White rust (*Albugo tragopogonis*) of sunflower in South Africa

by

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Submitted to the Faculty of Natural and Agricultural Sciences
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In partial fulfilment of the requirements for the degree of
MSc (Plant Pathology)

October 2003

Acknowledgements

It is a pleasure to thank and acknowledge the following people and institutions for their co-operation in making this dissertation possible.

Prof. T.A.S. Aveling, Department of Microbiology and Plant Pathology, University of Pretoria, for her enthusiastic support and valuable advice, not only during the preparation of this dissertation, but also throughout my student days at the University of Pretoria.

Dr. Altus Viljoen, Forestry and Agricultural Biotechnology Institute (FABI), for introducing me to sunflower and *Albugo tragopogonis*. Thank you for all your encouragement, advice, information and assistance during my time at FABI and the writing up period.

The fabulous FABI team, for the many good memories and practical support.

Staff of the Electron Microscopy Unit, University of Pretoria, for their technical assistance and advice.

Family Bandounas and van den Bout, your names will live forever in this book. We continue with life, love, happiness and everything else which is to follow.

Last but not least I am indebted to my husband, Iman, for all his support, understanding and help during my Masters.

Keywords

South Africa, *Helianthus annuus*, sunflower, *Albugo tragopogonis*, white rust, sporangia, inoculation technique, preservation, susceptible, tolerant, genotype, infection process.

Abbreviations

cm: centimetres

d: days

g: grams

h: hours

LSD_T: least significant difference of Tukey

min: minutes

ml: millilitres

µl: microlitres

mm: millimetres

µm: micrometres

mo: months

PAL: phenylalanine ammonia-lyase

SEM: scanning electron microscope

wks: weeks

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Chapter 1

General Introduction

1.1 Background

Sunflower is considered to be one of the most important oil seed crops and the 4th most important arable crop in South Africa (Pakendorf, 1997; Viljoen, 1997). The sunflower market has increased in the past few years with an annual growth of approximately 4% (Nel, 2001). The Orange Free State and the North West Province have become the largest sunflower growing areas producing the most seed per tonnes annually (SAGIS, 2001).

With the expansion of the local sunflower industry, various pests and diseases have become widespread and today affect production of sunflower in South Africa. These include several infectious bacteria, fungi and insects (Van Wyk, 1994). The relative importance of sunflower diseases varies annually depending on the biological and climatic factors (Viljoen, 1997). The presence or absence of certain diseases can also be linked to management strategies followed on farms where sunflower is cultivated.

In this thesis we have established an inoculation and preservation technique for *Albugo tragopogonis* (Pers.) S.F. Gray, an obligate pathogen that causes severe damage to sunflower crops in South Africa (Van Wyk *et al.*, 1995). These techniques will hopefully contribute to the study of *A. tragopogonis*, its biology and pathogenicity under greenhouse conditions.

1.2 Motivation for the Study

A wide range of fungal diseases have been reported worldwide on sunflower (Allen and Brown, 1980; Gulya *et al.*, 1997; Novotelnova, 1962; Piszker, 1995; Pernaud and Perny, 1995; Sackston, 1957; Sarasola, 1942; Zimmer and

Hoes, 1978). In South Africa, the production of sunflower has not been seriously affected by most of these diseases. The exceptions are Sclerotina stem rot, Sclerotina head rot and rust caused by *Puccinia helianthi* Schw, all of which are predominantly present during wet periods (Parkendorf, 1997). White rust has never been considered an economically important disease and thus little research has been done on *A. tragopogonis* of sunflower. However, with the expansion of the local sunflower industry, white rust has become more widespread and has resulted in extensive damage and yield loss (Viljoen, 1997). Due to the obligate nature of the pathogen, studies have been limited to field trials and observations. Greenhouse trials will greatly contribute to the current understanding of the disease.

1.3 Fundamental Objective

To establish an artificial inoculation technique and methods for maintaining sporangia of *A. tragopogonis* on sunflower for use in greenhouse trials.

1.4 Specific Objectives

- Development and evaluation of a reliable artificial inoculation technique under laboratory and greenhouse conditions that would imitate the infection process of the pathogen in the field.
- Development of a reliable storage method where sporangia would be available all year round (independent of the sunflower growing season).
- After having met the two criteria mentioned above, examination of the infection process of the pathogen on a known susceptible and tolerant sunflower genotype.

1.5 Chapter Outline

Chapter 2: White rust of sunflower, with special reference to the South African situation

This chapter is a literature review. The distribution and importance of white rust in South Africa is discussed, followed by a review of the pathogen, the disease cycle and epidemiology of the pathogen. Various symptoms associated with the disease are also reviewed. Possible measures for control are discussed under the following topics: cultural practices, breeding for resistance, chemical control and biological control.

Chapter 3: Developing an artificial inoculation technique for *Albugo tragopogonis* on sunflower

In this study the aim was to develop a practical and effective greenhouse inoculation technique. The effect of sporangial concentration on germination and white rust development was determined. This was followed by examination of the effect of leaf age, and light and darkness on the development of white rust.

Chapter 4: Investigating methods for maintaining sporangia of *Albugo tragopogonis*, causal agent of white rust of sunflower

The aim of this study was to evaluate different techniques that could be used to preserve sporangia of *A. tragopogonis.* The effect of storage on sporangial germination and on disease incidence was examined.

Chapter 5: Infection of susceptible and tolerant sunflower plants with *Albugo tragopogonis*

In this study the infection of *A. tragopogonis* on a susceptible and tolerant sunflower genotype was examined using light and scanning electron microscopy.

Chapter 6: General Discussion

This chapter includes a general discussion, the author's interpretation of and comments concerning the experiments conducted and recommendations.

Chapter 7: Summary

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Chapter 2

White rust of sunflower, with special reference to the South African situation

2.1 Introduction

White rust has been known in several sunflower (*Helianthus annus* L.) producing countries for many years, but was never considered an important disease. Under certain conditions, however, white rust appeared to become damaging. A new symptomology associated with the fungus causing white rust has been observed in several countries, including South Africa, Australia, Argentina and France (Pernaud and Perny, 1995; Van Wyk *et. al.*, 1995a; Allen and Brown, 1980, Sarasola, 1942). However, it was during the 1993/94 season that these new symptoms resulted in severe damage to late season plantings (Van Wyk et al., 1995a), thereby establishing itself as the most important disease of sunflower in South Africa.

Sunflower is the fourth largest arable crop cultivated in South Africa (Pakendorf, 1997; Viljoen, 1997). The industry has grown considerably in the past 10 years and has thus indirectly contributed to the spread of the white rust pathogen to new areas (Viljoen, 1997). The pathogen has become established on almost all commercial hybrids available to the market, and as a result is threatening local commercial sunflower growers (Viljoen, 1997). Because of the world-wide insignificance of white rust, very little information is available on the disease. This review, therefore, aims to summarise the available information, and hopes to propose research strategies for the future.

2.2 Production of Sunflower

The sunflower plant is a member of the Compositae family, and consists of 67 species in the genus *Helianthus*, all native to the Americas (Berglund, 1994).

The American Indians used the plant for food, medicinal products and dyes for body painting and pottery before colonisation of the new world. Thereafter, the plant was taken by Spanish explorers to Europe where it was grown as a common garden flower. The cultivated sunflower (*H. annuus*) was developed as a premier oil-seed crop in Russia, and today, several high-oil varieties and hybrids are widely grown in the world. Apart from the oil-seed varieties, sunflower is also used for confectionery and as a bird feed.

The major production areas of sunflower include the former Soviet Union, Argentina, Eastern Europe, USA, China, France and Spain (Berglund, 1994). These countries produce about 84% of the world's oilseed and non-oilseed sunflower, comprising more than 20 million metric tons in 1992. In comparison to the major sunflower producing countries, South Africa is a minor producer, even though it produces 81% of Africa's sunflower seed (Vorheis, 2001). Production increased from approximately 100 000 tons in the 1965/66 season to 600 000 tons at the end of the century (SAGIS, 2001). The majority of sunflower seed since 1993 is produced in the North West and Free State provinces. This was not always the case, as Mpumulanga was considered the major sunflower production area until the severe outbreaks of Sclerotinia infections destroyed the industry in the province.

Sunflower seed is the source of 82% of all eligible oil production in South Africa (Nel, 2001). From the seed produced between 1989/90 and 1998/99, approximately 219 00 tons of oil was extracted (Nel, 2001). Still, sunflower was only considered as an alternative crop to maize production, that is, if a maize crop was not successfully produced during the first part of the growing season (Pakendorf, 1997). However with the freeing of the market economy and the inclusion of sunflower oil cake (a by-product of sunflower oil extraction) as a source of protein for animal feed blends (Ebedes, 1997; Nel, 2001), sunflower gradually developed into a crop in its own right (Parkendorf, 1997). South Africa is still considered to be a net importer of vegetable oils (Nel, 2001) even though exceptions exist. For example, in 1999 South Africa exported 56 000 tons and 764 000 tons were sold locally.

Sunflower production can be badly affected by diseases and pests. In South Africa, sunflower production was not adversely affected by diseases, with the exception of *Sclerotinia sclerotiorium* (Lib.) De Bary and *Puccinia helianthi* Schw., which were present during periods of high rainfall in the Mpumulanga Province (Parkendorf, 1997). With the increase in local sunflower production, white rust and downy mildew became more prevalent and may in the future have a large impact on the market (Viljoen and van Wyk, 1997)

2.3 White Rust of Sunflower

White rust of sunflower has been reported from several countries such as the U.S.A. (Gulya *et al.*, 1997), Uruguay (Sackston, 1957), Russian Federation (Novotelnova, 1962), Hungary (Piszker, 1995), France (Pernaud and Perny, 1995), Argentina (Sarasola, 1942), Australia (Allen and Brown, 1980) and other countries (Zimmer and Hoes, 1978). The most important of these include Argentina, Australia and South Africa. In most of these countries, white rust was considered to be of minor importance. Middleton (1971) summarised this opinion by stating that yield reduction was likely to be small and, for that reason, control of the disease was never attempted.

Sackston (1957) first recognised the potential of white rust to cause severe damage under favourable conditions in Uruguay. Surveys that were conducted during the 1978/79 growing season by Allen and Brown (1980) in New South Wales, found unique symptoms to be common in late season sunflower plantings. They concluded that the pathogen was more important than previously anticipated. Similar symptoms were later reported from several other countries, and in some instances caused substantial losses of sunflower (Middleton, 1971; Allen and Brown, 1980; Delhey and Kiehr-Delhey; 1985).

White rust was first reported in South Africa by Verwoerd in 1929 (Viljoen, 1997). According to Potgieter *et al.* (1997), white rust occurred sporadically in the Mpumalanga Province during the 1970's, and no loss in yield was

reported in commercial crops. White rust, therefore, was not considered to be an economically important disease. The first severe outbreak of white rust occurred in a breeding nursery in Potchefstroom in 1979 (Potgieter *et al.* 1997). During the 1980's, the disease became more prevalent on late season plantings. Potgieter *et al.* (1997) mentioned that, with the expansion of the sunflower industry, the disease was introduced into new sunflower growing areas. As a result it became a problem in the Free State and North West provinces. In the 1989/1990 growing season, leaf infections were observed in early, middle and late season plantings (Malan, 1989). Unique symptoms of white rust on sunflower stems (grey stem spot) were observed for the first time in the 1991/1992 planting season (Potgieter *et al.*, 1997). In 1993, the effect of lodging resulted in stem breakage exceeding 80% in some sunflower growing fields (Van Rooyen, 1995; Van Wyk *et al.*, 1995a).

Leaf symptoms have become severe on commercial hybrids in South Africa since 1993. However, significant yield losses appear to be uncommon (Viljoen, 1997). Petiole greying has become more common since 1994 with plants becoming more susceptible after flowering (Viljoen, 1997). Both petiole greying and grey stem spot are followed by defoliation of infected plants, thereby reducing the yield (Potgieter *et al.*, 1997). Since 1993, yield losses were lower.. In 1996 levels of lodging were severe due to the extremely wet season (Viljoen, 1997).

2.4 The Pathogen

In 1801, *Albugo tragopogonis* was described from stems and leaves of *Tragopogon porrifolius* L. and was named as *Uredo candida* β *tragopogi* by Persoon (Whipps and Cooke, 1978). According to Whipps and Cooke (1978), *U. candida* β *tragopogi* was renamed *Uredo tragopogi* by de Candolle in 1815. In 1821, during the renaming of *Uredo* species, Gray changed the name of the pathogen to *Albugo tragopogonis* (Pers.) S.F. Gray. *Albugo tragopogi* (Pres.) Schroet. was listed in Biga's description of species of *Albugo* (Biga, 1955). However, the epithet 'tragopogi', which refers to the type host, was an

orthographic error which was conserved in the early literature. *Albugo tragopogonis* (Pers.) S.F. Gray is the proper form and this name persists to the present day (Whipps and Cooke, 1978).

In his description of *Albugo* spp., Biga (1955) classified isolates linked to different hosts into five different biotypes. Experimental evidence by Whipps and Cooke (1978) and Hartmann and Watson (1980) showed that *A. tragopogonis* was not cross-infective on four different Composite species. Whipps and Cooke (1978), therefore, suggested that only one *formae specialis* of *A. tragopogonis* per host exists. According to Mukerjii and Brown (1976), *A. tragopogonis* infects members of Compositae (Asteraceae) only. Gulya *et al.* (1997) stated that sporangia originating from weedy Composites were likely not to infect wild or cultivated sunflower and that to date no one has investigated the host range of *A. tragopogonis* isolates from cultivated sunflower.

Albugo tragopogonis has both a sexual and asexual life cycle (Agrios, 1988; Nowell and Viljoen, 1997). The asexual stage becomes visible when sporangia are produced in pustules on the lower surfaces of leaves. Individual sporangia are hyaline to light yellow in colour and cylindrical to spherical with flattened ends (18-24 μm x 12-20 μm) (Gulya *et al.*, 1997; Hamilton-Attwell *et al.*, 1995; Mukerjii and Brown, 1976). Pustules are produced by mycelium growing intercellularly below the epidermal cells towards the abaxial surface and finally giving rise to short, club-shaped sporangiophores that produce a number of sporangia in rows (Hamilton-Attwell *et al.*, 1995; Mukerjii and Brown, 1976). These exert a pressure on the host epidermis and causes it to rupture (Viljoen and Van Wyk, 1996; Zimmer and Hoes, 1978).

The sexual stage is characterised by spherical dark brown to black oospores (Gulya *et al.*, 1997; Mukerjii and Brown, 1976). On the onset of cooler weather, sexual structures called oogonia and antheridia are produced in the intercellular spaces of the host tissue. There is no detail available in literature

about which method of the cytological fertilisation is used by *A. tragopogonis* in sunflower. In general, an oogonium and antheridium fuse result in an oospore (Alexopoulos *et al.*, 1996). The oospores are visible as grey lesions on stems, petioles, receptacles, involucral bracts and veins on leaves (Allen and Brown, 1980; Van Wyk *et al.*, 1995a).

2.5 The Disease Cycle and Epidemiology

Albugo tragopogonis overwinters as oospores in the soil (Gulya *et al.*, 1997; Pernaud and Perny, 1995; Viljoen and Van Wyk, 1996). The oospores serve as primary inoculum that infects young sunflower seedlings. With the first rainfall, oospores are water-splashed onto leaves of young seedlings (Gulya *et al.*, 1997; Viljoen and Van Wyk, 1996; Viljoen, 1997) (Fig. 2.1). The oospores then germinate and release their motile zoospores. The zoospores enter stomata of leaves, encyst within the substomatal cavity and germinate to produce intercellular hyphae (Kajornchaiyakul and Brown, 1976; Pernaud and Perny, 1995) (Fig. 2.1). Hyphae infect cells and eventually produce white pustules on the bottom of sunflower leaves. These pustules rupture and sporangia are disseminated by wind and water splashed to initiate secondary infections (Gulya *et al.*, 1997; Pernaud and Perny, 1995; Viljoen and Van Wyk, 1996) (Fig. 2.1).

Infection is dependant on the presence of water (Gulya *et al.*, 1997; Potgieter *et al.*, 1997; Viljoen, 1997). Sporangia and oospores require water in order to germinate and release zoospores. All phases of infection are also influenced by temperature (Kajornchaiyakul and Brown, 1976; Pernaud and Perny, 1995). Sporangia germinate between 4 and 35°C to form zoospores (Kacjornchaiyakul and Brown, 1976; Pernaud and Perny, 1995). The optimum temperature for sporangial germination is 15°C (Kajornchaiyakul and Brown, 1976). According to Kajornchaiyakul and Brown (1976), zoospores that are released remain viable in temperatures between 4 and 20°C. Optimum temperature for infection is between 10 and 15°C and optimum

temperature for disease development is between 20 and 25°C (Kajornchaiyakul and Brown, 1976).

Secondary infection of sunflower leaves plays a very important role in the build-up of the inoculum in the field and in the spread of the disease during the growing season (Viljoen, 1997). However, according to Potgieter *et al.* (1997), no information is available on the amount of inoculum necessary to cause primary and secondary infection. The rate at which the disease can spread under favourable conditions has also not been determined either (Potgieter *et al.*, 1997). Secondary infections also result in the infection of the stems, petioles and the leaf axillas (Van Wyk *et al.*, 1995a) (Fig. 2.1). Sporangia germinate in water, liberating 7-11 biflagellate zoospores per sporangium (Fig. 2.1) (Kacjornchaiyakul and Brown, 1976). Zoospores are motile and enter the stems and petioles through stomata (Gulya *et al.*, 1997; Van Wyk *et al.*, 1995a) (Fig.2.1).

Sporangia are important for the spread and not for the survival of the pathogen (Viljoen, 1997). At the onset of cooler weather (usually at the end of the growing season), mycelium matures in the cells to produce oospores. These structures are formed through sexual reproduction of antheridia and oogonia (Allen and Brown, 1980; Pernaud and Perny, 1995; Viljoen and Van Wyk, 1996). Fungal hyphae and oospores weaken the host cells and cause the stems and petioles to become susceptible to lodging and defoliation (Krüger *et al.*, 1999). Oospores present in petioles, stems and sunflower heads end up in the soil (Fig. 2.1) where they can survive for nine years and longer (Potgieter *et al.*, 1997) to complete the life cycle of the fungus. Oospores have been reported in the pericarp of seeds (Van Wyk *et al.*, 1999; Viljoen *et al.*, 1997), and this may be responsible for the introduction of the fungus into new areas. However, Viljoen *et al.* (1997) and Van Wyk *et al.* (1999) concluded that the incidence of seed transmission of the white rust fungus was expected to be low.

