CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The Host: Oilseed Sunflower

The genus *Helianthus* L. contains about 50 annual and perennial species all of which are herbs originating in North America (Schilling and Heiser, 1981). The basic chromosome number is $n = 17$ and diploid, tetraploid and hexaploid species exist (Rogers, Thompson and Seiler, 1982).

The development of sunflower as a major Australian and World crop has been a protracted process. Archaeological evidence (Smith, 1965; Lawton, Wilke, DeDecker and Mason, 1976) and more recent observations (Putt, 1978) suggest that the common annual sunflower (*Helianthus annuus* L.) was used by some of the aboriginal cultures of North America over 5000 years ago. Domestication probably commenced in the midwest of the present United States (Rieseberg and Seiler, 1990). The sunflower provided a source of oils for cosmetics, medicines and dyes. Ground sunflower seed meal was especially prized as a high energy food that sustained warriors and hunters when travelling (Schneider and Schneider, 1993) Some of the land races of sunflower such as 'Arikara', 'Havasupi', 'Hopi' and 'Seneca' still exist (Heiser, 1976*a*). They are generally tall monocephalic forms in contrast to the highly branched wild species.

Sunflowers were introduced from the Americas to Europe during the 16th century and grown as ornamentals. Cultivation then spread across Europe. Selection for enhanced production of edible oil commenced in Russia in 1860 (Heiser, 1976*b*). Breeding programs aimed at developing improved cultivars followed. Taxonomically the cultivated sunflowers were divided between the ornamentals *Helianthus annuus* ssp. *annuus* and the agricultural cultivars *H. annuus* var *macrocarpus* [DC.] Ckll. (Heiser, Clevenger and Martin, 1969). During the present century Russian lines of *H. annuus* var. *macrocarpus* have been distributed around the world. The crop has been successfully grown from tropical areas of Australia and Africa to cool temperate areas of Argentina, Canada and Europe. The incorporation of high oil germplasm into sunflower breeding programs internationally and the wide adaptability of the crop has resulted in sunflower becoming one of the top four edible oil producing crops in the world (FAO, 1984).

Further impetus to sunflower cultivation came with the discovery of hybridisation systems. Leclerq(1969) reported the discovery of cytoplasmic male sterility (*cms*) in crosses between *Helianthus petiolaris* Nuttall and *H. annuus*. Nuclear fertility restoration genes for this PET1 *cms* (F.A.O. Code) were reported in 1971 (Leclerq, 1971; Vranceanu and Stoenescu, 1971). Commercial hybrids were subsequently released during the 1970's and quickly adopted by farmers because of the higher yields and improved crop uniformity they provided. Other *cms* systems have been discovered but the PET1 system is still used to produce all commercial hybrids (Crouzillat, de la Canal, Perrault, Ledoigt, Vear and Serieys, 1991).

Sunflower has been traditionally considered as a premium source of polyunsaturated oil with a fatty acid composition of around 70% linoleic and 20% oleic acids (Robertson, 1972; Fick, 1989). The popularity of polyunsaturated oils increased rapidly following research linking intake of saturated animals oils and fats with cardiovascular diseases (Deeth, 1992). The polyunsaturated essential fatty acids such as linoleic acid are also important in human physiology (Horrobin, 1992). Recently, public interest has moved from polyunsaturated to monounsaturated oils because they are able to better withstand oxidation in storage and cooking and after ingestion can aid in the lowering of blood cholesterol levels without reducing the levels of beneficial high-density lipoproteins (Purdy, 1985; Deeth, 1992). Sunflower breeders have been able to respond to this demand for monounsaturated oil by developing breeding lines producing oil with a fatty acid content of over 80% oleic acid. These lines were derived through mutagenesis (Soldatov, 1976). Wild species of *Helianthus* may also provide genetic variants producing high oleic oil (Seiler, 1985).

The future of sunflower as a crop may be further ensured by developing cultivars producing renewable sources of industrial oils or other products. Genes may come from sunflower species which produce oil with higher levels of palmitic and/or stearic acid than commercial sunflower (Thompson, Zimmerman and Rogers, 1981). Alternatively, genetic manipulation may be used to transfer the genes encoding for the oil of interest from undomesticated plant species to cultivated sunflower (Murphy, 1992). The sunflower species *Helianthus agrestis* Pollard, *H. occidentalis* Riddell, *H. radula* (Pursh) T. & G., *H. californicus* DC and *H. resinosus* Small have all been identified as having natural rubber contents in their foliage of 14 to 20 $g.kg^{-1}$ (Stipanovic, O'Brien, Rogers and Hanlon, 1980; Stipanovic, Seiler and Rogers, 1982). These levels might be increased through selection. Sunflower capitula, as a by-product of oilseed production, have also been examined as a source of pectins (Alarcao e Silva, 1990). At capitula maturity, the pectin content reaches 20% of dry weight, it has a higher galacturonic acid content than apple pectin and superior gel properties when compared with commercial pectin (Campbell, Sosulski and Sabir,

1978; Zitko and Bishop, 1965). The Jerusalem artichoke (*Helianthus tuberosus* L.) and inter-specific hybrids with *H. annuus* may provide alternative forage crops in cool areas of northern Europe (Hay and Offer, 1992).

In Australia, *H. annuus* was introduced as an ornamental (M^{c}Alpine, 1906). At various times during the present century attempts were made to promote sunflowers as a crop. Early protagonists could find no fault with the crop. Boyd(1915) boldly stated that sunflowers '*will grow in almost any soil, and in any climate. It will withstand cold or heat, drought or rain. It is subject to no disease, and to no climatic disqualification*'. The crop was promoted as having multiple uses with the seed used as a source of edible oil or for animal feed, the leaves as cattle silage or as a vermicide in horses, the stem pith in life preservers and any trash convertible to protein-rich ash (Anon., 1916*a*; Boyd, 1915; Keane, 1915, 1920). Interest in the crop was heightened during the two World Wars when other edible oils could not be imported (Anon., 1916*b*; Anon., 1941). The absence of mills for the extraction of oil hindered the development of the crop.

The imposition of the wheat quota system to Australia in 1969 forced farmers to experiment with alternative crops (Lovett and Lazenby, 1979). The combination of the wheat quota's, the advent of high yielding sunflower hybrids and increasing health consciousness among consumers of edible oil products led to the revival of interest in sunflower as a crop. The area sown to sunflower in Australia increased from 6000ha in the 1967/68 season to 294000ha in the 1971/72 season (Australian Bureau of Agricultural Economics, Canberra). Since that time the area sown has varied from 100000 to 350000ha annually depending on climatic and market fluctuations. The crop is usually grown under rainfed conditions except for small areas in Victoria where flood irrigation is practised. In the Australian marketplace the crop competes against sunflower seed and oil and other edible oils sourced from outside Australia. The crop therefore often offers a relatively low gross margin return to growers and any constraint to reliable production must be removed if sunflowers are to remain a viable cropping option in Australia.

