Fungicide x Concentration x Isolate	56	0.05190243	0.00092683	2.26	0.0001 **

[1]F Value = the ratio of variance associated with the particular effect measured to the natural variance

[2]Pr>F = the probability (or confidence level) of the effects concerning

NS = not significant

* = significant at 99 % confidence level

** = significant at 99.9 % confidence level

Appendix 14 Relationship between fungicide and spray season with regard to mean stem scores of *Elsinoë* spp. infection on *Protea* L. cultivar Pink Ice

Source	DF	Type III SS	Mean Square	F Value[1]	Pr>F[2]
Fungicide	4	3.03701000	0.75925250	62.36	0.0001 *
Season	1	0.01056250	0.01056250	0.87	0.3599 NS
Season x Fungicide	4	0.48925000	0.12231250	10.05	0.0001 *

[1]F Value = the ratio of variance associated with the particular effect measured to the natural variance

[2]Pr>F = the probability (or confidence level) of the effects concerning

NS = not significant

* = significant at 99.9 % confidence level