Environmental conditions in South Africa favour infection of white rust of sunflower. Optimum infection takes place when night-time temperatures drop

bellow 15°C and adequate moisture of 90% relative humidity is present (Potgieter *et al.*, 1997). In midsummer (December-February), night temperatures can drop to 15°C following thunderstorms. Daytime temperatures of 20-25°C are also suitable for the development of the disease (Kajornchaiyakul and Brown, 1976). At the end of the season (March-May), environmental conditions become unfavourable to the pathogen (Potgieter *et al.*, 1997). The drop in temperatures results in the formation of oospores on petioles, stems, receptacles and involucral bracts (Potgieter *et al.*, 1997).

2.6 Symptoms

Symptoms associated with white rust of sunflower can be divided into two categories (Nowell and Viljoen, 1997). The first category includes symptoms expressed during the asexual phase of the pathogen's life cycle. The second, category includes symptoms associated with the sexual phase of the life cycle.

Symptoms expressed during the asexual phase become visible as white pustules on the infected plant parts during local and systemic infections (Nowell and Viljoen, 1997; Van Wyk *et al.*, 1995a). Symptoms associated with the sexual phase are manifested on the sunflower plants as grey, bruise-like lesions of sunflower leaf veins, stems, petioles and heads(Van Wyk *et al.*, 1995a).

2.6.1 Leaf spots

Early symptoms of white rust consist of chlorotic leaf spots present on the upper surface of the leaves (Gulya *et al.*, 1997; Zimmer and Hoes, 1978). Directly below these spots, white to creamish blister-like swellings called pustules are formed (Gulya *et al.*, 1997; Zimmer and Hoes, 1978). Sporangia are produced in pustules (Hamilton-Attwell *et al.*, 1995; Mukerjii and Brown, 1976). The sporangia exert a pressure on the host epidermis causing it to

bulge and eventually rupture, thereby exposing the white sporangia, which become air-borne as secondary inoculum (Viljoen and Van Wyk, 1996; Zimmer and Hoes, 1978). During heavy infections, pustules have also been observed on involucral bracts and petioles of sunflower (Nowell and Viljoen, 1995). Severely infected leaves become necrotic, turn brown and give the plant a blighted appearance (Zimmer and Hoes, 1978).

2.6.2 Systemic infections

Systemic and semi-systemic leaf infections by *A. tragopogonis* were reported in South Africa since the 1992/1993 growing season (Viljoen and Van Wyk, 1996). According to Van Wyk *et al.* (1995a), sporangia on these plants were not produced in localised blisters, and lesions were spread over large areas bordering the veins of apical leaves. It is unknown whether systemically infected plants result from seed-borne and/or soil-borne inoculum (Nowell and Viljoen, 1997; Van Wyk *et al.*, 1995a).

2.6.3 Petiole greying

Petiole greying is caused by numerous black oospores of *A. tragopogonis* that are produced in the cortex of the host tissue (Allen and Brown, 1980; Van Wyk *et al.*, 1995a). The symptom seems to originate at the base of the petioles and may later be observed along the rest of the petioles (Nowell and Viljoen, 1997; Van Wyk *et al.*, 1995a,b). Petiole greying was first described in Australia (Allen and Brown, 1980) and in Argentina (Gulya *et al.*, 1997; Van Wyk *et al.*, 1995a). According to Allen and Brown (1980) and Van Wyk *et al.* (1995a), susceptible plants develop petiole greying after flowering and petal fall phase of plant growth.

According to Krüger *et al.* (1999), parenchymatous tissues in infected petioles are heavily colonised by fungal hyphae. Infected tissue cells deteriorated and finally collapsed. Infected petioles become necrotic, dried up and defoliation occurred (Van Wyk *et al.*, 1995a). Nowell and Viljoen (1997) stated that great

loss in yield could be expected if petiole greying and premature defoliation occurred before seed development was fully completed.

2.6.4 Grey stem spot

Grey stem spot is grey, bruise-like lesions that form individually or coalesce on the entire length of stems. Closer examination of the stem lesions showed that hyphae occurred intercellularly in the cortex, cambium, vascular rays, and pith of stems (Krüger *et al.*, 1999). The middle lamellae of cells in infected tissue was dissolved, cells degenerated and eventually collapsed (Krüger *et al.*, 1999). This eventually resulted in lodging of stems (Krüger *et al.*, 1999) when the slightest force (example strong winds) was applied (Van Wyk *et al.*, 1995a). The white rust fungus has been associated with stem lesions on sunflower in the past (Allen and Brown, 1980; Gulya *et al.*, 1997), but lodging of stems was never observed.

Lodging of sunflower was reported for the first time in South Africa and was associated with the symptoms expressed by grey stem spot (Van Wyk *et al.*, 1995a, b; Van Wyk and Viljoen , 2000). Van Wyk *et al.* (1995a) stated that stem-breakage most often occurred 5-6 cm above the soil level, but that it can occur on the rest of the stem as well (Nowell and Viljoen, 1997; Van Wyk *et al.*, 1995b). Lodging was common in plants grown the mid and late seasons (Van Wyk *et al.*, 1995a, b). According to Van Wyk and Viljoen (2000), inoculum build-up of *A. tragopogonis*, susceptible cultivars, the tendency to over-use herbicides and favourable climatic conditions have probably all contributed to the development of lodging.

2.6.5 Greying of sunflower heads

Greying of sunflower heads occurs when oospores are formed in infected tissue. According to Gulya *et al.* (1997), *A. tragopogonis* caused greying at the base of the involucral bracts on sunflower heads in Argentina. In

Australia, greying of receptacles and involucral bracts was also reported (Allen and Brown, 1980). Oospores were also reported in the pericarp of sunflower seeds (Van Wyk *et al.*, 1999; Viljoen *et al.*, 1997). According to Viljoen *et al.* (1997) and Van Wyk *et al.* (1999), the pathogen appeared to enter the seed through the micropyle.

2.6.6 Greying of leaf veins

Oospores of *A. tragopogonis* have been found in veins of sunflower leaves. Nowell and Viljoen (1997) stated that this symptom might have an effect on photosynthesis. However, these symptoms seemed to be more common on breeding lines than on commercial hybrids (Nowell and Viljoen, 1997).

2.7 Control

Control measures for white rust have not been developed due to the sporadic occurrence of the disease and its limited economic importance in most countries (Viljoen and Van Wyk, 1996). Recent trials and field observation, however, have shown that several control strategies are effective.

2.7.1 Cultural practices

Various cultural practices can influence disease development. For example, planting date, herbicide treatment, spacing and type of cultivar can have an effect on the control of the disease (Van Wyk and Jooste, 1997). Unfavourable environmental conditions can also lower the level of inoculum, and, therewith, disease incidence. The absence of free water, low temperatures and young leaves can break the disease cycle of the fungus (Nowell and Viljoen, 1997; Potgieter *et al.*, 1997). Due to the obligate nature of the pathogen, it can only survive and multiply on living host tissue (Potgieter *et al.*, 1997).

Planting date: Planting date provides a good opportunity for white rust management, since temperature and rainfall play an important role in disease development (Viljoen and Van Wyk, 1996). Early plantings are subjected to low initial inoculum levels (Van Wyk and Jooste, 1997; Viljoen and Van Wyk, 1996). Environmental conditions during spring and early summer favour rapid growth of sunflowers. Plants are healthier and are subjected to fewer environmental stresses (Van Wyk and Jooste, 1997). In contrast, when sunflower is planted late in the season, the environmental conditions are below the optimum required for sunflower production, the inoculum levels are higher, and new infections are favoured by the cooler weather conditions (Van Wyk and Jooste, 1997; Viljoen and Van Wyk, 1996). The cooler conditions also result in the formation of oospores that weaken sunflower petioles and stems and result in defoliation and lodging.

Herbicide treatment: Herbicides affect sunflower plants and predispose them to infection by white rust (Van Wyk and Jooste, 1997). Van Wyk and Jooste (1997) also suggested that instructions recommended by the manufacturers for a certain herbicide must be followed precisely, especially information regarding the concentration of herbicide that must be used for a specific soil type.

Planting density: Planting density plays an important role for the management of lodging in sunflower (Van Wyk and Jooste, 1997). The normal planting density of sunflower is 30 000-40 000 plants per hectare (Van Wyk and Jooste, 1997). Stems in higher density plantings are thinner and more susceptible to lodging following infection by *A. tragopogonis* (Van Wyk and Jooste, 1997; Viljoen and Van Wyk, 1996).

Crop rotation and sanitation: Crop rotation has little effect on the management of white rust (Van Wyk and Jooste, 1997). The reason for this is that oospores can survive for up to nine years or longer in soil (Potgieter *et al.*, 1997). Van Wyk and Jooste (1997) suggest that an effective means of crop

sanitation may be deep ploughing, which can bury inoculum so deep that infections become less probable (Van Wyk and Jooste, 1997).

2.7.2 Breeding for resistance

Gulya *et al.* (2000) evaluated sunflower germplasm of the United States Department of Agriculture (USDA) for resistance to *A. tragopogonis* in South Africa utilising natural infection during late season plantings. Of these, 83% of the accessions were completely susceptible and 8% were rated as immune or highly resistant to leaf, stem and petiole infection (Gulya *et al.*, 2000). Gulya *et al.* (2000) observed that leaf pustules were restricted to lower leaves and expressed as small, localised lesions on some lines, while petiole and stem lesions would be large and widespread on the plant. Van der Merwe *et al.* (1997) also stated that there was a lot of variation in susceptibility among sunflower breeding lines and that this type of variation was suggestive of polygenic resistance to the disease. The exact number of genes involved and gene actions are still unknown and await inheritance studies.

Several sunflower genotypes were identified with high levels of resistance or immunity. To date, the only accession identified with near immunity to leaf, stem, petiole, bract and head lesions have been Ames 3430 (a breeding line from Russia designated as VIR 107).

2.7.3 Chemical control

No fungicide is registered for the control of *A. tragopogonis* on sunflower in South Africa. A recent trial conducted by Strauss and Viljoen (1997) indicated that sunflower plants sprayed with metalaxyl/mancozeb had white rust symptoms but the chemical had prevented the development of new infections. This fungicide, however, did not cure infected tissue from infection by *A. tragopogonis* (Viljoen and Van Wyk, 1996).

Metalaxyl inhibits RNA synthesis in Oomycetes and was applied because of its known activity against the Albuginaceae (Saharan and Verma, 1992; Strauss and Viljoen, 1997). Mancozeb is toxic to fungi as they are metabolised by the pathogen. The fungicide inhibits the production and function of a variety of amino acids and enzymes (Agrios, 1988). In South Africa, damage to sunflower is mostly restricted to lodging of plants. Therefore, successive spraying with metalaxyl would have to be applied throughout the growing season (Strauss and Viljoen, 1997). This is an expensive method of control and can only be financially justified for seed production and not for foliar sprays of commercial plantings (Strauss and Viljoen, 1997).

Metalaxyl has been successful in the control of the Oomycetes, and particularly important in the control of fungi within the order Peronosporales (Saharan and Verma, 1992). It has been effective against *Plasmopara halstedii* (Far.) Berl and de Toni, which causes downy mildew of sunflower (Melero-Vara *et al.*, 1982; Oros and Virányi, 1987). According to Mouzeyar *et al.* (1995), metalaxyl treatment on sunflower caused numerous histological changes in susceptible plants infected by *P. halstedii*. They concluded that metalaxyl might have activated genes involved in genetic resistance, by triggering host defence mechanisms, which led to those histological changes.

2.7.4 Biological control

Two beetle species, *Formicomus rubricollis* LaFerte (Coleoptera: Anhicidae) and *Astylus atromaculatus* Blanchard (Coleoptera: Melyridae) have been observed to feeding on pustules of *A. tragopogonis* (Viljoen *et al.*, 1996). *Astylus atromaculatus* plays an important role in decreasing the level of inoculum in the early part of the season, but it does not feed on the fungus when pollen becomes available (Viljoen and Van Wyk, 1996; Viljoen *et al.*, 1996). *Formicomus rubricollis* was not as an aggressive a feeder as *A. atromaculatus* (Viljoen *et al.*, 1996). However, this beetle did visit newly developed pustules and can also contribute to the decrease in incidence of

disease. Both beetle species reduced the inoculum levels in the early part of the season, but did not completely eliminate the fungus. They can, therefore, be considered as natural enemies of the fungus (Viljoen *et al.*, 1996).

2.8 Conclusion and Research Aims

The impact of white rust on South African commercial sunflower crops has not been determined. It is, however, clear that symptoms that cause premature defoliation and lodging can result in extensive damage and yield loss. Additionally, seed infections can potentially threaten local and international markets. Research needs to be done to find possible seed treatment that will keep the disease at bay and protect sunflower crops around the world.

To date, little work has been done on *A. tragopogonis* of sunflower. This is primarily due to the sporadic occurrence of white rust and its minor economical importance in the past. Due to the obligate nature of *A. tragopogonis*, studies of its biology, epidemiology and control have been limited to field trials and observations. Although the above-mentioned studies have contributed largely to the current understanding of the disease, greenhouse trials are also needed. Greenhouse trials can support studies examining host-pathogen interactions and even molecular studies on the population genetics of *A. tragopogonis* isolates from cultivated sunflower. The knowledge gained can be used for finding alternative methods of control. Pinpointing the weaknesses of the pathogen during the disease cycle on specific genotypes will also support future research in determining which chemicals, treatments and method of application could be useful for control.

In order to effectively carry out greenhouse studies, two criteria have to be met. Firstly, a reliable artificial inoculation technique needs to be developed which mimics the infection process of the pathogen as in the field. Secondly, sporangia need to be available all year round so that research can be done independent of the seasonal harvesting of sporangia in the field. Having met

these two criteria, it will be possible for researchers to investigate the infection process of the pathogen and determine possible strategies or control.

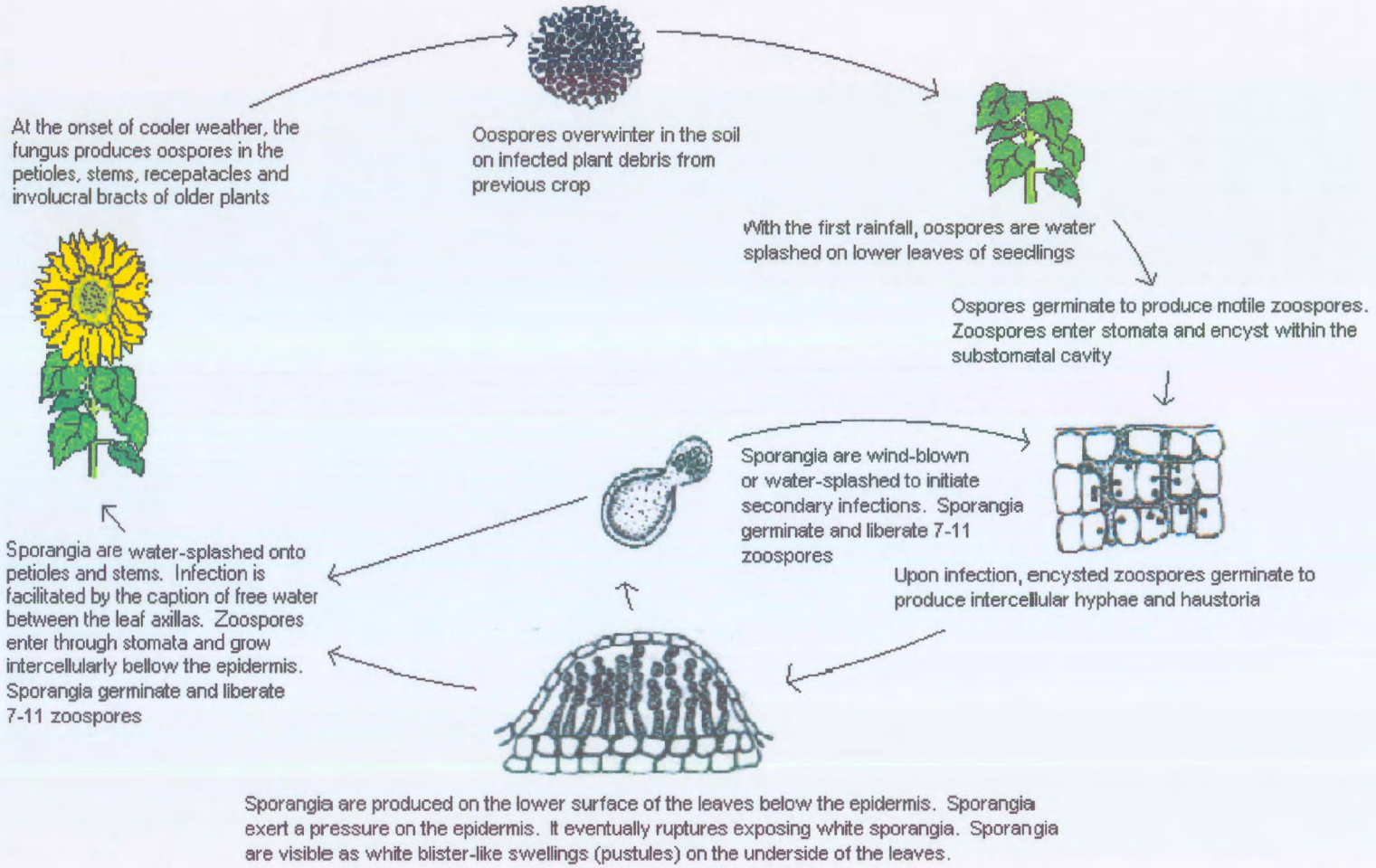

Figure 2.1 The disease cycle of *Albugo tragopogonis* on sunflower.

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Chapter 3

Developing an artificial inoculation technique for *Albugo tragopogonis* on sunflower

3.1 Introduction

Albugo tragopogonis (Pers) S.F. Gray is responsible for white rust of sunflower (Van Wyk and Rong, 1993; Van Wyk *et al.*, 1995). White rust is not considered a major disease of sunflower, but has been reported to be damaging to the crop in South Africa (Van Rooyen, 1995; Van Wyk *et al.*, 1995), Australia (Allen and Brown, 1980) and Argentina (Sarasola, 1942). As a result of its limited economic importance, relatively little research has been done on the disease and its causal agent.