The incidence of plant diseases can influence the attitude of farmers in the determination of potential cropping strategies. In Australia, the major diseases of sunflower are sunflower rust (*Puccinia helianthi* Schw.) and alternaria blight (*Alternaria helianthi* Tub. et Nish.). Diseases that may occur and cause economic damage depending on the environmental conditions prevalent during the culture of the crop include sclerotinia head rot (*Sclerotinia sclerotiorum* (Lib.) de Bary, rhizopus head rot (*Rhizopus arrhizus* Fischer, *R. oryzae* Went.), charcoal rot (*Macrophomina phaseolina* (Maubl.) Ashby) and stem base rots (*Sclerotium rolfsii* Sacc., *Sclerotinia sclerotiorum*, *S. minor* Jagger). Other diseases that occur but are not attributed with causing significant yield losses include septoria leaf spot

(*Septoria helianthi* Ell. & Kell.), grey mold (*Botrytis cinerea* Pers. ex Fr.), white blister (*Albugo tragopogonis*(Pers.)Schroet.), powdery mildew (*Erysiphe cichoracearum* DC), noogoora burr rust (*Puccinia xanthii* Schw.), verticillium wilt (*Verticillium dahliae* Klebahn), bacterial stem rot (*Erwinia carotovora* (Jones 1901) Bergey, Harrison, Breed, Hammer & Huntoon 1923), bacterial leaf spot (*Pseudomonas syringae* pv. *helianthi*(Kawamura 1934) Young, Dye & Wilkie 1978) and various stem spots (*Phoma* and *Phomopsis* species). Australia is distinct from other sunflower producing areas of the world since downy mildew (*Plasmopara halstedii* (Farl.)Berl & de Toni), stem canker or brown stem rot (*Phomopsis helianthi* Munt.-Cvet. *et al.*) and broomrape (*Orobanche cumana* Wallr.), considered to be serious in other parts of the world, have not been recorded here while stem rot caused by *Sclerotinia minor* Jagger only seems to be a problem in this country.

The increasing incidence of sclerotinia diseases is of concern to the sunflower industry in Australia. An understanding of these diseases will aid in the development of strategies to reduce their effects on the production of sunflower oil.

1.2 The Pathogens: *Sclerotinia sclerotiorum* and *S. minor.*

1.2.1 Introduction

Sclerotinia sclerotiorum is capable of infecting all organs of a sunflower plant. Soil-borne sclerotia of the fungus can germinate myceliogenically and infect host roots in close proximity. Decay of roots and lower parts of stems produce the characteristic symptoms of wilt and plant death (Jones, 1923). Sclerotia that are close to the soil surface may undergo carpogenic germination to form apothecia. Ascospores released from the apothecia can establish infections of leaf lamina and capitula. Infections of leaves can progress down the petiole and cause stem rot (Cuk, 1975). Ascospores that land on florets during anthesis can cause capitulum (or head) rot. Wilt caused by *S. sclerotiorum* is the predominant sclerotinia disease of sunflower in North America (Gulya, Vick and Nelson, 1989) and is also important in Yugoslavia (Chun and Maric, 1989). Head rot is important in other parts of Europe (Acimovic, 1984), Argentina (Bazzalo, 1982) and to an increasing extent in Australia.

Sclerotinia minor also incites a root and stem base rot of sunflower. Artificial inoculation shows that the fungus is capable of infecting sunflower leaves, stems and capitula. However natural infection of these organs has not been recorded presumably because apothecial formation in nature is rare or does not occur (Abawi and Grogan, 1979).

Germination of sclerotia may be by single hyphae or be eruptive when a mass of the mycelial contents of the sclerotium erupts through the rind. Sclerotia germinate over a wide range of temperatures, oxygen, carbon dioxide and ethylene concentrations and at soil moisture tensions as great as -15bars (Imolehin, Grogan and Duniway, 1980; Imolehin and Grogan, 1980*a*). Natural infections of sunflower by *S. minor* have been reported from Uruguay (Sackston, 1957), France (Gaudineau and Lafon, 1958), Argentina (Carranza, 1962), Canada (Creelman, 1965), and in the State of Victoria as well as on the Liverpool Plains area in the State of New South Wales in Australia (Clarke, 1982; Porter and Clarke, 1992). and, recently, from Romania (Iliescu, Ionita, and Jinga, 1988), South Africa (Baard and Los, 1989) and California (Gulya, Woods, Bell and Mancl, 1991). Under controlled conditions isolates of *S. minor* and *S. trifoliorum* collected from other hosts have been shown capable of infecting sunflowers (Comack, 1946; Thault and Tourvieille de Labrouhe, 1988).

1.2.2 Aetiology of Sclerotinia Diseases

Hyphae from germinating sclerotia can infect roots, stem bases or senescent leaves in contact with the soil surface (Purdy. 1958). The clump of mycelium that emerges during myceliogenic germination utilise the energy reserves in the sclerotia without the need to colonise some exogenous food source before infection (Adams and Tate, 1976; Adams and Ayers, 1979). However, the single hyphae that emerge through the sclerotial rind during hyphal germination must first proliferate on some food base before being able to infect healthy tissue (Adams and Tate, 1976). Hyphae from germinated ascospores can infect wound sites (Abawi and Grogan, 1975) or proliferate on dead flowers, senescent leaves, organic matter such as pollen, and organic exudates before infection (McLean, 1958; Sutton and Deverall, 1983; Sedun and Brown, 1987).

The infection processes of *Sclerotinia* species are generally similar irrespective of whether ascospores or sclerotia are the inoculum (Purdy, 1958). Lumsden and Wergin (1980) observed the following infection process on bean leaves inoculated with agar cultures of *S. minor*.

1 The tips of hyphae contacting the host cuticle developed into simple appressoria which branched to become complex infection cushions composed of multiple, truncated cells that were orientated perpendicular to the host surface,

2 Direct penetration of the cuticle was effected either by mechanical means or by enzymatic digestion (Tariq and Jeffries, 1987). A granular vesicle formed between the cuticle and epidermis (Lumsden and Dow, 1973). Plant cells beneath the infection pegs may collapse and the contents of neighbouring cells become disorganised suggesting that a toxin or toxins are released by the fungus during early stages of

penetration (Purdy, 1958). Lewis and Bibby (1989) observed the formation of crystals around the infection sites of *S. sclerotiorum* that they tentatively identified as being calcium oxalate. They suggested that the infection cushions exuded oxalic acid during infection.

3 Inflated infection hyphae (8.5 to 34.0μm wide) initially develop between the cuticle and epidermis and later develop intercellularly, before,

4 narrower ramifying hyphae developed inter- and intracellularly through the epidermis and underlying parenchyma. Histological observations have shown that disruption of the middle lamella and cells occurs in advance of invading hyphae (Lumsden, 1976). Hyphae may also enter the xylem vessels (Prior and Owen, 1964; Clarke, Porter and Woodroffe, 1993).

5 Finally, sclerotia form within the invaded tissue if there is sufficient space or on the exterior if the relative humidity is sufficient.

Species of *Sclerotinia* produce a wide array of plant cell macerating enzymes that can be attributed with formation of the soft rot symptoms. Plant tissue is notionally composed of individual cells enclosed by cell walls consisting of cellulose microfibrils embedded in a matrix of hemicelluloses (rhamnogalacturonans, galactans, arabinans, xylans), pectins and proteins (hydroxyproline-rich glycoproteins). The middle lamella between the cells is composed primarily of pectic compounds. Lignin may be deposited in the cell wall and middle lamella as the tissue matures (Goodwin and Mercer, 1983). Species of *Sclerotinia* are capable of degrading the principal plant structural polysaccharides causing the release of oligo- and monosaccharides. Vascular bundles and other lignified structures are left behind since lignin is not digested by *Sclerotinia* (Young and Morris, 1927). The diversity of extracellular enzymes reported to be produced by species of *Sclerotinia* and their cellular substrates are presented in Table 1.1.

1.2.3 Specific Taxonomy and Intra-specific Variation.

Sclerotinia Fuckel is the type genus of the family Sclerotiniaceae which was erected to describe stromatic inoperculate discomycetes (Whetzel, 1945). The genus had included up to 265 species until the systematic revisions by Kohn (1979*a*,*b*) and Willets and Wong (1980) led to the conservation of only a few species, *Sclerotinia sclerotiorum*(Lib.) de Bary, *S. trifoliorum* Erikss., *S. minor* Jagger and a few species of dubious taxonomy. The lectotype of *Sclerotinia* is *S. sclerotiorum* which was also described as *S. libertiana* by Fuckel to avoid a supposed tautonym (Kohn, 1979*a*) and *Whetzelinia sclerotiorum* by Korf and Dumont (1972) since the fungus *S. candolleana* transferred to the genus *Ciborinia* by Whetzel (1945) had precedence for the generic name. Arguments for the conservation of *S. sclerotiorum* as the lectotype of *Sclerotinia* have been presented by Kohn (1979*a*,*b*) and

adopted.

Table 1.1 Extracellular Enzymes produced by *Sclerotinia* spp. and their substrates[3].

E.C. Code[4]	Common Name	Substrate and Activity[2]	pH optima	Reference
E.C. 3.1.1.4	Phospholipase A_2	hydrolysis of phosphatidylcholine in cell membranes	4.0	Lumsden (1970)
EC 3.1.1.11	pectin methylesterase	de-esterfication of pectins to form pectate		Hancock (1966); Lumsden(1976)
E.C. 3.2.1.4	endo-1,4-β-D-glucanase (cellulase)	cellulose β-1,4-glucosidic linkages	3.6; 5.0	Lumsden (1969); Marciano, di Lenna and Magro(1983); Waksman (1988)
E.C. 3.2.1.15	endo-polygalacturonase	hydrolyses α-1,4 linkages of pectic acid	4.5-5.5, 4.0; 4.0	Lumsden(1976), Keon and Waksman(1990); Echandi and Walker (1957)
E.C. 3.2.1.21	β-glucosidase	cellobiose produced from cellulase activity on cellulose	5.0	Waksman(1988), Riou, Freyssinet and Fevre (1991)
E.C. 3.2.1.22	α-galactosidase	hydrolysis of terminal α-D-galactose residues from α-D-galactosides		Scott and Fielding(1982)
E.C. 3.2.1.23	β-galactosidase	hydrolysis of terminal β-D-galactose from β-D-galactosides		Riou *et al.* (1991)
E.C. 3.2.1.32	endo-1,3-β-D-xylanase	random hydrolysis of 1,3-β-D-xylosidic linkages in xylans	4.0, 5.0	Riou *et al.* (1991); Marciano, Di Lenna and Magro (1983)
E.C. 3.2.1.37	β-xylosidase	hydrolysis of 1,4-β-D-xylans		Scott and Fielding(1982), Riou *et al.* (1991)
E.C. 3.2.1.38	β-fucosidase	hydrolysis of terminal β-D-fucose from β-D-fucosides		Riou *et al.* (1991)
E.C. 3.2.1.55	α-L-arabinofuranosidase	arabinans in cell walls to release arabinosides	4.0-4.5, 3.6-5.8	Fuchs, Jobsen and Wouts(1965); Baker, Whalen, Korman and Bateman(1979); Scott and Fielding(1982)
E.C. 3.2.1.67	exo-polygalacturonase	hydrolysis of terminal α-1,4-linkages of polygalacturonic acid	4.5	Lumsden (1976); Riou, Freyssinet and Fevre (1992)
E.C. 3.2.1.88	α-arabinosidase	hydrolysis of α-L-arabinoside to release L-arabinose		Riou *et al.* (1991)
E.C. 3.2.1.89	endo-β-1,4 galactanase	endohydrolysis of β-1,4-galactosidic linkages in arabinogalactans	4.5	Bauer, Bateman and Whalen (1977)
E.C. 4.2.2.10	pectin lyase	catalysis of α-1,4 glycosidic linkages in pectin		Scott and Fielding(1982)

1. Many of these enzymes may exist as several isoforms as detected by iso-electric focusing (Keon and Waksman, 1990; Scott and Fielding, 1982; Marciano, Di Lenna and Magro, 1982, Riou *et al.*, 1991). 2. Pectin-degrading activity as summarised by Sakai (1992), other activities by Webb(1992). 3. Enzyme production *in vitro* may be constitutive or induced dependent on carbohydrate substrate provided (eg Riou *et al.*, 1991). 4. Enzyme Commission Code relating to activity.

The taxonomic status of the plant pathogenic species within the genus has also been confused since clear criteria for delineation have not been established. Approaches that have been used to delimit the plant pathogenic species include sclerotial size, host range, mycelial characteristics, mycelial interactions, number of nuclei in ascospores, haploid

chromosome number, size of ascospores, structure of the apothecia (particularly the nature of the ectal excipulum), electrophoretic patterns of enzymes (Wong and Willets, 1975) and, more recently, DNA fingerprinting through the use of restriction fragment length polymorphism (R.F.L.P.) analyses (Kohn, Stasovski, Carbone, Royer and Anderson (1991).