Due to the obligate parasitic nature of *A. tragopogonis*, studies of the biology, epidemiology and control of the disease has, until now, been limited to field trials and observations. Field trials have proved to be useful in evaluating breeding lines and hybrids for disease resistance (Van der Merwe *et al.*, 1997), testing fungicides (Strauss and Viljoen, 1997) and biological control agents (Viljoen and Wyk, 1996; Viljoen *et al.*, 1996), and understanding the epidemiology of the fungus (Potgieter *et al.*, 1997). Although some studies have addressed the biology *of A. tragopogonis* (Bandounas *et al.*, 2000; Hamilton-Attwell *et al.*, 1995; Kajornchaiyakul and Brown, 1976; Mukerji and Brown, 1976; Whipps and Cooke, 1978b), there is still no clear understanding of the infection process, resistance mechanisms, and population biology of the pathogen.

Greenhouse trials need to be developed to evaluate sunflower seedlings for white rust resistance, treatment with fungicides, understanding the infection process, and examining the resistance mechanisms in the plant. This study, therefore, is an attempt to develop a practical and effective greenhouse inoculation technique to study the biology and pathogenicity *of A.*

tragopogonis. The effect of sporangial concentration on germination and severity of the pathogen was first determined. The effect of leaf age on the infection of sunflower leaves by *A. tragopogonis* was then examined. Lastly the effect of light and darkness on the severity of A. *tragopogonis* was investigated.

3.2 Materials and Methods

3.2.1 Plant material and trial design

The sunflower accession RHA 358, obtained from the USDA Plant Introduction Station at Iowa State University in the United States, was used in all experiments. This accession proved to be highly susceptible to *A. tragopogonis* in previous studies (Gulya *et al.*, 2000). In all the experiments, 4-5 seedlings were grown in pots containing 'Braak' potting soil, which is composed of plant moss, peat bark, volcanic material, river sand, milled oasis and plant food additives. At the 4-leaf stage the number of seedlings were thinned to three per pot. Ten pots were used for each treatment, and the pots were arranged in a completely randomised block design with a factorial arrangement of treatments. After inoculation, greenhouse temperatures were adjusted to day/night time temperatures of 25/18°C, and treated plants incubated until symptom development. The sunflower seedlings were exposed at daytime with a light intensity of 22 000 lux for 12 h. Sunflower seedlings were watered every second day.

3.2.2 Collection and germination of inoculum

Freshly collected sporangia of *A. tragopogonis* were used for inoculation of sunflower seedlings in all experiments. Initially, leaves infected with *A. tragopogonis* were collected from sunflower plants at the Grain-Crops Institute in Potchefstroom, South Africa. The leaves containing white rust pustules

were kept fresh by placing them in plastic bags in a cooler box, and with their petioles submerged in ice water. Sporangia collected from the white rust pustules of field plants were then inoculated onto the leaves of seedlings in the greenhouse. The sporangia contained in the white rust lesions that developed on seedling leaves were used as inoculum throughout the duration of this study. Fresh sporangia, therefore, were used in all inoculation studies.

Sporangia were collected from sunflower leaves by using a vacuum pump before suspension in sterile distilled water. The sporangial suspensions were then diluted to 1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 sporangia per ml by using a Kovac Glasstic hemocytometer. Each dilution was incubated at 10°C for 3 h in the dark. During the incubation period, spore suspensions were examined every 30 min to determine when the zoospores would be released, encyst and germinate. This information was required to determine what the optimum period of incubation would be.

At the end of the 3 h incubation period, germination of the sporangia was assessed. A sporangium was considered germinated if it lacked cytoplasm (Verma and Bhowick, 1991). Suspensions were also stained with lactophenol-cotton blue and examined microscopically. The average percentage germination of each dilution was determined from the average of three random fields.

3.2.3 The effect of sporangial concentration on white rust development

Sunflower seedlings were inoculated by spraying the five different concentrations of *A. tragopogonis* spores on the adaxial surface of the leaves with a small hand held sprayer until drip-off. The inoculated seedlings were then placed at 12-15°C and covered with plastic bags to maintain a high humidity. The plastic bags were removed ±16 h after inoculation, and incubated in a greenhouse until symptom development.

3.2.4 The effect of leaf age on white rust development

In order to determine if leaf age had any effect on white rust development, sunflower leaves at several stages of plant development were inoculated with *A. tragopogonis*. Sunflower seedlings were grown in the greenhouse to 2-, 4-, 6-, 8- and 10-leaf stage (Fig. 3.1), and sprayed with a sporangial suspension to the concentration of 10^5 sporangia/ml. The leaves within each age category were inoculated with a hand held garden sprayer bottle at dusk and incubated at 12°C. Plants were covered with plastic bags, and these were removed ±16 h after inoculation.

3.2.5 Ratings on adaxial/abaxial cotyledon surfaces of various sunflower lines

A visual white rust rating scale was developed by Fox and Williams (1984) for *A. candida* on cotyledons of *Brassica campestris* L. The rating scale measures the development of pustules on both surfaces of the cotyledons. Pustules were also evident on both surfaces of cotyledons in sunflower, and for this reason we decided to use the scale. The cotyledons were inoculated using the hand held garden sprayer described previously.

3.2.6 The effect of time of day on infection of sunflower leaves by *A. tragopogonis*

Two sets of sunflower seedlings were inoculated at the 4-leaf-stage by spraying a spore suspension of 10^5 sporangia/ml onto the adaxial surface of the leaves with a small hand held sprayer until completely wet. One set of sunflower seedlings was inoculated 4 h after sunrise, and the other set was inoculated at dusk. Both sets of seedlings were placed at 12-15°C and covered with plastic bags to maintain a high humidity. The plastic bags were removed ±16 h post-inoculation, and the plants remained in a greenhouse until symptoms developed.

3.2.7 The effect of inoculation method on white rust development

Four methods of inoculating sunflower leaves with *A. tragopogonis* were compared. In each, a sporangial concentration of 10^5 sporangia/ml was used. After inoculation, the plants were incubated until symptom development. The four methods were:

- Seedlings were carefully uprooted and their leaves submerged into a sporangial suspension for either 1.5 or 3 h. Seedlings were then replanted. For each time period, one set of plants was covered with plastic bags and the other set remained uncovered.
- Drops of inoculum (10 µl) were placed onto each leaf of seedlings at the 4-leaf stage with a calibrated micropipette. One set of plants was covered with plastic bags, and the other set remained uncovered.
- A sporangial suspension was brushed on the underside of leaves with a camelhair brush. One set of inoculated plants was covered with plastic bags and the other set remained uncovered.
- Seedlings were inoculated using a small hand held garden sprayer. One set of plants were sprayed with the zoospore suspension on the adaxial surface of the leaves only, while the other set of plants were sprayed on both the adaxial and abaxial surfaces of the leaves. For both treatments, one group of plants was covered with plastic bags while the other group remained uncovered.

3.2.8 Evaluation of sunflower varieties for disease resistance

Six sunflower seedling accessions were included in a trial to determine whether the small plant screening method would discriminate between susceptible and resistant plants. The accessions include RHA 358, RHA 357, RHA 247, HV 3037, PAN 7392, HYS 333. During field evaluation HV 3037 and PAN 7392 slightly tolerant and HYS 333 highly tolerant to white rust.

3.2.9 Assessment of infection levels

Infection levels of sunflower leaves were assessed 10-14 d after inoculation. A scale of 1-5 developed by Siddiqui *et al.* (1975) was used, with 1 indicating resistance and 5 indicating severe infection.

3.2.10 Statistical analyses

White rust incidence in all trials was determined using the SYSTAT programme. The General Linear Model was used to analyse the variation within the different sporangial concentrations. Wherever the F-values were significant for the above analyses, the least significant difference (LSD_T) of Tukey was calculated to indicate which means differed significantly from each other. The tables with statistical values for each trial can be found in Appendix A.

3.3 Results

3.3.1 Germination of inoculum

About 30 min after sporangia of *A. tragopogonis* were placed in water, a papilla formed at the apex of the sporangia (Fig. 3.2a). The papilla would develop until undifferentiated protoplasm was released from the sporangium through an opening at the papilla approximately 1.5-2 h after it was first placed in the water (Fig. 3.2b). The protoplasm differentiated into zoospores that were swimming actively 2.5-3 h after being placed in water. Zoospores had encysted within 6 h (Fig. 3.2c), and between 8-12 h, all the zoospores had germinated and produced one –to two germ tubes (Fig. 3.2d).

3.3.2 The effect of concentration on sporangial germination and white rust development

A sporangial concentration of 10^5 sporangia/ml showed the highest germination percentage of 25% but did not differ significantly from 10^3 (Fig. 3.3). Sporangial concentrations of 10^4, 10^3 and 10^2 sporangia/ml had germination percentages of 19.4, 21.7 and 18.0%, respectively. Sporangial concentration at 10^6 showed a significantly low percentage germination of 10.8% (Fig. 3.3).

White rust development was most significant when seedlings were inoculated with *A. tragopogonis* suspensions at concentrations of 10^4 and 10^5 sporangia/ml. At both these concentrations, disease severity was significantly more than in plants inoculated with sporangial suspensions of 10^2 and 10^3 sporangia/ml, and the control (Fig. 3.4). No significant differences were found between the control and the severity of plants inoculated with concentrations of 10^2, 10^3, 10^6 sporangia/ml.

3.3.3 The effect of leaf age on white rust development

Sunflower leaves at establishment stage (G2), i.e. 2- and 4-leaf stage, were found to be most susceptible to infection by *A. tragopogonis* (Fig. 3.5). The susceptibility of these leaves did not differ significantly from severity of infection of the cotyledons (level 1). Young sunflower leaves at the establishment stage, however, differed significantly in their susceptibility to infection from the old, vegetative stage (G1) sunflower leaves, with the exception of level 7 and 11, the first alternative leaves formed at 6-leaf and 8-leaf stage respectively (Fig. 3.5). On the same plant, leaves also became more susceptible as they aged. On the 8-leaf plants, for example, the first true leaves (level 8) proved to be less susceptible than the next pair of opposite leaves (level 9), and level 9 leaves less susceptible than level 10 leaves, although they did not differ significantly.

3.3.4 Ratings on adaxial/abaxial cotyledon surfaces of various sunflower lines (Fox and Willams, 1984)

There were no significant differences between the treatment means of the six genotypes. The rating scale seemed to differentiate between susceptible and tolerant lines, with RHA 358 producing a scale rating above 3 and HYS 333 a scale rating well bellow 1 (Fig 3.6). RHA 358 is a known susceptible and HYS 333 a known tolerant plant (Viljoen, personal communication).

3.3.5 The effect of time of day on the infection of sunflower leaves by *A. tragopogonis*

No significant difference was found in disease severity between plants inoculated 4 h after sunrise and those inoculated at dusk. Plants inoculated at dusk produces a mean severity rating of 4.0, while those plants inoculated in the morning produced a mean severity rating of 1.5 (Fig. 3.7).

3.3.6 The effect of inoculation method on disease development

White rust symptoms developed best on sunflower seedlings that were inoculated by spraying leaf surfaces with *A. tragopogonis*, and dipping of the plants in a spore suspension (Fig. 3.8). Placing drops on leaves, or using the camel-hair brush resulted in low symptom development. Dipping the plants for 3 h resulted in better infection than dipping it for 1.5 h only (Fig. 3.8). Uprooting the plants and submerging them in the spore suspension, however, stressed the plants tremendously, and the number of plants that died was in excess of 30%. A significantly higher infection level was also consistently achieved when leaves of seedlings were covered after inoculation, in contrast to those not covered (Fig. 3.8).

3.3.7 Evaluation of sunflower varieties for disease resistance

The rating scale seemed to differentiate between susceptible and tolerant genotypes. There were significant differences between RHA 358, a known susceptible plant that produced a severity of 4, and the tolerant HYS 333, which produced a severity rating of well bellow 1 (Fig. 3.9).

3.4 Discussion

In this study a reliable artificial inoculation technique was developed for studies on white rust of sunflower. This technique was optimised to determine the optimum spore concentration, method of inoculation, the optimum age of seedlings, and was sensitive enough to distinguish resistance among sunflower varieties. This inoculation technique can in future be used to study several aspects of white rust biology and pathology.

A number of interesting observations were made regarding the biology and pathogenicity of *A. tragopogonis* during the development of a young-plant inoculation technique. Young leaves of sunflower proved to be more susceptible to infection by *A. tragopogonis* than old leaves. Nowell and Viljoen (1997) and Potgieter *et al.* (1997) observed similar results in the field. According to Agrios (1988) plants can defend themselves against pathogen attack by a combination of structural or metabolic defence mechanisms. Within the same host and pathogen, the combinations of structural and biochemical defences vary depending on the age of the plant, the tissue attacked, condition of the plant and weather conditions. The reasons for young leaves being more susceptible to infection by *A. tragopogonis* than older leaves could be attributed to the presence of structural characteristics in older leaves that act as a physical barrier to the pathogen and thus prevent entrance and spread through the plant. Lignification, callose deposits around haustoria and the development of necrotic zones have been reported to be responses in sunflower to infection by *Plasmopara halstedii* (Berl) Farl. Et de Toni (Mouzeyar *et al.*, 1995). Biochemical reactions are usually related to the

production of various substances that are toxic to the pathogen or that inhibit growth of the pathogen by the plant (Agrios, 1998). Accumulation of phytoalexins have been reported in sunflower plants when infected by *P. halstedii* (Mouzeyar *et al.*, 1995).

Another reason for why younger leaves could be more susceptible to attack by *A. tragopogonis* can be explained by Salisbury and Ross (1992). They stated that respiration is influenced by the age of a plant. Salisbury and Ross (1992) found that in sunflowers, respiration remained high during the period of most rapid vegetative growth and dropped before flowering. During respiration, stomata are open at night, thus allowing light sensitive zoospores to penetrate and successfully germinate within substomatal cavities in young sunflower leaves Respiration starts to drop 22 d after germination. In the field it has also been observed that the youngest leaves of sunflower plants become infected up until flowering and then infection stops (Viljoen personal communication).

Disease severity was highest when plants were inoculated with 10^4 and 10^5 sporangia/ml. Lower concentrations probably resulted in fewer zoospores being able to penetrate and infect through stomata on the leaves. The dilution with 10^6 sporangia/ml also showed a very low percentage in germination. This low germination percentage may be due to the effect of competition per sporangia/ml of water.

Cotyledons are not usually used as an indicator for susceptibility. We used a scale developed by Fox and Williams (1984) to examine whether we could differentiate between various sunflower genotypes. No significant differences were found between the six genotypes used in the experiment. However, the rating scale seemed to differentiate between susceptible and tolerant lines, with RHA 358 producing a scale rating above 3 and HYS 333 a scale rating well bellow 1. RHA 358 is a known susceptible and HYS 333 a known tolerant plant (Viljoen, personal communication). The visual white rust scale used on the cotyledons was successful in measuring variability of white rust development of sunflower genotypes. However, the technique was developed

for *B. campestris* cotyledons and unfortunately was not sensitive enough for sunflower cotyledons showing much variation between the treatment means. We recommend for future research that another scale, based on the same principles as Fox and Williams technique, be developed to measure the rate of susceptibility of sunflower cotyledons in the greenhouse.

It was imperative that inoculation occurs at low temperatures and light intensities and, therefore, inoculation in this study was done at dusk. As encysted zoospores are vulnerable to high temperatures and light (Kajornchaiyakul and Brown, 1976), the motile zoospores would have a better chance to penetrate stomata and encyst within the substomatal cavities in the dark. More stomata are open at night for respiration, and this can greatly influence the rate of penetration and infection by the fungus. Additionally, respiration rates are high in sunflowers between seed germination and 22 d after germination (Salisbury and Ross, 1992).

White rust symptoms developed best on sunflower seedlings that were inoculated by spraying leaf surfaces with *A. tragopogonis* using the sprayer bottle technique and dipping the plant in the sporangial suspension. According to Zimmer and Hoes (1978), there are more stomata present on the lower surface of the leaves. Spraying both surfaces increased the probability of infection. This could also explain why submerging the sunflower seedlings into the zoospore suspension was successful. However, the latter technique stressed plants as it involved uprooting the plants.

A significantly higher infection was achieved when seedlings were covered with plastic bags after inoculation. All the treatments used seedlings of 4-leaf stage, which is equivalent to 4 wks growth after planting. At 4 wks, respiration rates are high and this would cause the plant to transpire (Salisbury and Ross, 1992). Water is essential for encystment and germination of zoospores, the plastic bag therefore compensated for water loss by maintaining a high humidity.

In conclusion, an inoculation technique was developed to assist scientists working on white rust of sunflower with their greenhouse studies. Based on the results obtained in this study, it is suggested that sunflower seedlings between cotyledon stage and four-leaf-stage be inoculated with freshly collected sporangia from just opened or fallen pustules. The zoospore suspension should be prepared by allowing 10^4 or 10^5 sporangia/ml to germinate in distilled water for 3 h at 10°C. The zoospore suspension should then be sprayed onto both the adaxial and abaxial surfaces of the leaves until the leaves are completely wet. Inoculated seedlings must be covered with plastic bags to maintain high humidity and incubated at 12°C for ±16 h in darkness. Infection levels can be assessed 10-14 d after inoculation using the scale developed by Siddiqui *et al*. (1975).