Sclerotinia trifoliorum is a cosmopolitan species largely restricted in its host range to forage legumes. *S. minor* was first described in 1920 by Jagger as a pathogen of vegetables. Purdy (1955) concluded that these species represented a natural group within *S. sclerotiorum* since near-continuous variation occurred in cultural characteristics, size of sclerotia, asci and ascospores. Differences in anatomy, morphology and cytology in sclerotia and apothecia lead to the revision of the genus and readoption of the three species (Kohn, 1979*a,b*). This delineation was supported by Willets and Wong (1980) in their review of morphological, cytological and physiological studies up to 1980. More recent studies have questioned the conclusions reached by Kohn (1979) and Willets and Wong (1980). Often this has been because the characters used originally to separate species were later considered to be too indistinct to be useful. Jayachandran, Willets and Bullock (1987) reported difficulty in using the nature of the ectal exipulum of apothecia to delimit the species as suggested by Kohn (1979*a,b*). Similarly, the reliability of using the presence of short, tomentous hyphae arising from globose cells in the rind of sclerotia to separate *S. trifoliorum* from *S. sclerotiorum* (Kohn, 1979a,b) was questioned by Pratt, Dabney and Mays(1988).

The inter-relationships between the plant pathogenic species of *Sclerotinia* are also confused. Uhm and Fuji(1986) reported that the haploid chromosome number of *S. sclerotiorum* and *S. minor* was 8 while that of *S. trifoliorum* was 9. However, Wong and Willets (1979) reported 8,4 and 8, respectively for the three species. Tariq, Gutteridge and Jeffries (1985) found that analysis of *in vitro* growth rates, mycelial interactions between isolates, gross sclerotial characteristics, polyacrylamide gel electrophoresis of sclerotial proteins and Curie-point pyrolysis mass spectrometry supported the retention of *S. minor* as a distinct species but did not distinguish between *S. sclerotiorum* and *S. trifoliorum*. Scott (1981) concluded, on the basis of immuno-electrophoretic comparisons, that a closer relationship existed between *S. minor* and *S. sclerotiorum* than between either and *S. trifoliorum*. Serological behaviour of lectins isolated from *Sclerotinia* species in double immunodiffusion and ELISA assays demonstrated that the proteins from *S. minor* and *S. trifoliorum* were closely related but distinct from those of *S. sclerotiorum*. The lectins from three *S. sclerotiorum* isolates were identical (Kellens, Goldstein and Peumans, 1992). *Sclerotinia minor* was reported by Willets and Wong (1980) to be antagonistic to *S. sclerotiorum* and *S. trifoliorum* causing lysis of the hyphae of the latter species. This

antagonism was used as a characteristic to confirm the identification of *S. minor* by Baart and Los(1989).

The advent of new molecular-based methods for 'fingerprinting' cellular DNA has allowed mycologists to examine taxonomic problems with a new level of resolution (Kohn, 1992). Application of restriction fragment length polymorphism (R.F.L.P.) technology to 39 isolates of *S. sclerotiorum*, *S. trifoliorum*, *S. minor* and three new or putative species *S. ficariae*, *S. asari* Wu and C.R. Wang and *Sclerotinia* n. sp. supported the contention that *S. sclerotiorum*, *S. trifoliorum*, *S. minor*, *S. asari* and the undescribed species are distinct species and *S. ficariae* is synonymous under *S. sclerotiorum* (Kohn, Petsche, Bailey, Novak and Anderson, 1988). Most of the polymorphisms found in ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) restriction fragment lengths were between the putative species rather than within species.

Physiologic races (*sensu* Robinson, 1976) based on the qualitative differential interaction of host and pathogen isolates have not been detected within *Sclerotinia* species. However, Price and Colhoun (1975) demonstrated that quantitative differences occurred in the ability of different isolates of *Sclerotinia sclerotiorum* to infest different plant species. Studies of natural intraspecific variation for other characters such as colony morphology or pattern of production of sclerotia often give inconclusive results (Torés, 1990). The use of mutagenesis (Miller, Ford and Sands, 1989) and protoplast regeneration (Boland and Smith, 1991) methods have demonstrated that variants for sclerotial production, cultural morphology and pathogenicity can be generated from a single isolate of *S. sclerotiorum*.

Intraspecific variation has, however, been found for other characters. Mycelial compatibility (or incompatibility) groups have been described within *S. sclerotiorum* (Kohn, Stasovski, Carbone, Royer and Anderson, 1991). These groupings are based on the observations that mycelia of different species or isolates interact in a variety of ways when co-cultured. Willets and Wong(1980) identified three interactions; i) hyphae freely intermingle and form occasional anastomoses, ii) hyphae in the contact zone are raised and darken as hyphal cells of either of the colonies lyse and, iii) rapid formation of a brown zone as interacting cells rapidly lyse. The identification by Kohn *et al.*(1991) of 32 mycelial compatibility groups among isolates of *S. sclerotiorum* isolates from two small fields of canola (*Brassica napus* L.) provides evidence of the potential for great genetic heterogeneity within a species in a relatively small area. R.F.L.P. analyses of these and further isolates from across Canada showed that the mycelial compatibility groups had a genetic basis that could be demonstrated through 'DNA fingerprinting' (Kohli, Morral, Anderson and Kohn, 1992).

1.3 Control of Sclerotinia Diseases in Sunflower.

1.3.1 Cultural Methods

The longevity of soil-borne sclerotia and the small inoculum densities required to initiate severe disease are the greatest difficulties in the control of sclerotinia diseases through cultural practices. Sclerotia of *Sclerotinia* species are adapted for survival in the soil. The melanized sclerotial rind allows water and water soluble nutrients into the sclerotium while preventing the penetration of hyperparasites (Trevethick and Cooke, 1973; Merriman, 1976). A small proportion of sclerotia of *S. minor* and *S. sclerotiorum* may survive in soil for several years (Adams, 1975; Adams and Ayers, 1979). The presence of sclerotia of *S. sclerotiorum* at an average density of 0.03 per 1.55 kg of dried field soil was associated with incidences of sunflower wilt of between 12 and 79% (Huang, 1980; Holley and Nelson, 1986; Chun and Maric, 1989). Inoculum densities must therefore be reduced to very low levels to control sclerotinia wilt. Cultural practices such as crop exclusion, time of sowing, plant population density and crop nutrition may provide means of avoiding, escaping or lessening the likelihood that the diseases will occur. Other methods such as cultivation, soil solarization, soil amendments and soil flooding are potential means of actively reducing the level of inoculum in soil.

i Sunflower Exclusion

Farmers have the potential of growing crops other than sunflower. Crops such as sorghum, maize, cotton, and millet are examples of alternative summer crops that are not susceptible to sclerotinia diseases which may be grown in place of sunflower. Sunflower has however established a place in Australian agriculture because of its broad adaptability and ease of culture. Its withdrawal would reduce the cropping options available to farmers and could lead to heavier imports of edible oils.

ii Time of Sowing

Sowing times can sometimes be manipulated to avoid coincident periods of maximum host susceptibility with an environment that favours growth of the pathogen and disease development. The incidence of wilt caused by either *S. sclerotiorum* or *S. minor* is greatest after budding (Growth Stage R2, Schneiter and Miller, 1981). Clarke, Porter and Woodroofe(1993) found that in Victoria the incidence of wilt caused by *S. minor* was lower in sunflower crops sown during October (spring) than those sown during November and December (early-summer). Increased rates of shoot and root growth in the later sown crops were attributed with increasing the probability of infection. Cooler soil temperatures when the later sown crops reached the growth stages most prone to the disease may have

also favoured sclerotial germination and infection by *S. minor*. Chun and Maric (1989) found that earlier sown crops in Yugoslavia had a higher incidence of wilt caused by *S. sclerotiorum*. This was attributed to the later sown crops maturing under drier conditions which did not favour disease development.

A knowledge of historical weather data, host characteristics, and epidemiological information about a pathogen can allow the construction of models that can be used to predict the probability of a disease occurring. CETIOM (1981) published an agrometeorological approach to forecasting outbreaks of sclerotinia head rot of sunflower in France. The model was based on the critical factor that ascospores require a relative humidity greater than 95% for at least 40h to establish infections of sunflower heads. From meteorological records it was possible to determine the frequency that these conditions occurred during the growing seasons in the different areas of France. As well, the barometric pressure maps that preceded and coincided with the periods of high humidity were also studied. The model could be used to warn farmers when conditions conducive to infection were imminent. A simple forecasting system was used by Slatter (1992) to recommend the latest dates for sowing certain sunflower hybrids in areas of Australia where sclerotinia head rot can be a problem. Sowing of late maturing hybrids (based on days from sowing to anthesis) should be completed at earlier dates than quick maturing hybrids.

iii Crop Population Density

The incidence of sclerotinia wilt in crops can increase through spread of the fungus across root contacts between neighbouring plants (Huang and Hoes, 1980). It may be possible therefore to decrease incidence of the disease by establishing a plant population where inter-plant spread does not occur but yield potential is not compromised by the presence of too few plants. Hoes and Huang(1985) recommended that plants be spaced at least 36cm apart. Subsequent work, however, has not demonstrated consistent relationships between plant populations and incidence of wilt even at plant spacings greater than 36 cm (Chun and Maric, 1989; Nelson, Hertsgaard and Holley, 1989). The conflicting results may reflect a non-uniform distribution of high densities of sclerotia in the test sites resulting in infection of large numbers of individual plants. The differences could also have been compounded by the later authors using hybrid cultivars which may have a more extensive root system than the open-pollinated cultivar Krasnodarets used by Huang and Hoes (1980) and Hoes and Huang (1985).

Sclerotinia wilt in Australia is most frequent in the irrigation areas of northern Victoria. The soil in these areas is quite shallow and consequently to prevent lodging sunflower crops are grown at populations of 80000-100000 ha^{-1} so that head size and weight is

reduced and neighbouring plants can physically support each other. Humidity levels at soil level in such crops can be sufficient to allow mycelium of *S. minor* to grow over the soil surface between plants. Hybrids of short stature that do not lodge easily may allow lower plant populations to be grown with greater drying of the soil surface. This may lead to a reduced incidence of wilt.

Lower plant populations which allow greater air movement between plants may enhance drying of the soil surface and foliage. This may reduce formation of apothecia and subsequent middle stem and head infections. The incidence of head rot was 72% higher in sunflowers grown at a row spacing of 50cm compared to those grown at a spacing of 80cm. This was attributed to soil drying at the wider spacing inhibiting formation of apothecia of *S. sclerotiorum* (Peres, Regnault and Allard, 1989).

iv Crop Nutrition

Nutrition can influence the reaction of plants to diseases (Huber, 1989). Applications of higher levels of nitrogenous fertilisers were found to increase the susceptibility of artificially inoculated sunflower (Heber, Bazzalo and Stamppacchio, 1985) and naturally infected plants (Chun and Maric, 1989) to sclerotinia wilt. Similarly, the incidence of head rot increased with increased applications of nitrogen (Peres *et al.*, 1989). Calcium levels were found to be higher in sunflower cultivars that were less susceptible to head rots caused by *S. sclerotiorum* and *Botrytis cinerea* (Antonova, Zaichhuk and Kalinchenko, 1984). Application of calcium carbonate to an acidic soil in pots alleviated aluminium toxicity symptoms and decreased the susceptibility of sunflower to *Sclerotinia sclerotiorum* wilt (Orellana, Foy and Fleming, 1975).

Application of zinc and copper salts as foliar sprays reduced the incidence of sclerotinia blight of peanuts caused by *S. minor* (Hallock and Porter, 1981). Potassium, calcium, magnesium, phosphorous, manganese, iron, sulphur and chloride did not suppress blight. Similarly, the application of ammonium bicarbonate failed to reduce the growth of *S. minor in vitro* or suppress the incidence of blight in field plots (Brenneman, Csinos and Phipps, 1990). The compound did however inhibit the production of sclerotia by *Sclerotium rolfsii*. The latter fungus is known to be sensitive to ammonia (Punja and Grogan, 1982).

v Flooding

Moore(1949) showed that continual or cyclic flooding for 45 days resulted in complete decay of sclerotia of *S. sclerotiorum* Stoner and Moore (1953) went on to recommend the growing of flooded rice as a cultural control for *S. sclerotiorum* in Florida. Sclerotia of *S. minor* maintained at 0 matric water potential were not viable after 56 days (Abawi, Grogan

and Duniway, 1985). Heritage (1988) suggested that flooding might be used to eradicate *S. minor* from the irrigated sunflower fields of Victoria. Porter *et al.* (1989) subsequently found that flooding for 6 weeks reduced the number of viable sclerotia of *S. minor* by 50% but the viability of sclerotia in the check banks or floating on the water was not reduced.

vi Thermal Inactivation

Sclerotia have a high tolerance to heating but can be killed if temperatures are high enough. Sclerotia of *S. sclerotiorum* were killed by exposure to dry heat of 120°C for 20 minutes (Dueck, 1979). Gilbert (1991) described the destruction of sclerotia of *S. sclerotiorum* in the burning of lucerne (alfalfa) stubble. The sclerotia were mostly on the soil surface and in the stubble and were therefore exposed to maximum heating. It is unlikely that sunflowers produce enough biomass to support a fire of sufficient intensity to destroy sclerotia particularly those produced on the roots.