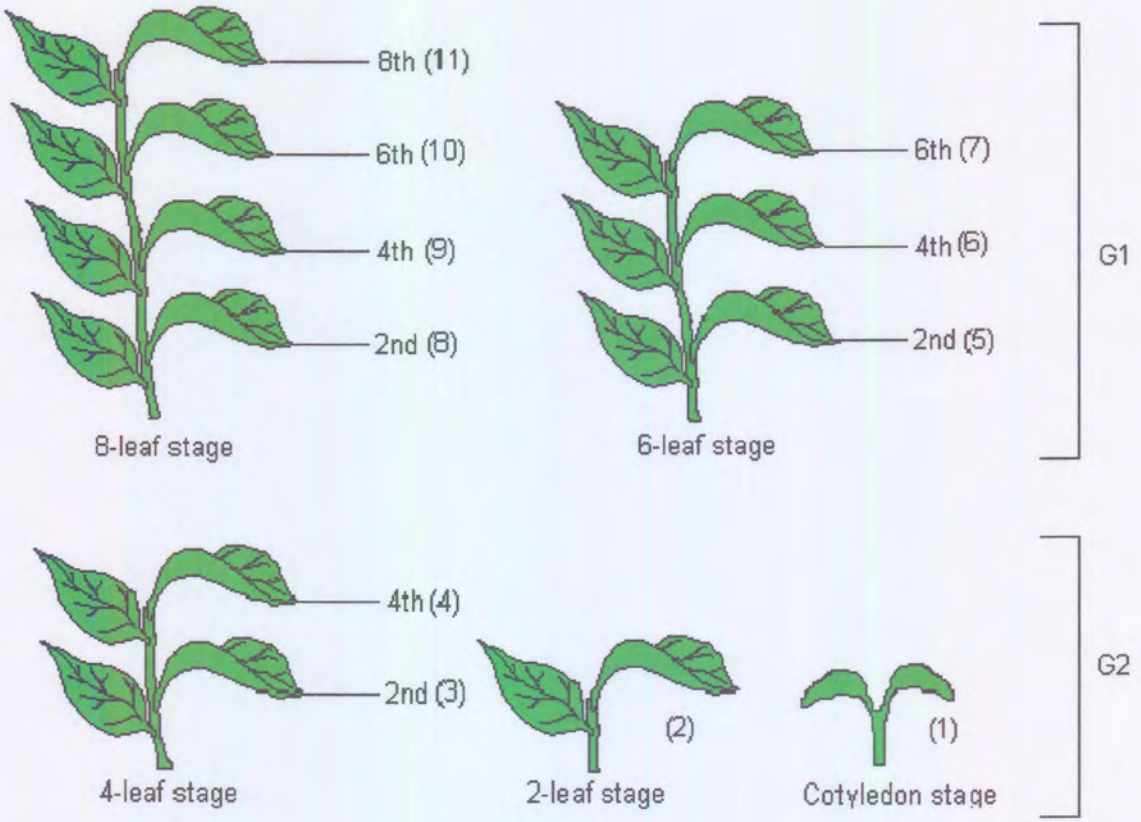

Figure 3.1 Diagram representing the various leaf age categories as defined by Siddiqui *et al.* (1975). G1=Vegetative stage: from the formation of the first leaf showing spiral phyllotaxy to the appearance of the inflorescence head, G2=Establishment stage: from emergence of the cotyledons to the formation of the last pair of leaves showing opposite phyllotaxy. Level 1= cotyledons, level 2= 2-leaf stage – 1st pair of opposite leaves formed, level 3 and 4= 4-leaf stage – 2nd pair of opposite leaves formed, level 5, 6 and 7= 6-leaf stage – first alternate leaf formed, level 8, 9, 10 and 11= 8-leaf stage – second alternate leaf formed.

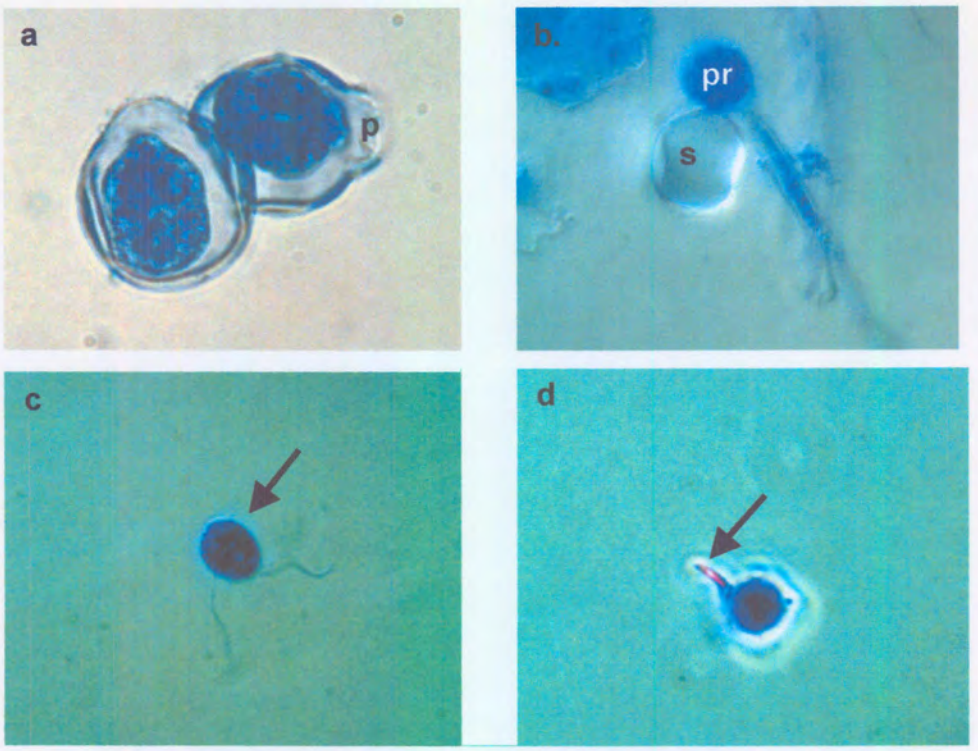

Figure 3.2 Stages of the germination of sporangia of *Albugo tragopogonis*:

a. A papilla (p) is formed at the apex of the sporangia.

b. At 1.5-2 h after sporangia (s) were placed in water, undifferentiated protoplasm (pr) was released from the sporangium through an opening at the papilla.

c. Biflagellate zoospore (arrow) had encysted within 6 h after being placed in water.

d. Between 8-12 h, the zoospore germinated and producing one to two germ tubes (arrow).

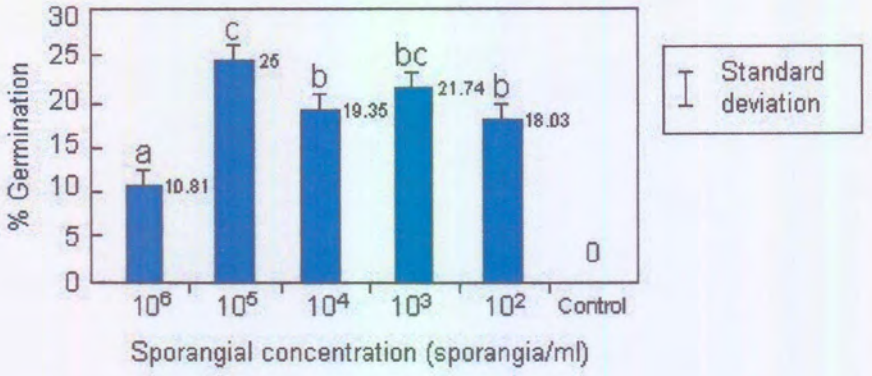

Figure 3.3 The effect of sporangial concentration on the germination of *Albugo tragopogonis*.

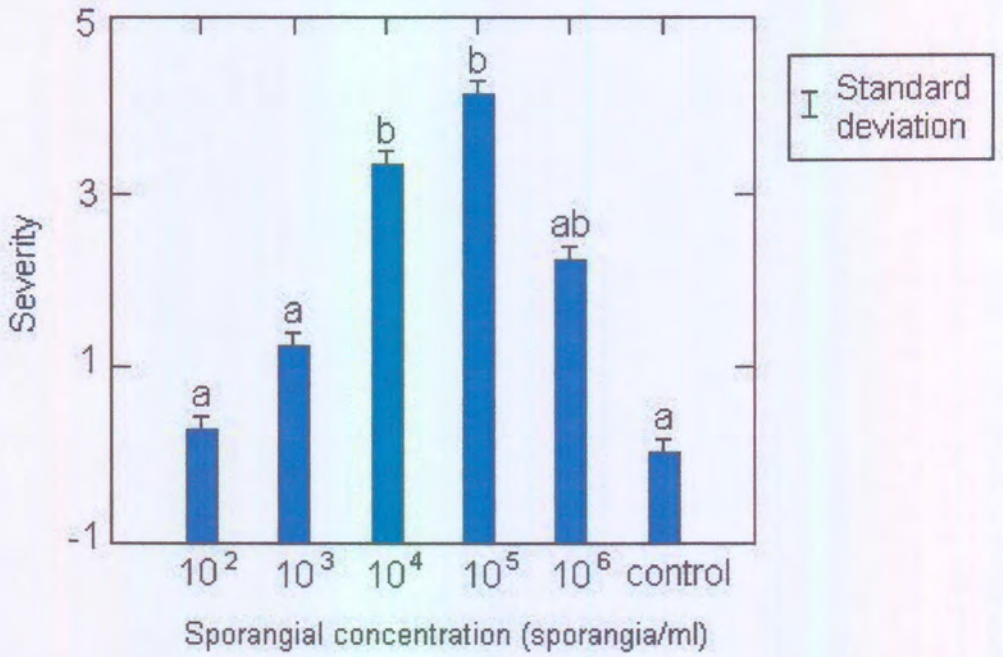

Figure 3.4 The effect of sporangial concentration on the severity of white rust development on sunflower seedlings. A severity rating of 1 indicates a low, and 5 a high rate of infection. Bars that have the same lower case letter do not differ significantly according to the LSD_T value (P=0.05).

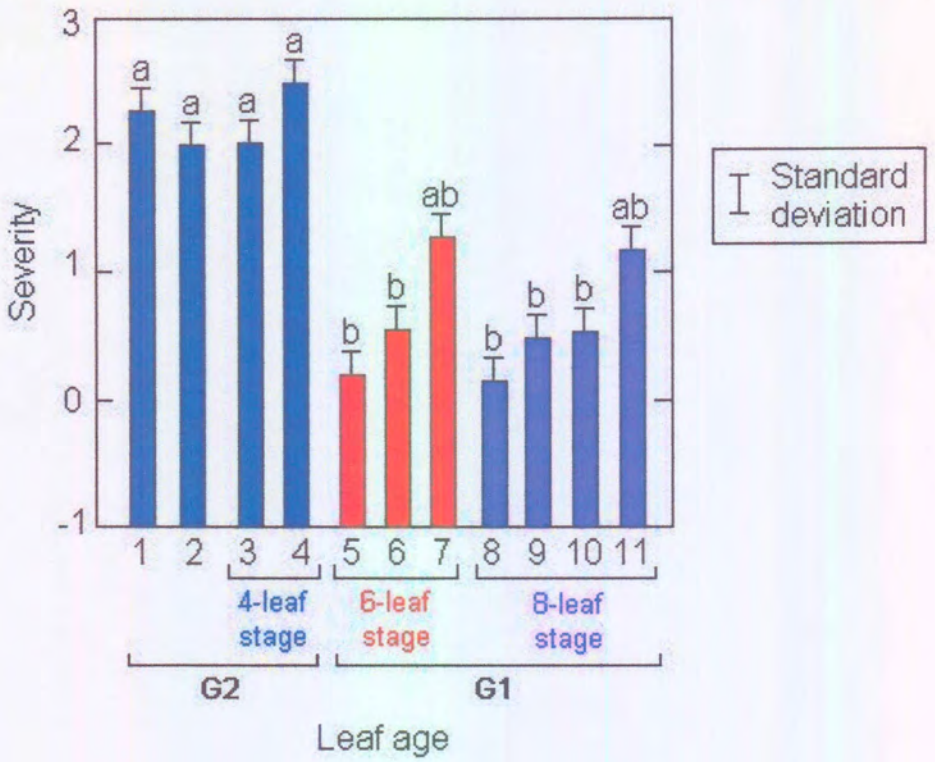

Figure 3.5 The effect of leaf age on the severity of white rust on sunflower seedlings. G1 represents the vegetative growth stage, and G2 the establishment stage (refer to Fig. 3.1). A severity rating of 1 indicates a low, and 5 a high rate of infection. Bars that have the same lower case letter do not differ significantly according to the LSD_T value (P=0.05).

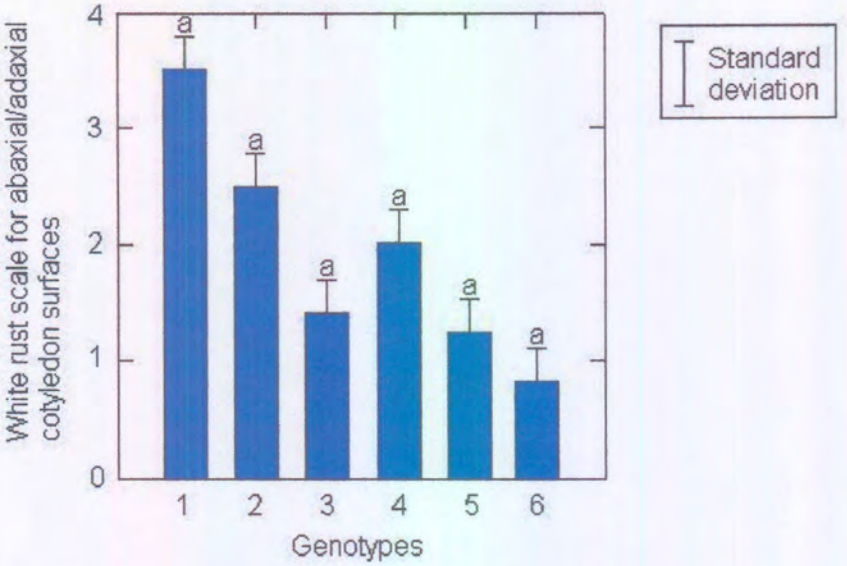

Figure 3.6 The means of the effect of infection of cotyledons by *Albugo tragopogonis* on six sunflower genotypes, 1=RHA 358, 2=RHA 357, 3=RHA 247, 4=HV 3037, 5-PAN 7392, 6=HYS 333. Bars that have the same lower case letter do not differ significantly according to the LSD_T value of 2.259 (P=0.05).

Figure 3.7 The effect light and darkness on disease development of sunflower seedlings inoculated with *Albugo tragopogonis*. A severity rating of 1 indicates low, and 5 a high rate of infection. Bars that have the same lower case letter do not differ significantly according to the LSD_T value of 1.140 (P=0.05).

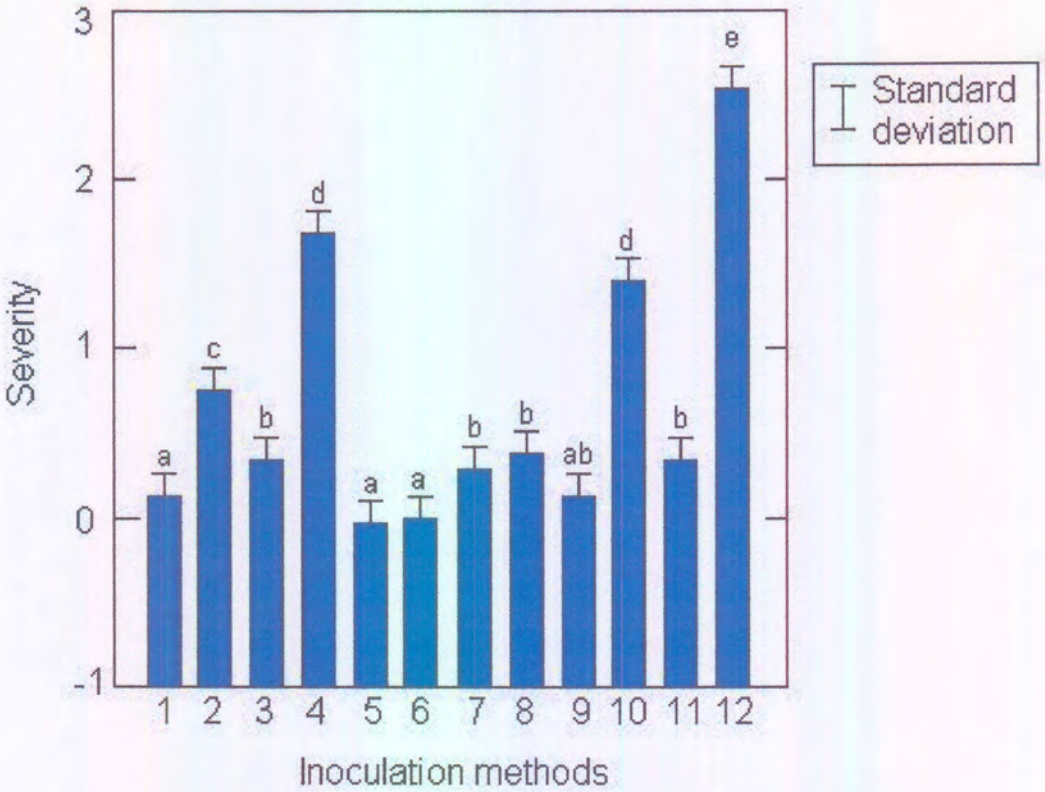

Figure 3.8 The effect of inoculation techniques on white rust development in sunflower seedlings inoculated with *Albugo tragopogonis*. 1=Dipped for 1.5 h, 2=Dipped for 1.5 h and covered with plastic bag, 3=Dipped for 3 h, 4=Dipped for 3 h and covered with plastic bag, 5=Drop inoculum, 6=Drop Inoculum and covered with plastic bag, 7=Camel-hair brush, 8=Camel-hair brush and covered with plastics bag, 9= Sprayed adaxial surface only, 10= Sprayed adaxial surface only and covered with plastic bag, 11=. Sprayed both surfaces, 12= Sprayed both surfaces and covered with plastic bag. A severity rating of 1 indicates low, and 5 a high rate of infection.