Soil solarization is one means of increasing the temperatures of soil to a level lethal to soil-borne pests. Phillips(1990) reported that less than 15% of sclerotia of *S. sclerotiorum* in the upper 5cm of soil survived solarization. The loss of sclerotial viability was attributed to the combined action of thermal damage and the antagonistic activity of thermotolerant species such as *Aspergillus terreus* Thom. The incidence of sunflower wilt in treated plots was reduced. Solarization is presently considered to be too expensive for 'broad acre' control of *Sclerotinia* spp.

vii Soil Amendments

The addition of suitable organic composts or the incorporation of green manure crops can be used to suppress plant pathogens. Suppression may result from the increased activity of antagonistic microorganisms or the release of toxic gases such as ammonia as the amendment decomposes. In one study, Lumsden, Millner and Lewis (1986) reported that the addition of 7-10t.ha^{-1} of composted sewage sludge suppressed the infection of lettuce by *S. minor*. Sclerotial survival was unaffected but growth of mycelia was inhibited. Sun and Huang (1985) described the use of S-H Mixture ^{TM} (bagasse, rice husks, oyster shell powder, urea, potassium nitrate, calcium superphosphate and mineral ash) as an amendment for the control of various soil-borne pathogens . The mixture was also found to reduce the survival and germination of sclerotia of *S. sclerotiorum in vitro* (Huang and Sun, 1991). Unfortunately, application of S-H Mixture was also found to reduce the soil populations of *Coniothyrium minitans* Campbell which is a known mycoparasite of *Sclerotinia* spp (Huang, 1992).

Future research may focus on the use of special green manure crops. Singh, Ram and Tewari (1990) reported that chickpea (*Cicer arietinum* L.) mulched with small amounts of

leaf of bael (*Aegle marmelos* (L.)Co rr.) was less severely affected by *S. sclerotiorum* than untreated plants. The mechanism involved was not discussed but it is possible that fungitoxic essential oils (Singh, Bhatt and Sthapak, 1983; Pandey, Chandra, Tripathi and Dixit, 1983) released from the bael provided systemic protection against the fungus when absorbed by the chickpea plants. Other plants have also been shown to contain natural compounds that are fungitoxic to *S. sclerotiorum* at concentrations comparable to efficacious fungicides such as carbendazim (Singh, Pathak, Khare and Singh, 1979; Yegen, Berger and Heitefuss, 1992). Intercropping sunflower with plant species resistant or antagonistic to *Sclerotinia* spp should be trialed as a means of reducing the incidence of sclerotinia wilt. Experimental evidence revealed that sunflower yields were not depressed when intercropped with lentil (*Lens esculenta* L.)(Kandel and Schneiter, 1993). *Lens* and other members of the leguminous tribe Vicieae are known to exude β-(Isoxazolin-5-on-2-yl)-alanine from their roots. This non-protein amino acid has broad antifungal activity (Schenk, Lambein and Werner, 1991).

Green manure crops must be chosen carefully. The stubble of crops that are not normally hosts to *Sclerotinia* may be colonised by the fungus and new sclerotia formed (Dillard and Grogan, 1985).

vii Cultivation

The incidence of sclerotinia diseases can be influenced by the position of sclerotia in the soil. Cultivation of infected crops to bury sclerotia of *S. minor* and *S. sclerotiorum* has been attributed with reducing the incidence of lettuce drop and of peanut blight (Adams and Tate, 1975; Baard and Los, 1989) by preventing infection of senescing plant tissue by sclerotia present in debris on the soil surface or near the soil surface. Deep burial of sclerotia prevents these contacts. Apothecia of *S. sclerotiorum* usually develop from sclerotia in the top 4cm of soil (Anas and Reeleder, 1985) and deeper burial prevents stipes from reaching the soil surface. Head and middle stem rots of sunflower will not develop if ascospores are absent. Cultivation may not be effective against sclerotinia wilt of sunflower because infections are primarily of the roots 5-10cm deep (Huang and Hoes, 1980).

Burial can also influence the survival of sclerotia. Imolehin and Grogan (1980*b*) found that the formation and subsequent survival of sclerotia of *S. minor* on naturally infected lettuce crowns decreased with increased depth of burial. No sclerotia were recovered after 3 months at depths equal or greater than 10cm. It must be noted that these results were derived from the use of infected plant material that might also act as a food base for mycoparasites. The survival potential of isolated sclerotia at depth is still uncertain.

1.3.2 Chemical Control

Many chemicals show activity against *Sclerotinia*. These include a number of fungicides as well as a few compounds registered for use as herbicides. Gyula Oros (Plant Protection Institute, Budapest; *pers. comm.*) tested 37 fungicides in vitro for toxicity to a range of sunflower pathogens. Sixteen compounds had ED_{50} of <50mg.L-1 against *Sclerotinia sclerotiorum*. Active compounds included the benzimidazoles (eg benomyl, thiabendazole), triazoles (eg triadimephon), and dicarboximides (eg procymidone, vinclozolin, iprodione). A number of experimental fungicides have been identified which show increased fungistatic and fungitoxic activity against *S. minor* in *in vitro* and field evaluations (Smith, Phipps and Stipes, 1991). Chemicals can be used in several ways in the control of sclerotinia diseases.

i Seed Treatments

Sclerotinia sclerotiorum may be introduced into new areas as sclerotial contamination of planting seed. Sackston (1960) found mycelia of *S. sclerotiorum* in seed that was apparently healthy. Enzyme-linked immunosorbent assay (ELISA) methods can be used to detect the presence of *S. sclerotiorum* within sunflower tissue (Walcz, Pasca, Emödy and Szabo, 1985). If detected then chemical applications of benomyl, vinclozolin, iprodione and procymidione applied to sunflower seed as acetone infusions can be used to control *Sclerotinia* infections (Herd and Phillips, 1988). At present, only thiabendazole is registered in Australia for application as an aqueous slurry to control *Sclerotinia* in sunflower seed lots (Beavis, Simpson, Syme and Ryan, 1991).

ii Soil Fumigants

These are designed to kill sclerotia present in the soil. 'Fumigation' (fumigant + irrigation) with metham-sodium can significantly reduce the viability of sclerotia of *S. minor* and *S. sclerotiorum* in soil (Krikun and Frank, 1981; Ben-Yephet, Bitton and Greenberger, 1986) but is considered too expensive for use with low value agricultural crops such as sunflower (Heritage, 1988). Dazomet is another broad spectrum fumigant active against *Sclerotinia* spp (Beavis *et al.*, 1991). This treatment, however, is even more expensive than metham-sodium (Heritage, 1988). It would require several disease-free sunflower crops to recover the expense of fumigation and since fumigation may not kill all sclerotia in or below the treated zone it is likely that disease levels would quickly increase if susceptible crops are grown. A high mortality of natural antagonists caused by fumigation may also allow the pathogen to dominate the soil micro-flora. A positive feature of fumigation is that other soil-borne pathogens may also be controlled.

iii Soil Sprays

Soil sprays can be applied in-furrow at sowing, as side-bands during cultivation or as soil drenches. Soil sprays have been examined as means of controlling sclerotinia diseases of peanuts (*Arachis hypogaea* L.) (Porter, 1980; Dougherty, Sarojak and Locher, 1983) and onions (Porter, 1989). Preliminary evaluation of the efficacy of soil sprays to control sunflower wilt caused by *S. minor* has been conducted in Victoria (Porter *et al.*, 1989). Disease development was delayed but final mortality of plants was still greater than 50%.