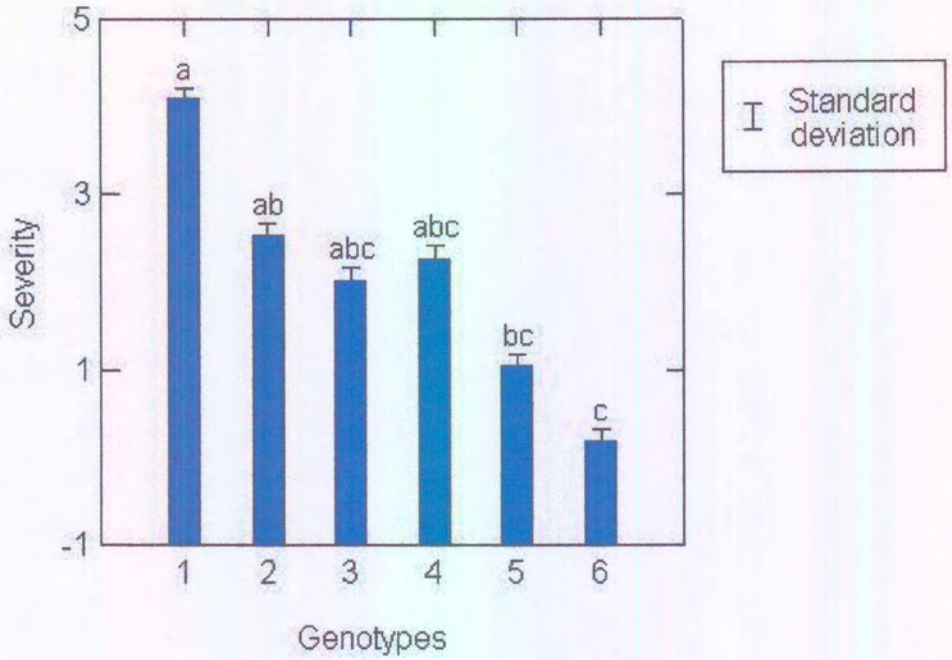

Figure 3.9 The susceptibility of six sunflower genotypes to *Albugo tragopogonis*. 1=RHA 358, 2=RHA 357, 3=RHA 247, 4=HV 3037, 5-PAN 7392, 6=HYS 333. Bars that have the same lower case letter do not differ significantly according to the LSD_T value (P=0.05).

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Chapter 4

Investigating methods for maintaining sporangia of *Albugo tragopogonis*, causal agent of white rust of sunflower

4.1 Introduction

Albugo tragopogonis (Pers.) S.F. Gray is an obligate fungal parasite that causes white rust of sunflower. The fungus survives in living plant tissues or as dormant oospores in the soil (Whipps and Cooke, 1978). Because of its obligate parasitic nature, it is not possible to maintain *A. tragopogonis* on artificial culture media. Maintaining the pathogen on a susceptible host by inoculating healthy sunflower leaves with freshly collected sporangia from infected greenhouse material is tedious and requires a lot of greenhouse space. Consequently, research on *A. tragopogonis* is often limited to field and greenhouse experiments during the growing season only. To perform laboratory experiments under controlled greenhouse conditions throughout the year, a method needs to be developed whereby spores of *A. tragopogonis* can be stored for extended periods.

In a study to investigate the infection process and factors affecting infection of sunflower by *A. tragopogonis*, Kajornchaiyakul and Brown (1976) found that sporangia of *A. tragopogonis* remained viable for 6-12 mo when stored at -18°C. Verma and Bhowmik (1991) found that *Albugo candida* (Pers.) Kuntze sporangia on *Brassica juncea* L. leaves, placed between blotting paper and dried for 24 h, then stored at 0, 4, 15, 20 and 25°C, showed very low germination percentages, with those stored at 20 or 25°C not germinating.

The aim of this study was to evaluate various techniques that could be used to preserve sporangia of *A. tragopogonis*. An effective storage method would allow researchers to successfully inoculate white rust of sunflower in the laboratory during all seasons.

4.2 Materials and Methods

4.2.1 Collection of infected plant material from the field

Leaves infected with *A. tragopogonis* were collected from sunflowers planted in an experimental field at the Grain-Crops Institute in Potchefstroom, South Africa. The leaves were covered with plastic bags and placed in a cooler box with their petioles submerged in ice water. Some infected leaves were placed in paper bags and allowed to dry for 24 h.

4.2.2 Spore collection and storage

Sporangia of *A. tragopogonis* were collected from white rust pustules using a vacuum device (Fig. 4.1a). During the collection, sporangia were sucked from pustules into gelatin capsules (Fig. 4.1b), as described by Pound and Williams (1963). Since the collection of sporangia using the vacuum device was laborious and tedious, a method was devised by pressing the abaxial surface of infected leaves onto a piece of black cardboard paper. The resulting 'sporangial print' was then placed into gelatin capsules, either by folding the cardboard in half and carefully tipping sporangia into capsules, or by brushing sporangia with a toothbrush or paintbrush to the center of the page. Thereafter sporangia were collected using the vacuum device.

Capsules containing sporangia were immediately stored in glass screw cap vials at -20°C, -70°C, in liquid nitrogen, and at -70°C following desiccation for 24 h. In the liquid nitrogen treatment, no thawing process was carried out on the gelatine capsules, instead they were placed immediately into the distilled water. Sporangia were also collected from the leaves that were placed in paper bags, which were slightly dry at the time of collection, and stored at -70°C. All capsules containing sporangia were stored for periods of 3, 5, 9, 12 or 15 mo.

4.2.3 The effect of storage on spore germination

The incidence of germination of sporangia of *A. tragopogonis* was determined after storage at different time intervals. After recovery, sporangia were suspended in 500-ml Erlenmeyer flasks containing 250 ml double-distilled water. Flasks were covered with parafilm and shaken. The sporangial suspensions were adjusted to 10^4-10^5 sporangia/ml using a Kovac Glasstic hemocytometer, and incubated at 10°C for 2-3 h on ice to maintain zoospore mobility. The suspension was stained with lactophenol-cotton blue according to the method described by Verma and Bhowick (1991). A sporangium was considered germinated if it lacked cytoplasm. Three random fields from 10 slides per storage method were used to determine the average percentage germination.

4.2.4 The effect of storage on disease incidence

Two sunflower lines obtained from the United States Department of Agriculture (USDA), namely RHA 357 and RHA 358, were grown in pots containing Braak potting soil. This potting soil is composed of plant moss, peat bark, volcanic material, river sand, milled oasis and plant food additives. The plants were used to measure the infection potential of the stored sporangia. Sporangia were suspended in sterile distilled water to a concentration of 10^4-10^5 sporangia/ml for 3 h, after which the sporangial suspension of *A. tragopogonis* was inoculated onto healthy sunflower seedlings. Seedlings were inoculated at the 4-leaf stage by spraying spore suspensions on the adaxial surface of the leaves with a small hand held sprayer until leaves were completely wet. Sunflower seedlings were then kept at 12°C in the dark. Inoculated seedlings were covered with plastic bags to maintain humidity, and the bags were removed ±16 h post-inoculation. The plastic bags were removed in the morning and greenhouse temperatures were adjusted at day/night time temperatures of 25/18°C. The sunflower seedlings were exposed at daytime with a light intensity of 22 000 lux for 12 h. Sunflower seedlings were watered every second day. Infection levels of *A. tragopogonis*

on leaves of sunflower seedlings were assessed according to the scale developed by Siddiqui *et al.* (1975). In this scale, 1 indicates no infection and 5 indicates severe infection. Infection levels were assessed 10-14 d after inoculation.

4.2.5 Statistical analysis

Four to five seedlings were inoculated per pot with each treatment. Ten pots were used per storage method over the various period intervals and arranged in a randomised block design. The variation of viability of stored sporangia over the 15 moperiod was analyzed using SYSTAT Version 7.0.1 (Copyright 1997, SPSS Inc.). The F-values and the LSD_T values for the main effects and interaction were calculated to indicate where significant differences occurred between the treatment means. The tables with statistical values for each trial can be found in Appendix A.

4.3 Results

4.3.1 The effect of storage on sporangial germination

Sporangia that were desiccated for 24 h and stored at -70°C resulted in the best germination rate of between 20 and 25% after 3, 5 and 9 mo (Fig. 4.2). Thereafter, germination gradually decreased to 5.8% after 15 mo. Similarly, sporangia collected from dried plant material and stored at -70°C had a good germination rate between 12 and 15% after 3, 5 and 9 mo (Fig. 4.2), with germination rate dropping to 5.9% after 15 mo (Fig. 4.2). Germination of the control treatment that included fresh sporangia collected from infected greenhouse material, varied between 19 –and 21%.

A good germination rate of 20 and 15% for sporangia stored at -20 and -70°C, respectively, was obtained after 3 mo. The germination rate, however, showed a gradual decline in viability from 3 to 15 mo, with both treatments having

approximately 1% germination after 15 mo (Fig. 4.2). Sporangia stored in liquid nitrogen directly after collection showed the lowest germination rate over the entire period, with no germination occurring after 12 mo of storage (Fig. 4.2). Observation under the microscope revealed that most of the sporangia that had been placed in liquid nitrogen prior to storage had burst.

4.3.2 The effect of storage on disease incidence

White rust symptoms developed on both sunflower cultivars following storage between 3 and 15 mo. More symptoms, however, consistently developed on the more susceptible cultivar RHA 358, than on RHA 357. The best infection rate after 15 mo was achieved when sporangia were stored at -70°C following desiccation (Fig. 4.3). Surprisingly, the level of infection by these spores was higher than that obtained with fresh sporangia. Infection of sunflower seedlings with sporangia stored at -70°C following collection from dried material showed similar results to the control. Both fresh sporangia, and sporangia stored at -70°C following desiccation caused significantly more infection on RHA 357 than sporangia stored in liquid nitrogen ($P=0.05$), but not on RHA 358 (Fig. 4.3). There was, however, no significant difference in disease severity following any of the other storage methods.

Disease severity of white rust caused by sporangia stored at -70°C following desiccation dropped slowly between 3 and 9 mo of storage. Thereafter, however, the infection levels decreased significantly to less than 5% disease incidence after 15 mo, with the exception of RHA 357 at 9 mo (Fig. 4.4).

A positive correlation was found between the percentage germinating sporangia over 15 mo of storage and the severity of these stored sporangia on both sunflower genotypes (Fig. 4.5). The percentage germination at $X=0$ shows that RHA 357 is more resistant to infection than RHA 358 and that at least 2.7% germination is required before any infection occurs on RHA 357. From Fig. 4.5, it can be predicted when germination is at 20% one can obtain a severity level between 3.5 and 4 when using the inoculation method described in the materials and methods. Additionally, when germination is at

5.0%, one can also obtain a in a severity rating of 1 on a susceptible genotype such as RHA 358 when using the inoculation method described.

4.4 Discussion

In this study, we were able to maintain sporangia of *A. tragopogonis* that remained viable for more than 15 mo. We further proved that the sporangia also maintained their fitness to infect sunflower leaves, and that the level of infection could be predicted on susceptible and tolerant sunflower genotypes according to their level of germination.

From our studies it appears that desiccating sporangia of *A. tragopogonis* before storage is an important factor in the viability of the sporangia. Similar to findings by Kachornchaiyakul and Brown (1976), it became evident that sporangia collected from field infected sunflowers and stored at low temperatures without desiccation rapidly lost their viability after 9 mo. Microscopic analysis of sporangia stored in liquid nitrogen might explain this, as many sporangia appeared to rupture as evident by the frozen cell contents. While desiccated sporangia can be better maintained at equally low temperatures, the absence of a significant difference in germination rate and infectivity levels suggest that further optimisation of the storage of sporangia is needed. It may also be of interest, for future research, to examine the effect of desiccation of sporangia prior to exposure to liquid nitrogen, storage at –20°C or other temperatures.

Drying the sporangia at different hours may also help to increase viability. For example Mishra and China (1963) did a study on the effect of slow and fast drying upon *Albugi bliti* (BIV.) KZE germination. Their results showed that in the case of fast desiccation, optimum percentage of germination (58%) was obtained at 30 min. The highest percentage germination (i.e. 49%) of slow desiccation was obtained after 120 min. The average percentage germination was higher for sporangia subjected to fast-drying of infected leaves. Thus, a combination of desiccation and freezing of sporangia may play an important

role in maintaining viability of sporangia. A refinement of the desiccating and freezing technique may call for future investigation.

Maintaining viability in sporangia of *Albugo* spp. is dependant on several factors such as the time period between collection and storage, the tempo of decreasing water content before storage, and the water content of the sporangia upon storage. Saharan and Verma (1992) observed that sporangia of *A. candida* from *B. juncea* lost their viability 18 h after collection from infected detached leaves. Raabe and Pound (1952) found that germination of sporangia of *Albugo occidentallis* G. Wils. collected from desiccated spinach leaves that were oven dried at 100°C for 2-3 h, reached 30-40% germination, while sporangia from the control leaves only reached 1-7% germination. However, the amount of desiccation needed to increase germination varied considerably from leaf to leaf. Raabe and Pound (1952) stated that work done by Maude Naper in 1933 reported that sporangia had to lose 30% of their moisture content before they would germinate.

This study will contribute substantially in the development of much needed greenhouse inoculation studies of sunflower with *A. tragopogonis*, a technique that was previously either not attainable or reliable. Greenhouse inoculation studies will not only aid in future studies on the control of white rust of sunflower by means of disease resistance or chemical control, but also on the biology and taxonomy of the responsible pathogen.

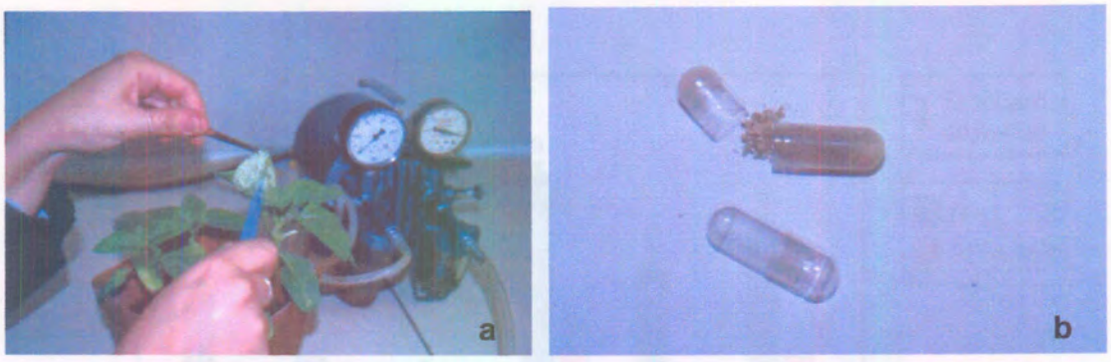

Figure 4.1 a) Sporangia of *A. tragopogonis* collected from sunflower leaves using a vacuum device, b) Gelatine capsules containing sporangia.

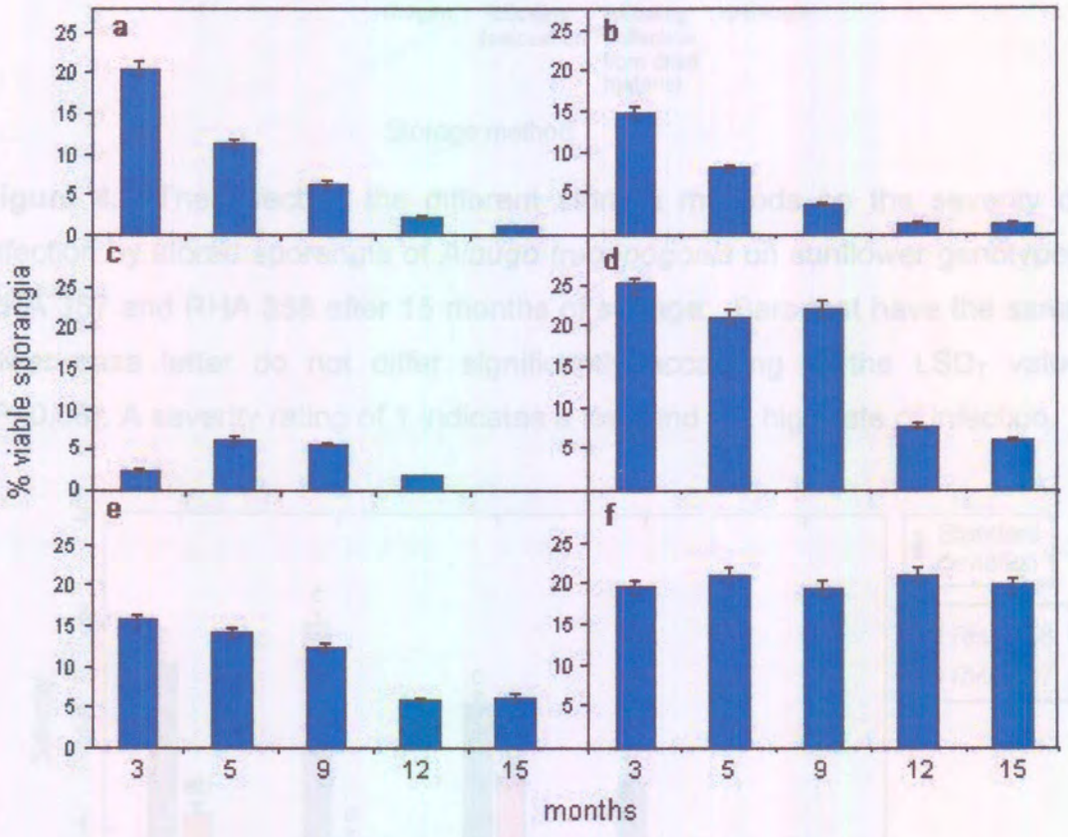

Figure 4.2 The effect of different storage methods on the viability of sporangia of *Albugo tragopogonis* over time. a=-20ºC, b=-70ºC, c=liquid nitrogen, d=-70ºC following desiccation, e=-70ºC following collection from dried material, f=Control (Fresh sporangia).

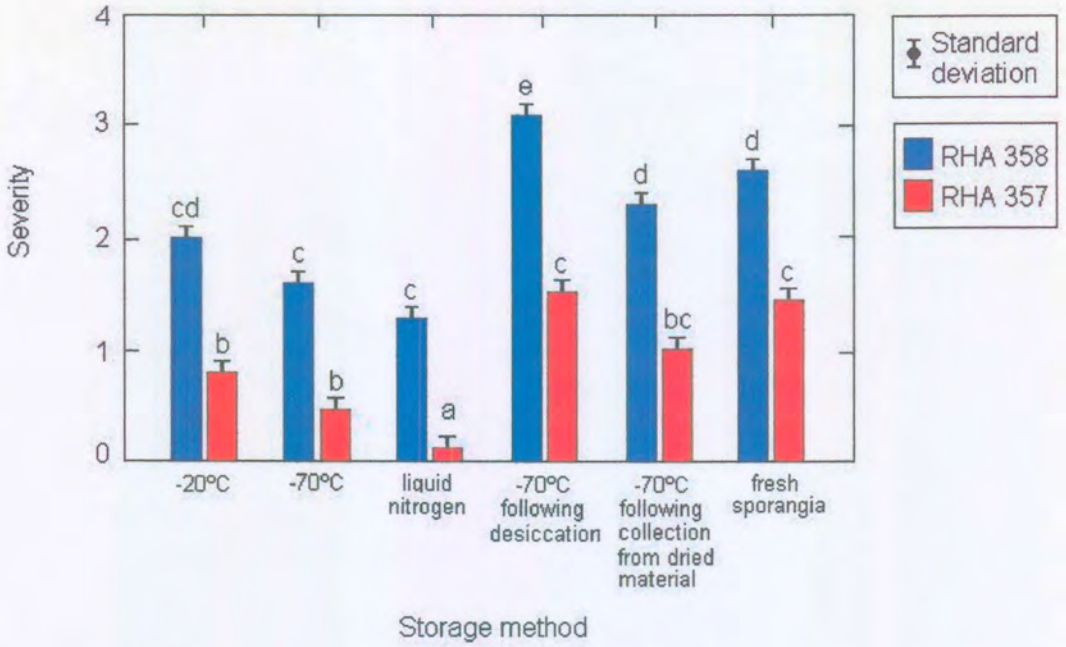

Figure 4.3 The effect of the different storage methods on the severity of infection by stored sporangia of *Albugo tragopogonis* on sunflower genotypes RHA 357 and RHA 358 after 15 months of storage. Bars that have the same lower case letter do not differ significantly according to the LSD_T value (P=0.05). A severity rating of 1 indicates a low, and 5 a high rate of infection.

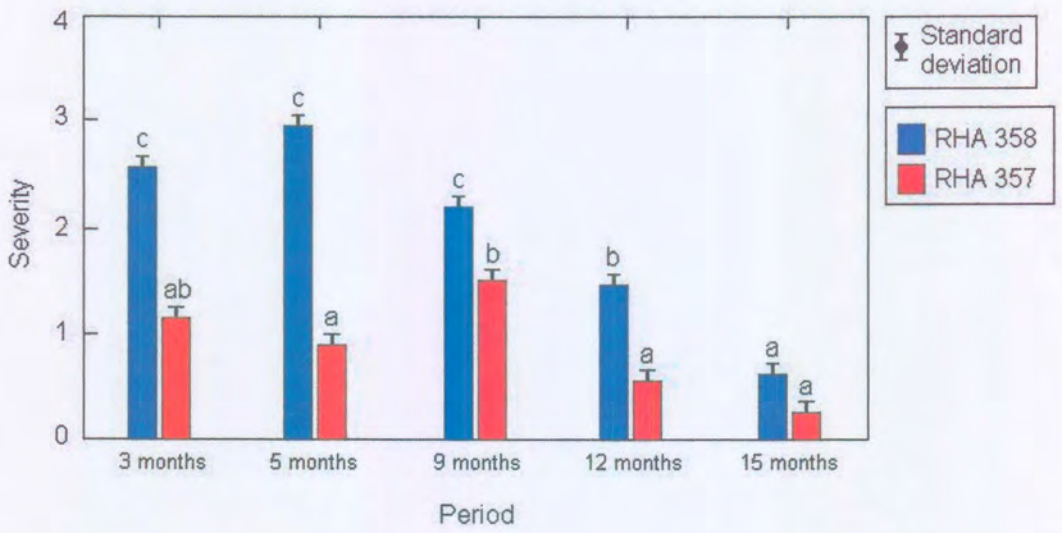

Figure 4.4 The effect of time on the severity of infection on RHA 357 and RHA 358 by sporangia of *A. tragopogonis* stored at –70°C after desiccation. Bars that have the same lower case letter do not differ significantly according to the LSD_T value (P=0.05).

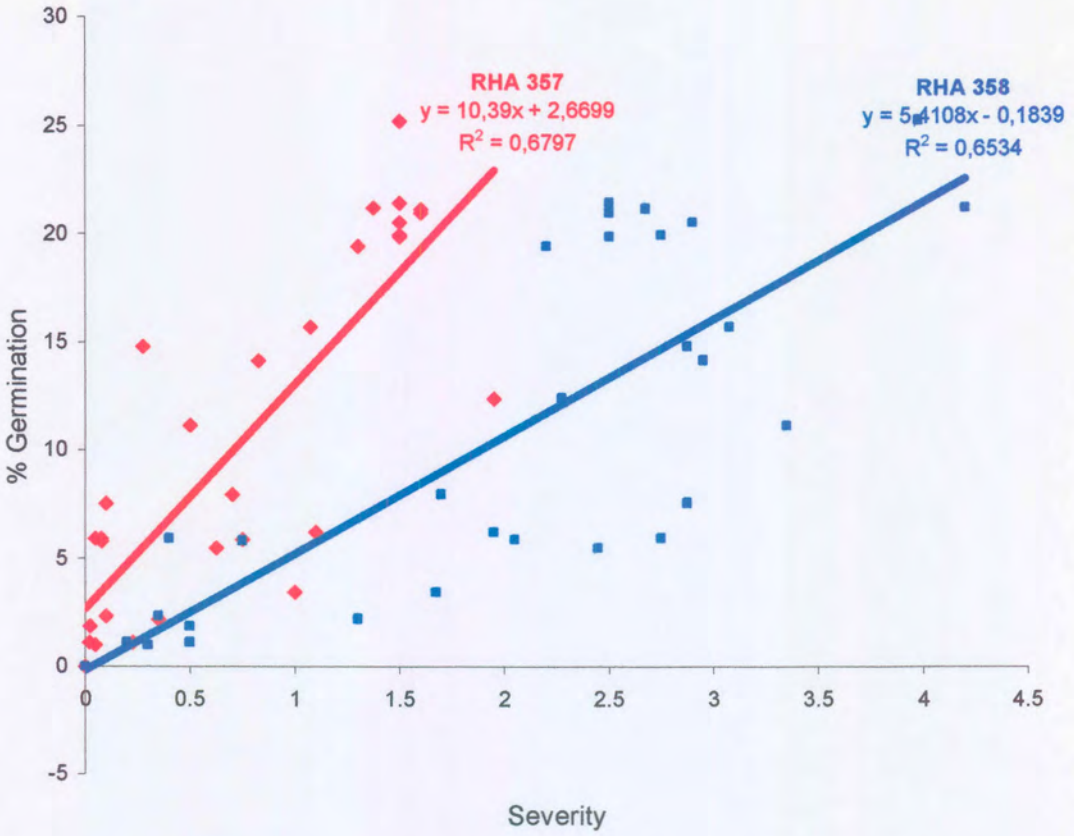

Figure 4.5 Correlation between percentages germinated sporangia and the level of infection by *Albugo tragopogonis* on two sunflower genotypes. A severity rating of 1 indicates a low, and 5 a high rate of infection.

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Chapter 5

Infection of susceptible and tolerant sunflower plants with *Albugo tragopogonis*

5.1 Introduction

Albugo tragopogonis (Pers.) S.F. Gray causes white rust of *Helianthus annuus* L. in many sunflower producing countries around the world (Gulya *et al.*, 1997, Van Wyk *et al.*, 1995). The most notable symptoms associated with the disease are chlorotic leaf spots produced on the upper surface of the leaves. Directly below these spots, white to creamish blister-like swellings called pustules are visible (Gulya *et al.*, 1997; Zimmer and Hoes, 1978). *Albugo tragopogonis* is an obligate pathogen, and for this reason screening susceptible or tolerant lines have been limited to field trials and evaluations.

Much of the information found in literature contributes to factors affecting infection or development of the disease, and no published research exists on the interaction between *A. tragopogonis* and a susceptible/tolerant sunflower host. The aim of this study was to use different microscopy techniques to study infection by *A. tragopogonis* of susceptible and tolerant sunflower genotypes, and possibly pinpointing the weaknesses of the pathogen during infection. This information could support research for future control.

5.2 Materials and Methods

5.2.1 Plant material

Six sunflower genotypes were selected to study their responses upon infection by *A. tragopogonis*. These genotypes include HYS 333, RHA 358, RHA 357, RHA 247, HV 3037 and PAN 7392.For each genotype, four to five

seeds were planted per pot (12 cm x 12 cm) containing 'Braak' potting soil (composed of plant moss, peat bark, volcanic material, river sand, milled oasis and plant food additives). The plants were maintained at 25/18°C day/night temperatures, with a daytime light intensity of 22 000 lux for 12 h.

5.2.2 Inoculation of sunflower seedlings

Leaves of seedlings at the 4-leaf stage, i.e. the second pair of opposite leaves formed, were inoculated with freshly collected sporangia from infected greenhouse plants. A zoospore suspension was prepared by allowing 10^5 sporangia/ml to germinate in distilled water for 3 h at 10°C. The zoospore suspension was then sprayed onto both the adaxial and abaxial surfaces of the leaves until run-off using a small handheld garden sprayer. Inoculated seedlings were incubated in humidity chambers at 12°C. The seedlings were removed after 16 h and plants were maintained at 18/25°C day/night temperatures until symptom development. Infection levels were assessed 10-14 d after inoculation.

5.2.3 Variation in size of white rust pustules on six sunflower genotypes

Pustule sizes of white rust lesions that developed on the sunflower genotypes were measured using a Zeiss stereo microscope at 2.5 x magnification. A visual classification was developed with 1 indicating a small pustule between 0.5-1 mm in diameter and 3 indicating a large pustule between 2.5-5 mm in diameter.

5.2.4 Light microscopy

Infection of the sunflower varieties HYS 333 (tolerant to *A. tragopogonis*) and RHA 358 (susceptible to *A. tragopogonis*) was investigated by means of light and electron microscopy. Leaves used for light microscopy were cut into 20

mm^2 segments and those for scanning electron microscopy into 5x5 mm pieces at 2, 4, 6, 8, 10, 12, 24, 36, 48 (2 d), 72 (3 d), 96 (4 d), 120h (5 d), 144 (6 d) and 168 h (7 d) time intervals after inoculation.

5.2.4.1 Whole-leaf clearing and staining technique

The whole-leaf clearing and staining technique described by Shipton and Brown (1962) involved the immersion of infected leaves in 10-15 ml of alcoholic lactophenol cotton blue (one part lactophenol cotton blue to two parts 95% alcohol). The solution containing the leaves was brought to boiling-point and simmered for 1 min. After the leaves sank, the solution was brought to the boil again for 30 sec. The leaves remained in the stain for approximately 48 h, after which they were removed, rinsed in water and placed in chloral hydrate for 30-50 min. The leaves were mounted on a microscope slide in 50% glycerin and viewed with a Zeiss light microscope with phase contrast condensers.

5.2.4.2 Lactophenol-ethanol-analine blue technique

In the lactophenol-ethanol-analine blue technique described by Whipps and Cooke (1978). Leaf sections were boiled in a lactophenol mixture (10 g phenol; 10 ml lactic acid; 10 ml glycerol; 50 ml ethanol) for 2 min and then left in lactophenol at room temperature for 24 h. Some sections were stained in lactophenol cotton blue and examined under a Zeiss light microscope with phase contrast condensers. The remaining sections were stained in aniline blue for 1 h and viewed under a Zeiss epiflourescence microscope (UV-H 365 type of excitation; Exciter filter BP 365/12; Barrier filter LP 397; Chromatic beam splitter FT 395; Mercury UV bulb).

5.2.4.3 Sectioning with freeze microtome

Leaf segments were fixed in 2.5% glutaraldehyde. Each piece was removed from the fixative and blotted dry for 2-3 min on a paper towel. The material was placed in a watch glass with water for a few minutes prior to sectioning. Samples were cut into cross-sections of 20-30 μm thickness using a freeze-microtome. Sections were stained for 1 h in aniline blue. Sections were viewed under a Zeiss epiflourescence microscope.

5.2.5 Scanning electron microscopy (SEM)

The leaf sections were fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4-7.6) for 2 h, rinsed in the same buffer and post-fixed in 0.25% aqueous osmium tetroxide for 2 h. This was followed by three successive washes in distilled water, dehydration in an ascending acetone series, critical point drying in a Bio-rad critical point dryer and mounted on SEM stubs. The epidermis, palisade parenchyma and spongy parenchyma were chronologically stripped using the double-sided tape method of Hughes and Rijkenberg (1985) in order to study the infection process beneath the epidermis and within the leaf (Bandounas *et al.*, 2000). All samples were coated with gold in a Polaron sputter coater and examined within a Joel JSM 840 scanning electron microscope operating at 5 Kv.

5.2.6 Statistical analysis

Variation of pustule size was determined using SYSTAT Version 7.0.1 (Copyright 1997, SPSS Inc.). A randomised block design with a factorial arrangement of treatments was used. If the F-value was significant, the least significant difference (LSD_T) of Tukey was calculated to indicate which means differed significantly. The tables with statistical values for each trial can be found in Appendix A.5.3 Results

5.3.1 Variation in pustule size of *Albugo tragopogonis* on six sunflower genotypes using the sprayer inoculation technique

RHA 358 produced significantly larger pustules than PAN 7392 and HYS 333, but not RHA 357, RHA 247 and HV 3037 (Fig. 5.1). HYS 333 had the smallest pustules but did not differ significantly in size from the genotypes RHA 247, HV 3037 and PAN 7392.

5.3.2 Light microscopy

Both the whole-leaf clearing and staining and the lactophenol-ethanol-aniline blue techniques proved to be unsuitable for this study as most of the tissue was damaged by the boiling process. Sectioning with the freeze microtome was also unsuccessful as the specimens became too hard and brittle when frozen and disintegrated upon sectioning.

5.3.3 Scanning electron microscopy (SEM)

5.3.3.1 Infection of susceptible and resistant sunflower leaves

Motile zoospores penetrated stomata of RHA 358 6 h after inoculation (Fig. 5.2) and became encysted in the sub-stomatal cavity of the host. Zoospores and stomata varied in size ranging from 6-8 μm and 12-15 μm in diameter respectively. Motile zoospores also penetrated the stomata of HYS 333 6 h after inoculation and encysted within the sub-stomatal cavity of the host (Fig. 5.3). Zoospores varied in size ranging from 4-6 μm in diameter and stomata varied in size ranging from 6-10 μm in diameter.

In RHA 358 encysted zoospores germinated at12 h producing one (Fig. 5.4) or two germ-tubes (Fig. 5.5). Zoospores penetrated stomata on both surfaces of the leaves. Usually one or utmost two zoospores per stomata were

observed within a single sub-stomatal cavity (Figs 5.4 and 5.6). In the resistant genotype, HYS 333, encysted zoospores germinated at 12 h and resembled those of RHA 358 (Fig. 5.7).

At 24 h, encysted zoospores in the susceptible (Fig. 5.8) and resistant genotype (Fig. 5.9), both gave rise to intercellular hyphae. At 36 h and 48 h, intercellular hyphae reached the spongy parenchyma of RHA 358 (Fig. 5.10) and HYS 333 (Fig. 5.11), respectively, dividing dichotomously and proliferating in the large intercellular air spaces. The fungus produced inter- and intracellular haustoria (Fig. 5.12).

At 96 h and 144 h, mycelia resembled tubes or circles on the abaxial epidermis, inside the leaf of RHA 358 (Fig. 5.13) and HYS 333 (Fig. 5.14), respectively. Additionally, hyphae started to differentiate and gave rise to short club shaped sporangiophores producing a number of sporangia in rows just beneath the abaxial surface (Figs 5.15 and 5.16). Large coalescing pustules were produced on RHA 358 and were primarily confined to the lower surface. Small, localized pustules were visible at 168 h on the abaxial surface of the leaves and resembled those found in RHA 358.

5.4 Discussion

A correlation between the infection process of *A. tragopogonis* in the susceptible (RHA 358) and a tolerant (HYS 333) genotype was determined in this study. The infection process of *A. tragopogonis* in RHA 358 and HYS 333 was almost identical, and generally similar to that described by Kajornchaiyakul and Brown (1976). Zoospores of *Albugo ipomoeae-aquaticae* Sawada penetrated its host, *Ipomoea aquatica* Forsk, by germ-tubes from encysted zoospores passing through the stomata (Edie and Ho, 1970), and similarly zoospores of *A. tragopogonis* on *Senecio squalidus* L. encysted either on guard cells or directly over stomatal pores. Encysted zoospores of *A. tragopogonis* on sunflower were not observed to infect the host from the surface of the leaf. Whipps and Cooke (1978) stated that this

unique mode of infection could be due to the size of the zoospore (6-12 μm), which allows them to enter through stomata more easily.

Kajornchaiyakul and Brown (1976) reported *A. tragopogonis* zoospore encystment and germination 8 h after inoculation on sunflower. A possible reason for the different tempo of germination of zoospores when compared to this study could be attributed to the technique of submerging leaves into a sporangial suspension used by these authors to inoculate *A. tragopogonis* onto the leaves. Our studies indicated that penetration occurred on both surfaces of RHA 358 and HYS 333, which were consistent with the report by Kajornchaiyakul and Brown (1976). For both genotypes, spores were found near or on stomata. Kajornchaiyakul and Brown (1976) reported two or three, sometimes more, encysted zoospores within a single sub-stomatal cavity. Our results indicated one or a maximum of two zoospores were present in RHA 358 and only one zoospore per sub-stomatal cavity in the tolerant genotype HYS 333. This difference could be due to the size of stomata present on HYS 333, which were considerably smaller than those found on RHA 358. Kajornchaiyakul and Brown (1976) stated that the zoospores varied greatly in size ranging from 6-12 μm (mean 8.3 ± 1.6 μm) in diameter (Whipps and Cooke, 1978). Whipps and Cooke (1978) reported sizes ranging between 7.5–10 μm in diameter consistent with our measurements.

According to Agrios (1988), a plants first line of defense against pathogens is its surface, which the pathogen must penetrate to cause infection. On HYS 333, the size of the stomata of were considerably smaller to those observed on RHA 358. Size of stomata could play an important role in limiting penetration and infection of the pathogen. According to Agrios (1988), apart from the size of the stomata, location and shape of stomata could also limit penetration. Considering that infection is dependent on the availability of water (Kajornchaiyakul and Brown, 1976), trichomes may also be a structural defense mechanism by producing a water-repellent surface that could reduce infection. Whether stomata or trichomes play a role as structural defense

mechanisms on sunflower leaves was not determined in this study, but could be considered for future research.