There are a number of potential problems with the use of soil sprays. Applications of compounds at sub-lethal doses have been shown to increase the longevity of sclerotia in soil (Davet and Martin, 1985). Several examples of *in vitro* tolerance to fungicides have been reported during since Steadman (1979) noted that resistance to fungicides had not been reported in *Sclerotinia* spp. Porter and Phipps (1985) identified isolates of *S. minor* tolerant to procymidone. Brenneman, Phipps and Stipes(1987) identified further isolates of *S. minor* that had increased tolerance to two other dicarboximides, iprodione and vinclozolin. These isolates also possessed cross-resistance to the aromatic hydrocarbon fungicides dichloran and pentachloronitrobenzene.

Fungicides may also be subject to non-target microbial degradation in the soil. The failure of iprodione to control lettuce drop after repeated applications was attributed to increased microbial degradation of the compound (Martin, Vega, Bastide and Davet, 1990). A second compound may need to be applied to protect the compound active against *Sclerotinia* from degradation. Yoder, Ben-Yephet, Katan and Aharonson (1986) applied thiram in combination with benomyl to delay degradation of the latter compound.

iv Capitula Sprays

Infection of the capitula by ascospores of *S. sclerotiorum* occurs via the florets during the early stages of anthesis (Lamarque, Leconte, Berrier and Jaunet, 1985). It would seem feasible therefore to protect against infection by providing fungicidal protection during this brief 'window'. Unfortunately, the orientation of the floral face is vertical and eastward-facing. Fungicide penetration between the florets is difficult if wind conditions are unfavourable, for example, from the west. Also, a food base for the fungus is supplied by the many ancillary floral parts that senesce after pollination. These tissues will not translocate chemicals and cannot therefore be protected by systemic fungicides. Compounds such as fenpropimorphe which exhibit significant systemic activity in sunflower leaves may not show the same activity in capitula (Pierre and Regnault, 1989). Some success with aerial applications of fungicides against head rot have been reported.

Iliescu, Csep and Craiciu (1983) in Romania applied two sprays of benomyl, vinclozolin or iprodione, one at anthesis and the second 10-15 days later. The incidence of head rot was reduced from 56% in unsprayed plots to less than 7% in sprayed plots. Wu (1981) reported that spraying experimental plots of sunflower every 10 days with vinclozolin significantly reduced the incidence of middle stem and head rots.

v Herbicides

Herbicides can have several effects on plant diseases either by directly affecting the pathogen or by altering the reaction of the host (Altman, 1983). Alternatively, herbicides may have adverse effects on microbial antagonists of the pathogen (Adams and Wong, 1991). A number of herbicides that affect *Sclerotinia* have been identified. Cerkauskas, Verma and McKenzie (1986) examined the interaction of *S. sclerotiorum* and 21 herbicides *in vitro*. Many decreased mycelial growth, some increased the weight of sclerotia produced in culture but none affected carpogenic germination. Herbicides that have been identified as being fungitoxic include the triazines atrazine and simazine. Abnormal apothecia and unviable ascospores are produced from sclerotia incubated in the presence of these compounds (Casale and Hart, 986, Radke and Grau, 1986). Growth of mycelium is inhibited by another herbicide 4-(2,4-dichlorophenoxy) butyric acid (2,4-DB) (Porter and Rud, 1980). All these herbicides are designed for the control of broadleaf weeds and consequently are toxic and cannot be used in conjunction with sunflower. The herbicide most commonly used with sunflower in Australia at present is trifluralin which has been found to either stimulate or inhibit production of apothecia (Radke and Grau, 1986; Teo, Verma and Morral, 1992). Manipulation of herbicide usage may have a role in the integrated control of sclerotinia diseases.

1.3.3 Biological Control

The use of antagonists and mycoparasites to control sclerotial diseases has attracted considerable interest (Ghaffer, 1988; Adams, 1990). In the present review an antagonist is defined as a microorganism that can adversely affect the growth or survival of *Sclerotinia* through competition for nutrients and/or production and release of toxic metabolites. Mycoparasites(hyperparasites) feed directly on the hyphae and sclerotia. Sclerotia are penetrated via openings in the rind caused by wounding or areas where melanization is incomplete (Huang, 1985).

A diverse range of organisms that affect *Sclerotinia* have been reported in the literature (Table 1.2). It must be acknowledged that the antagonism or mycoparasitism exhibited by many test organisms has only been observed *in vitro*. Whether or not this activity extends

to field conditions is unknown.

Early experiments on the biocontrol of sclerotinia diseases of sunflower concentrated on the use of *Coniothyrium minitans*. This fungus has been considered a 'passive' mycoparasite because it will parasitise any sclerotia and hyphae it contacts but will not actively seek new sclerotial substrates (Adams, 1990). Huang(1977), for example, found that while sclerotia on the surface of sunflower stems were heavily colonised by *C. minitans* sclerotia within the stem escaped parasitism. The 'passive' nature of this mycoparasite means that high levels of inoculum must be applied to soil if sclerotia are to successively contacted and destroyed. Huang(1980) attempted to control sclerotinia wilt of sunflower by applying inoculum with seed at sowing at a rate equivalent to $1.8t.ha^{-1}$. The incidence of wilt was reduced from 43% to 24%. In another study, Huang and Kozub (1991) found that the incidence of sunflower wilt decreased after several years of sunflower monoculture. They attributed this decline to an increase in the *C. minitans* population in the soil. More recently, isolates of *Pseudomonas cepacia* have been shown to be capable of improving the emergence from sunflower seed sown in the presence of *S. sclerotiorum* (McLoughlin, Quinn, Bettermann and Bookland, 1992).

Adams (1990) suggested that the use of 'aggressive' mycoparasites might allow the application of smaller amounts of inoculum. *Sporidesmium sclerotivorum* was his example of an 'aggressive' mycoparasite that sends hyphae through the soil in search of the other sclerotia. A survey of soils in the United States considered to be 'suppressive' to development of sclerotinia wilt of sunflower revealed that *Sporidesmium sclerotivorum* was present in most (Gulya, Adams and Rashid, 1992). *S. sclerotivorum* was also attributed with reducing the severity of disease in fields mono-cropped to susceptible hosts over a number of years.

The use of antagonists has been proposed as a means of suppressing infections by ascospores of *Sclerotinia sclerotiorum*. Concentrated suspensions (10^6-10^7 conidia.ml^{-1}) of a number of fungi that are common inhabitants of the phylloplane have been used to reduce infections from ascospores on lettuce and beans (Mercier and Reeleder, 1987; Boland and Inglis, 1989; Zhou and Reeleder, 1989). Applications of fungal antagonists may be a feasible means of suppressing head rot of sunflower. Active growth of the fungi among the florets may provide protection superior to that of chemicals.