Pustule size was smaller and more localized on the tolerant genotype - in contrast to the large, coalescing pustules present on RHA 358, which measured between 2.5-5 mm in diameter. It is evident that one can differentiate between susceptible, moderately tolerant and tolerant lines when looking at pustule size. Small and localized pustules could be the result of fewer zoospores having been able to penetrate and infect HYS 333 due to structural defense mechanisms. Small and localized pustules could also be an indication of resistance such as defense through hypersensitivity. It has been observed in aerial infection of sunflower with zoospores of *Plasmopara halstedii* (Berl.) Farl. Et de Toni that the amount of phenylalnine ammonia-lyase (PAL), chitinase and ubiquitin increased considerably after infection, suggesting that these compounds should be good markers of resistance after infection by the pathogen (Mazeyrat *et al.*, 1999). The accumulations of phytoalexins have also been reported as a response to infection by *P. halstedii* on sunflower (Mouzeyar *et al.*, 1995). Whether the above-mentioned compounds are also produced by sunflowers as a biochemical response to infection by *A. tragopogonis* is unknown. Further investigation into possible biochemical or metabolic defense mechanisms is required to fully understand which resistance mechanisms used by the host.

Kajornchaiyakul and Brown (1976) described the presence of appressoria-like structures on the tip of each germ-tube, these structures were not observed in our study. Hamilton-Attwell *et al.* (1995) also observed extensive mycelial growth in the spongy parenchyma of sunflower leaves and stated that they looked like thin tubes or circles, which coincides with our results. For both RHA 358 and HYS 333, intercellular hyphae were observed to move through the palisade parenchyma and start divide dichotomously in the spongy parenchyma. Kajornchaiyakul and Brown (1976) did not report the latter observation. However Verma *et al.* (1975), observed extensive branching of *A. candida* hyphae in the intercellular air spaces on their susceptible *Brassica* species.

The large intercellular spaces of the spongy parenchyma allowed the fungus to grow and divide profusely. According to Zimmer and Hoes (1978), these air spaces facilitate photosynthesis. Fungal growth in these spaces can decrease photosynthesis resulting in the most notable symptom, i.e. chlorotic leaf spots. Long and Cooke (1974) stated that the photosynthetic rate in *Senecio squalidus* leaves was reduced with the infection of *A. tragopogonis*. The fungus is also known to fix CO_2 (Long and Cooke, 1974; Thornton and Cooke, 1970) further reducing the photosynthetic rate of the host and as a result leads to the formation of chlorotic leaf spot symptoms.

Haustoria were observed in both genotypes and function to absorb nutrients for the fungus, which in turn facilitate growth and differentiation of hyphae into sporangiophores and sporangia. In HYS 333, however, differentiation of hyphae into asexual structures occurred much later when compared with the infection of *A. tragopogonis* in RHA 358. Verma *et al*. (1975) examined the infection process of *A. candida* on susceptible and resistant cotyledons of *Brassica* species. In the susceptible lines the first haustorium was observed 16-18 h after inoculation while the first haustorium was only observed 48 h in the resistant lines. Additionally, the rate of penetration and reaching of intercellular spaces, occurred later, which is consistent with our results (Verma *et al*., 1975).

Similalry, Lui *et al*. (1989) concluded that there were no significant differences in fungal growth between resistant and susceptible *Brassica* lines. However, they did find that soon after the formation of the first haustoria that the invaded host cells became necrotic leading to the collapse of haustoria and cessation in further fungal growth. The latter response was not observed in our study of infected HYS 333 leaves. Sporangial and sporangiophore formation was similar in both genotypes, confined to the lower surface of the leaves, confirming previous reports (Lui *et al*., 1989; Verma *et al*., 1975).

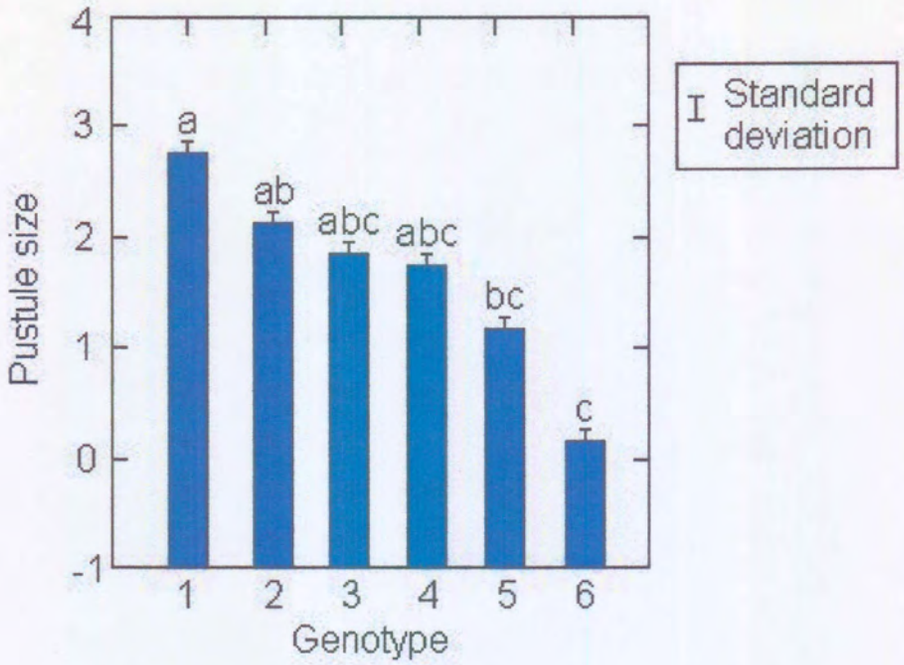
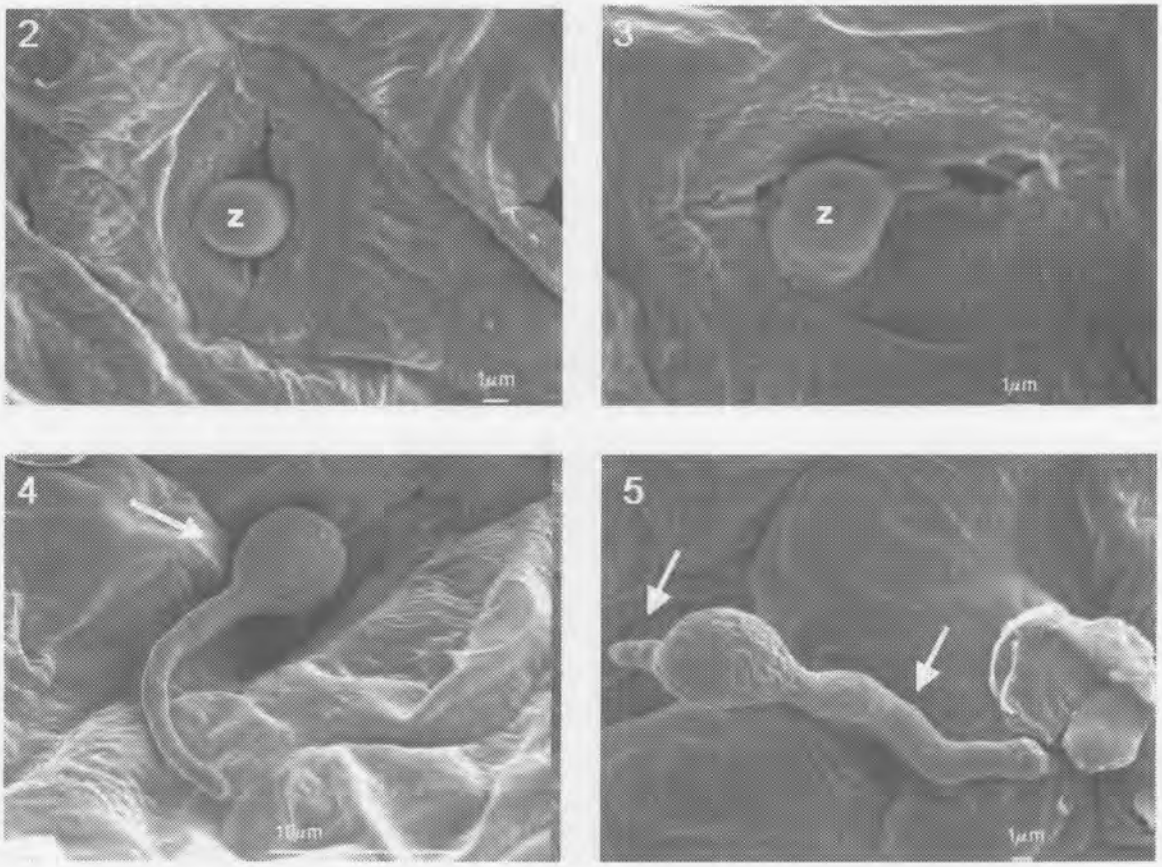


Figure 5.1 The means of the effect of white rust pustule size on six sunflower genotypes, 1=RHA 358, 2=RHA 357, 3=RHA 247, 4=HV 3037, 5-PAN 7392, 6=HYS 333.



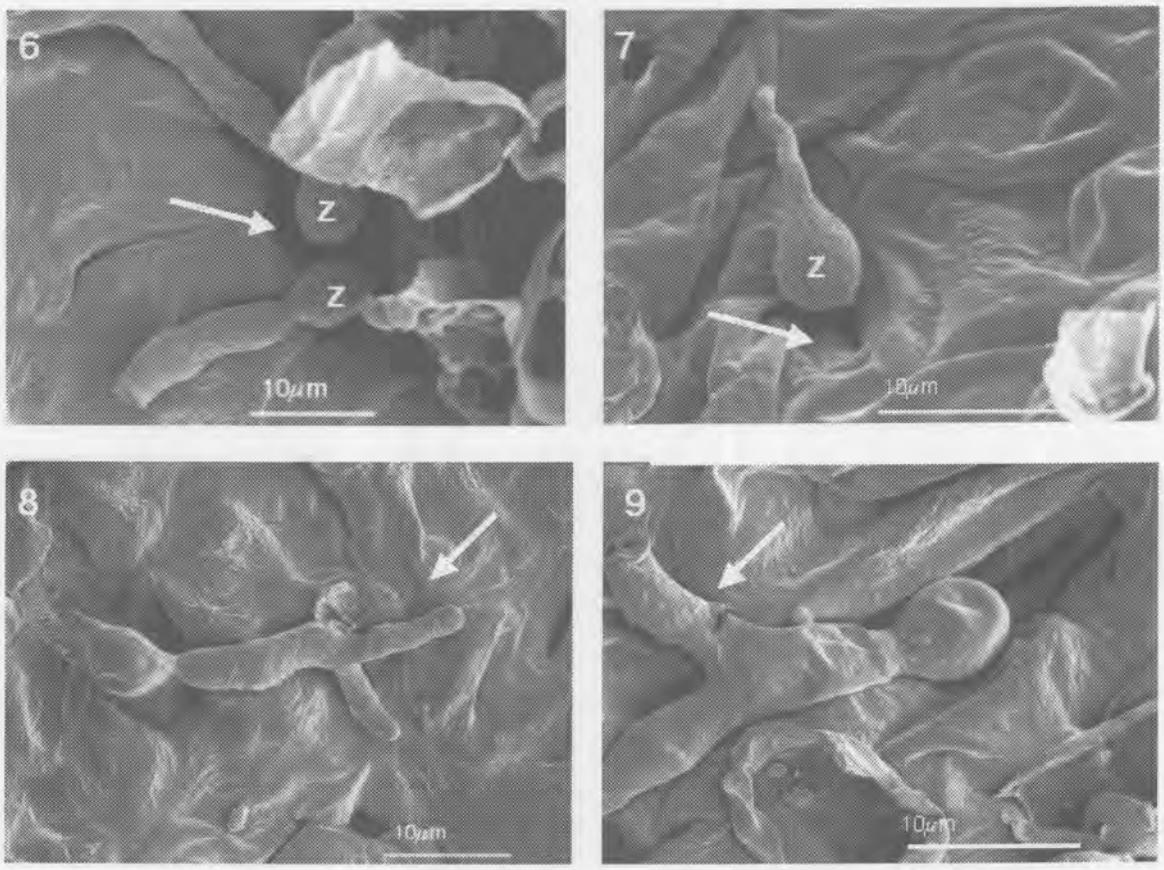
Figures 5.2 – 5.5. Micrographs of the post-penetration events of the infection of RHA 385 and HYS 333 leaves by *Albugo tragopogonis.*

Figure 5.2 Motile zoospore (z) penetrated stomata of RHA 358 at 6 h post inoculation.

Figure 5.3 Motile zoospores (z) penetrated stomata of HYS 333 at 6 h post inoculation.

Figure 5.4 A zoospore (arrow) has encysted and germinated to form a single germ-tube within the sub-stomatal cavity of RHA 358 at 12 h post inoculation.

Figure 5.5 An encysted zoospore has germinated producing two germ-tubes (arrows) within the sub-stomatal cavity of RHA 358 at 12 h post inoculation.



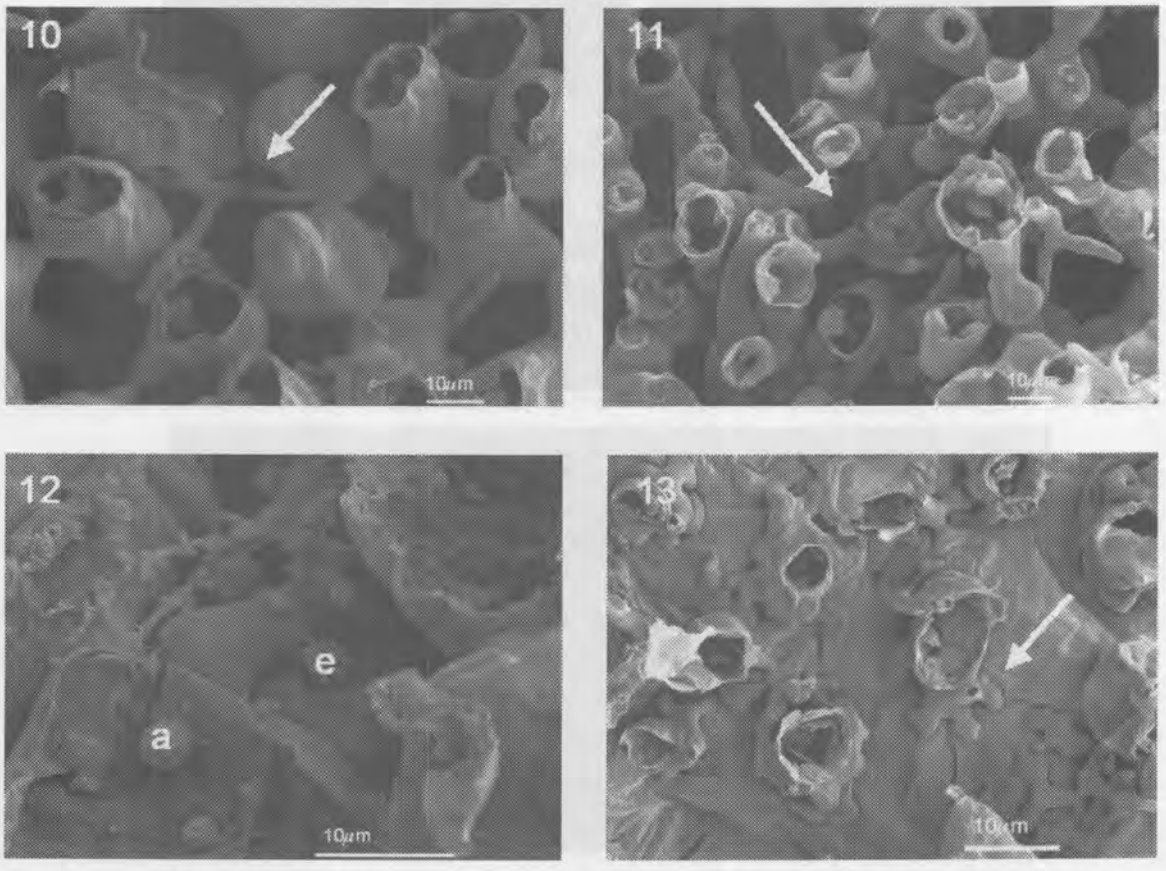
Figures 5.6 – 5.9. Micrographs of the post-penetration events of the infection of RHA 385 and HYS 333 leaves by *Albugo tragopogonis*.

Figure 5.6 Two zoospores (z) observed within a single substomatal cavity (arrow) of RHA 358 at 24 h post inoculation.

Figure 5.7 A zoospore (arrow) has encysted and germinated to form a single germ-tube within the sub-stomatal cavity of HYS 333 at 12 h post inoculation.

Figure 5.8 A germ-tube of a zoospore has branched dichotomously (arrow) within the sub-stomatal cavity of RHA 358 at 24 h post inoculation.

Figure 5.9 A germ-tube of a zoospore has branched dichotomously (arrow) within the sub-stomatal cavity of HYS 333 at 24 h post inoculation.



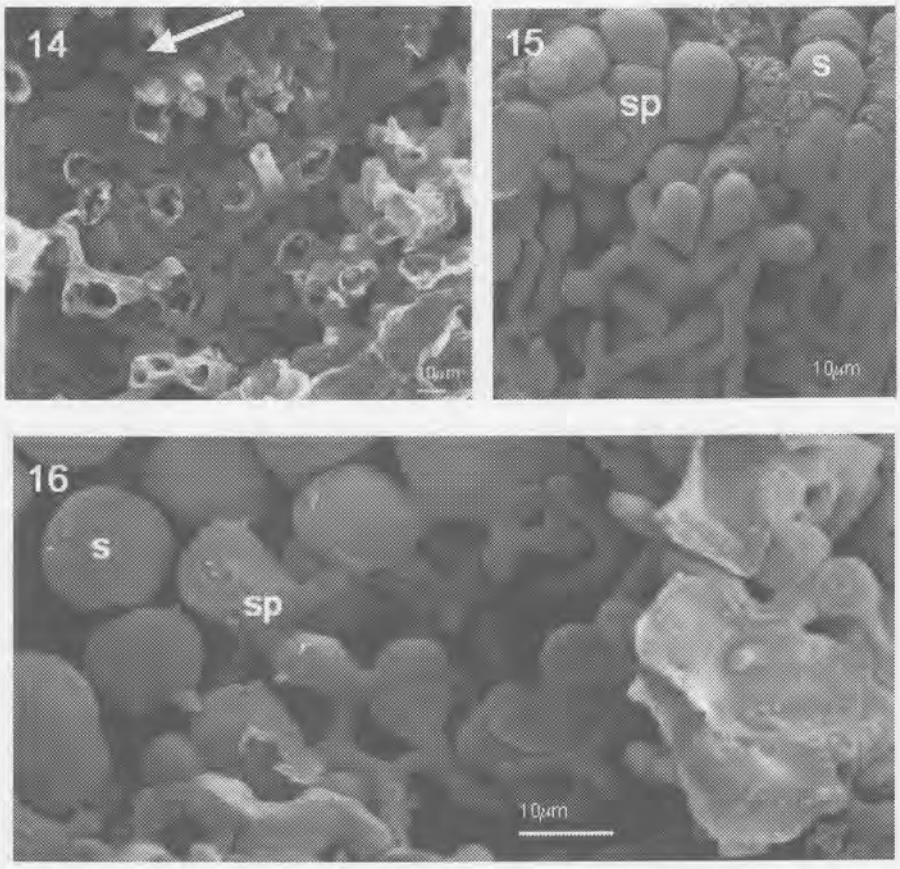
Figures 5.10 – 5.13. Micrographs of the post-penetration events of the infection of RHA 385 and HYS 333 leaves by *Albugo tragopogonis.*

Figure 5.10 Intercellular hyphae have divided dichotomously (arrow) in the spongy parenchyma of RHA 358 and proliferated in the large intercellular air spaces of the spongy mesophyll at 36 h post inoculation.

Figure 5.11 The fungus has proliferated throughout the spongy mesophyll of RHA 358 forming inter-(e) and intracellular (a) haustoria.

Figure 5.12 Intercellular hyphae have divided dichotomously (arrow) in the spongy parenchyma of HYS 333 and proliferated in the large intercellular air spaces of the spongy mesophyll at 48 h post inoculation.

Figure 5.13 Mycelia resemble tubes or circles (arrow) on the abaxial epidermis inside the leaf of RHA 358 at 96 h post inoculation.



Figures 5.14 – 5.16. Micrographs of the post-penetration events of the infection of RHA 385 and HYS 333 leaves by *Albugo tragopogonis.*

Figure 5.14 Mycelia have differentiated into sporangiophores (sp) and sporangia (s) within the abaxial surface of RHA 358 at 144 h post inoculation.

Figure 5.15 Mycelia resemble tubes or circles (arrow) on the abaxial surface of HYS 333 at 144 h post inoculation.

Figure 5.16 Mycelia have differentiated into sporangiophores (sp) and sporangia (s) within the abaxial surface of HYS 333 at 168 h post inoculation.

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Chapter 6

General Discussion

In this dissertation, techniques for the storage of sporangia of *A. tragopogonis* and inoculation of the pathogen on sunflower in the greenhouse were established. These techniques were used to aid with a study of the infection process.

Firstly, a reliable artificial inoculation technique was developed for studies on white rust of sunflower. This technique was optimised to determine the optimum spore concentration for infection, the best method of inoculation, effect of age of seedlings, and was sensitive enough to distinguish resistance among sunflower varieties.

Nowell and Viljoen (1997) and Potgieter *et al.* (1997) observed that young leaves of sunflower proved to be more susceptible to infection by *A. tragopogonis* than old leaves in the field. In chapter 3 similar results were obtained in greenhouse trials. Based on these results, all inoculations were performed on sunflower seedlings between cotyledon stage and four-leaf-stage.

Inoculation at different concentrations was examined. Results showed that disease severity was highest when plants were inoculated with 10^4 and 10^5 sporangia/ml. Lower concentrations probably resulted in fewer zoospores being able to penetrate and infect through stomata on the leaves. The dilution with 10^6 sporangia/ml also showed a very low percentage in germination.

Encysted zoospores are vulnerable to high temperatures and light (Kajornchaiyakul and Brown, 1976). Motile zoospores would have to penetrate stomata and encyst within the substomatal cavities in the dark. Therefore it is imperative that inoculation occurs at low temperatures and light intensities. For this reason, inoculation was performed at dusk.

According to Zimmer and Hoes (1978), there are more stomata present on the lower surface of the leaves of sunflower. An experiment was performed spraying both surfaces of sunflower leaves to test if this would increase infection probability. Submerging the sunflower seedlings into the zoospore suspension also was successful, but stressed plants as it involved uprooting the plants. Spraying both surfaces of the leaves proved to be less stressful to the plants while giving the same infection rates as dipping.

At 4 wks, respiration rates are high and plants transpire (Salisbury and Ross, 1992). Water is essential for encystment and germination of zoospores. Therefore, during inoculation seedlings were covered with plastic bags to compensate for water loss by maintaining a high humidity. In greenhouse trails, a significantly higher infection was achieved after inoculation when seedlings were covered with plastic bags compared to seedlings not being covered with plastic bags.

In conclusion, an optimum inoculation technique was developed in this study which imitates the infection process of the pathogen as in the field. The zoospore suspension should be prepared by allowing 10^4 or 10^5 sporangia/ml to germinate in distilled water for 3 h at 10°C. The zoospore suspension should then be sprayed onto both the adaxial and abaxial surfaces of the leaves until the leaves are completely wet. Inoculated seedlings must then be covered with plastic bags to maintain high humidity and incubated at 12°C for ±16 h in darkness. Infection levels can then be assessed 10-14 d after inoculation using the scale developed by Siddiqui *et al*. (1975).

Apart from developing a reliable artificial inoculation technique, the preservation of sporangia is also important. In this study it was possible to maintain viable sporangia of *A. tragopogonis* for more than 15 mo. We further proved that the sporangia also maintained their fitness to infect sunflower leaves, and that the level of infection could be predicted on susceptible and tolerant sunflower genotypes according to the percentage of germination of the sporangia.

Kachornchaiyakul and Brown (1976) found that sporangia collected from field infected sunflowers and stored at low temperatures without desiccation rapidly lost their viability. From our studies it appears that desiccating sporangia of *A. tragopogonis* before storage is an important factor in the viability of the sporangia. Our tests also included other storage techniques such as placing capsules containing sporangia immediately at -20°C, -70°C, in liquid nitrogen, at -70°C following desiccation for 24 h. Desiccating sporangia gave the best storage results. While desiccated sporangia can be better maintained at equally low temperatures, the absence of a significant difference in germination rate and infectivity levels suggest that further optimisation of the storage of sporangia is needed. It may also be of interest, for future research, to examine the effect of desiccation of sporangia prior to exposure to liquid nitrogen, storage at –20°C or other temperatures.

A comparison of the infection process of *A. tragopogonis* in the susceptible (RHA 358) and a tolerant (HYS 333) genotype was made in chapter 5. The infection process of *A. tragopogonis* in RHA 358 and HYS 333 was almost identical, and generally similar to that described by Kajornchaiyakul and Brown (1976). Zoospores of *A. tragopogonis* on *Senecio squalidus* L. encysted either on guard cells or directly over stomatal pores. In the present study, encysted zoospores of *A. tragopogonis* on sunflower were not observed to infect the host from the surface of the leaf, instead they would enter through stomata. This was explained by Whipps and Cooke (1978), who stated that this unique mode of infection could be due to the size of the zoospore (6-12 μm), which allows them to enter through stomata more easily.

The present study indicated that penetration occurred on both surfaces of RHA 358 and HYS 333, which was consistent with the report by Kajornchaiyakul and Brown (1976). For both genotypes, spores were found near or on stomata. Kajornchaiyakul and Brown (1976) reported two or three, sometimes more, encysted zoospores within a single sub-stomatal cavity. The results of the present study indicated one or a maximum of two

zoospores were present per sub-stomatal cavity in RHA 358 whilst only one zoospore was present in the tolerant genotype HYS 333.

According to Agrios (1988), a plants first line of defense against pathogens is its surface, which the pathogen must penetrate to cause infection. On HYS 333, the size of the stomata are considerably smaller than those observed on RHA 358. Size of stomata could play an important role in limiting penetration and infection of the pathogen. Additionally Agrios (1988), indicated that apart from the size of the stomata, location and shape of stomata could also limit penetration. Considering that infection is dependent on the availability of water (Kajornchaiyakul and Brown, 1976), trichomes may also be a structural defense mechanism by producing a water-repellent surface that could reduce infection. Whether stomata or trichomes play a role as structural defense mechanisms on sunflower leaves was not determined in this study, but could be considered for future research. Further investigation into possible biochemical or metabolic defense mechanisms is also required to fully understand which resistance mechanisms used by the host.

Verma *et al.* (1975) examined the infection process of *A. candida* on susceptible and resistant cotyledons of *Brassica* species. In the susceptible lines the first haustorium was observed 16-18 h after inoculation while the first haustorium was only observed 48 h in the resistant lines. Additionally, the rate of penetration and reaching of intercellular spaces, occurred later (Verma *et al.*, 1975)., which is consistent with our results . In HYS 333 differentiation of hyphae into asexual structures occurred much later when compared with the infection of *A. tragopogonis* in RHA 358. Sporangial and sporangiophore formation was similar in both genotypes, confined to the lower surface of the leaves, confirming previous reports (Lui *et al.*, 1989; Verma *et al.*, 1975).

This study will contribute to the development of much needed greenhouse studies of sunflower with *A. tragopogonis.* Greenhouse studies will not only aid in future studies on the control of white rust of sunflower by means of disease resistance or chemical control, but also on the biology and taxonomy of the responsible pathogen.

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Summary

Albugo tragopogonis is responsible for white rust of sunflower. It was first observed in 1929 in South Africa. Recently however, white rust has resulted in lodging exceeding 80% in some sunflower growing areas. Due to the obligate nature of the pathogen, studies of the biology, epidemiology and control of the disease has until now been limited to field trials and observations. Greenhouse trials are needed to understand the infection process, and to examine any resistance mechanisms used by the plant to defend itself against the pathogen. Presently, there is no practical artificial inoculation technique available and effective storage of the fungus is difficult. The purpose of these studies was to find new storage and inoculation techniques. Once the inoculation technique was optimized, the infection process of *A. tragopogonis* on susceptible and tolerant sunflower genotypes was examined.

Infected leaves were collected from sunflower seedlings at the Grain-Crops Institute in Potchefstroom. Infected leaves were covered with plastic bags and freshly cut stems were placed in a cooler box filled with ice water. Some of the infected leaves were also placed in paper bags and allowed to dry for 24 h. Sporangia were collected using a vacuum device and stored in gelatin capsules at -20°C, -70°C or in liquid nitrogen directly after collection or following desiccation for 24 h. Sunflower seedlings at the four-leaf-stage were inoculated with freshly collected sporangia, or sporangia stored for 3, 5, 9, 12 and 15 mo.

A zoospore suspension was prepared by allowing 10^5 sporangia/ml to germinate in distilled water for 3 h at 10°C. The zoospore suspension was then sprayed onto leaves until they were completely wet with a hand held garden spray bottle. Inoculated seedlings were covered with plastic bags to maintain high humidity and placed at 12°C for 16 h and incubated in a greenhouse until symptom development. Infection levels were assessed 10-14 d after inoculation, using a scale of 1-5, with 1 indicating resistance and 5 indicating severe infection. Infection with fresh sporangia proved to be very

consistent. Sporangia stored in capsules immediately after collection at -70°C after desiccation, produced the highest infection. Low levels of infection resulted from storage in liquid nitrogen or directly at -70°C. It is evident that successful storage may be obtained if the sporangia are dried before storage. These techniques to store and inoculate *A. tragopogonis* have proven to be reliable.

Susceptible and tolerant genotypes were inoculated, using the spray bottle inoculation technique described above, to examine the difference in infection of *A. tragopogonis.* Leaves used for light microscopy were cut into 20 mm^2 and those for scanning electron microscopy were cut into 5x5 mm pieces at 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h time intervals after inoculation. The epidermis, palisade parenchyma and spongy parenchyma were chronologically stripped using the double-sided tape method. The material for the light microscope was prepared using the whole-leaf clearing and staining technique, the lactophenol-ethanol-analine blue technique and sectioning with freeze microtome. The material for SEM was prepared according to standard procedures and examined with a JEOL 840 SEM at 5 kV.

Both the whole-leaf clearing and staining and the lactophenol-ethanol-aniline blue techniques proved to be unsuitable as most of the tissue was damaged by boiling. Sectioning with the freeze microtome was also unsuccessful. The SEM gave the most transparent results. This method gave us the ability to compare results with previous literature and to compare the infection process between of *A. tragopogonis* in the susceptible (RHA 358) and the tolerant (HYS 33) genotype.

Appendix A

Chapter 3

Table 3.1 Analysis of variance of sporangial concentration on the severity of leaf infection (N: 180)

		Severity			
Source	df	Sum-of-Squares	Mean-Square	F-ratio	P(0.05)
Main Effects					
Concentrations	5	422.711	84.542	123.720**	2.29
Blocks	9	35.200	3.911	5.724*	1.96
Interaction					
Conc.*Blocks.	45	85.400	1.898	2.777*	1.483
Error	120	82.000	0.683		

(Conc=Concentrations)

Durbin-Watson D Statistic; 2.843, First Order Autocorrelation; -0.421, Multiple R: 0.932, Squared multiple R: 0.869

Table 3.2 Analysis of variance of leaf age on the severity of leaf infection (N: 330)

		Severity			
Source	df	Sum-of-Squares	Mean-Square	F-ratio	P(0.05)
Main Effects					
Leaf Age	10	226.824	22.682	30.677**	1.88
Blocks	9	7.515	0.835	1.129ns	1.83
Interaction					
Leaf Age*Blocks	90	162.085	1.801	2.436*	1.27
Error	220	162.667	0.739		

Durbin-Watson D Statistic; 2.471, First Order Autocorrelation; -0.241, Multiple R: 0.842, Squared multiple R: 0.709

Table 3.3 Analysis of variance of occurrence of pustules on the adaxial/abaxial cotyledon surfaces on six sunflower genotypes (N: 180)

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P(0.05)
Main Effect					
Genotypes	140.711	5	28.142	15.444**	2.29
Blocks49.867	9	5.541		3.041*	1.96
Interaction					
Genotypes*Blocks	60.067	45	1.335	0.733ns	1.483
Error	218.667	120	1.822		

Durbin-Watson D Statistic: 1.698, First Order Autocorrelation: 0.150, Multiple R: 0.731, Squared multiple R: 0.534

Table 3.4 Comparison of the effect of light and darkness on disease severity and pustule size (N:60)

Source	df	Sum-of-Squares	Mean-Square	F-ratio	P(0.05)
Main Effects					
Treatments	1	58.017	58.017	124.321**	4.08
Blocks	9	23.483	2.609	5.591*	2.12
Interaction					
Treatment*Blocks	9	24.817	2.757	5.909*	2.12
Error	40	18.667	0.467		

Durbin-Watson D Statistic: 2.542, First Order Autocorrelation: -0.310, Multiple R: 0.681, Squared multiple R: 0.464

Table 3.5 Analysis of variance in white rust severity of the different inoculation methods (N: 360)

		Severity			
Source	df	Sum-of-Squares	Mean-Square	F-ratio	P(0.05)
Main Effects					
Inoculation Methods	11	214.967	19.542	32.571**	1.79
Blocks	9	17.400	1.933	3.222*	1.84
Interaction					
Inoculation*Blocks	99	87.533	0.884	1.474ns	1.945
Error	240	144.000	0.600		

Durbin-Watson D Statistic: 2.323, First Order Autocorrelation:-0.164, Multiple R: 0.830, Squared multiple R: 0.690

Table 3.6 Analysis of variance in the severity of white rust on 6 sunflower genotypes (N: 180)

		Severity			
Source	df	Sum-of-Squares	Mean-Square	F-ratio	P(0.05)
Main Effects					
Genotypes	5	267.650	53.530	87.595**	2.29
Blocks	9	8.894	2.099	3.435*	1.96
Interaction					
Genotypes*Blocks.	45	131.072	2.913	4.766*	1.483
Error	120	73.333	0.611		

Severity = Durbin-Watson D Statistic; 2.385, First Order Autocorrelation; 0.192,Multiple R: 0.922; Squared multiple R: 0.851

Chapter 4

Table 4.1 Analysis of variance of white rust severity ratings of RHA 357 and RHA 358 infected leaves (N: 1200 per genotype)

Source	df	RHA 357				RHA 358			
		Sum-of-Squares	Mean-Square	F-ratio	P (0.05)	Sum-of-Squares	Mean-Square	F-ratio	P (0.05)
Main Effects									
Periods	4	37.378	9.345	29.407**	2.37	188.164	47.041	50.337**	2.37
Storage Methods	5	62.077	12.415	39.071**	2.21	94.428	18.885	20.209**	2.21
Pot numbers	9	2.199	0.244	0.769 ns	1.88	6.503	0.723	0.773 ns	1.88
Interactions									
Periods*Storage's	20	16.069	0.803	2.528*	1.57	97.541	4.877	5.219*	1.57
Periods*pot no.	36	14.430	0.401	1.261 ns	1.394	16.132	0.448	0.480 ns	1.394
Storage's*pot no.	45	12.448	0.277	0.871 ns	1.335	47.777	1.062	1.136 ns	1.335
Error	180	57.197	0.318			168.213	0.935		

RHA 357:Durbin-Watson D Statistic: 2.033, First Order Autocorrelation: 0.020, Multiple R: 0.847, Squared multiple R: 0.717

RHA 358:Durbin-Watson D Statistic: 1.324, First Order Autocorrelation: 0.336, Multiple R: 0.853, Squared multiple R: 0.728

Chapter 5

Table 5.1 Analysis of variance in pustule size of *Albugo tragopogonis* on six sunflower genotypes (N: 180)

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P (0.05)
Main Effects					
Genotypes	121.028	5	24.206	96.822**	2.29
Blocks	8.450	9	0.939	3.756*	1.96
Interaction					
Genotypes*Blocks	44.583	45	0.991	3.963*	1.483
Error	30.000	120	0.250		

Durbin-Watson D Statistic: 2.481, First Order Autocorrelation: -0.241, Multiple R: 0.924, Squared multiple R: 0.853