The discovery of an association between the presence of virus-like particles composed of double-stranded ribonucleic acids (dsRNA) and hypovirulence of *S. sclerotiorum* by Boland (1992) provides a new approach for biocontrol. Hypovirulent strains of pathogens able to transmit the causal 'mycovirus' through anastomoses can be increased and used to inoculate fields. Unfortunately, this approach may not be effective with *Sclerotinia*

because of the existence of mycelial incompatibility groups that preclude the formation of anastomoses (Kohn *et al.*, 1991).

Table 1.2. Recent records of antagonists, mycoparasites, mycoviruses and predators of *Sclerotinia* species.

Antagonists	Authors
Acremonium implicatum(Gilman & Abbott)W. Gams	Singh(1991)
Alternaria alternata (Fr.)Keiessler	Boland and Hunter(1988)
Aspergillus niger Van Tieghem	Singh(1991)
Cladosporium cladosporioides (Fres.)de Vries	Boland and Hunter(1988)
Drechslera spp.	Boland and Inglis (1989)
Epicoccum purpurascens Ehrenheb.	Mercier and Reeleder(1987)
Erwinia herbicola	Tenning, van Rijsbergen, Zhao and Joos(1993)
Fusarium graminearum Schwabe	Boland and Inglis(1989)
Fusarium heterosporum Nees(Fr.)	Boland and Inglis(1989)
Fusarium aquaeductuum(Radlk.& Rabenh.) Lagerh.	Lüth, Pfeffer and Schulz(1992)
Fusarium polyphialidicum Marasas, Nelson. Toussoun & Van Wyk	Phillips(1989)
Gliocladium roseum Bain.	Phillips(1989)
Gliocladium virens Miller & Foste	Phillips(1989)
Pseudomonas cepacia Palleroni & Holmes 1981	McLoughlin, Quinn, Betterman and Brookland (1992)
Mycoparasites[1]	
Coniothyrium minitans Campbell	Huang and Kokko(1987), Phillips(1989), Lüth *et al.* (1992)
Dictyosporium elegans Corda	McCredie and Sivasithamparam(1985)
Fusarium solani (Mart.)Sacc.	Zazzerini and Tosi(1985)
Fusarium oxysporum Schlect.	Zazzerini and Tosi(1985)
Gliocladium catenulatum Gilman & Abbott	Zazzerini and Tosi(1985)
Gliocladium roseum	Zazzerini and Tosi(1985), Phillips(1989)
Gliocladium virens	Mueller *et al.* (1985), Phillips(1989)
Penicillium griseofulvum Dierchx.	Zazzerini and Tosi(1985)
Penicillium citrinum Thom	Akem and Melouk(1987)
Penicillium verrucosum(Westling)Samson *et al.*	Zazzerini and Tosi(1985)
Phoma nebulosa (Pers.:Fr.)Berk.	Whipps, Budge and Mitchell(1993)
Sporidesmium sclerotivorum Uecker, Ayers and Adams	Adams(1987, 1989)
Talaromyces flavus (Klocker)Stolk & Samson	McLaren, Huang and Rimmer (1986)
Talaromyces wartmanii (Klocker)Stolk & Samson	Zazzerini and Tosi(1985)
Terarosperma oligocladum Ueckers, Ayers and Adams	McLaren, Huang and Rimmer (1986)
Trichoderma hamatum (Bonord.)Bain	Lüth *et al.* (1992)
Trichoderma harzianum Rifai	Zazzerini and Tosi(1985), Phillips(1989), Singh (1991) , Knudsen, Eschen, Dandurand and Bin(1991)
Trichoderma viridae Pers. ex Fr.	Zazzerini and Tosi(1985)
Mycoviruses	
double stranded RNA associated with hypovirulence	Boland(1992)
Predators	
Bradysia sp. (dark-winged fungus gnat)	Anas and Reeleder(1987)

1. Presumed from the ability to reduce the viability of inoculated sclerotia. Some may also be antagonistic.

1.3.4 Breeding for Host Resistance

Quantitative differences in the susceptibility of sunflower lines to sclerotinia wilt have been recognised for more than 30 years (Putt, 1958). About the same time, it was reported that certain lines of *Helianthus tuberosus* had been found that were resistant to *Sclerotinia minor* and *S. sclerotiorum* (Gaudineau and Lafon, 1958). The introgression of resistance into cultivated sunflower through interspecific crosses proved difficult. The infrequent occurrence of sclerotinia diseases in most sunflower growing areas and the more immediate problems of sunflower rust and downy mildew meant that breeding for resistance to *Sclerotinia* spp was given low priority. This situation may have also been one of convenience since dominant genes for resistance to rust (Putt and Sackston, 1963) and downy mildew (Zimmer and Kinman, 1972) were readily identified and utilised.

The increasing popularity of sunflower as an oilseed crop around the world has been associated with increasing damage caused by *Sclerotinia sclerotiorum*. Pessimism was initially expressed about the possibility of finding and incorporating useful levels of resistance to sclerotinia diseases into sunflower. This pessimism was probably founded on the inconsistent experimental results obtained when screening sunflower lines for resistance. For example, the line CM392 has been described as, i) being highly susceptible, ii) susceptible with the possibility that selections for greater resistance could be made and iii) resistant to wilt caused by *Sclerotinia sclerotiorum* (Kolte, Singh and Tewari, 1976; Huang, 1981 ; Fick, Gulya and Auwater, 1983). From an examination of the literature, it would seem that many of the sunflower lines tested may have been genetically heterogeneous and this was a possible source of experimental variation. This could be true of the United States Department of Agriculture (U.S.D.A.) and Agriculture Canada inbred lines which were released at early stages of inbreeding so that further selections could be made at breeding stations receiving the seed. The sunflower lines CM497 and CM526 which were released as potential sources of resistance to sclerotinia wilt were described in the release notice as being heterogeneous for many traits (Huang and Dedio, 1982). The selections made and tested at one locality therefore may have been very different from those tested at another locality.

In a recent review of breeding sunflower for resistance to a number of diseases Sackston (1992*b*) stated that "*it does not seem reasonable to expect to find resistance within one of its host species, or even a genus, to a fungus with such a wide host range*" and thereby reinforced the idea that breeding for resistance to sclerotinia diseases in sunflower may not be possible. Certainly, complete resistance to any of the sclerotinia diseases of sunflower has not been reported. However, an understanding of the genetics of the quantitative

resistance to the diseases has been slowly increasing. In general, little relationship has been found between genetic control of resistance to wilt and genetic control of resistance to head rot (Vear and Tourvieille de Labrouhe, 1984; Skoric, 1988; Tourvieille de Labrouhe and Vear, 1990). Similarly, lines with good resistance to middle stem rot may be highly susceptible to head rot (Cuk, 1980). Inheritance of resistance to wilt, middle stem rot and head rot must therefore be examined independently.

Resistance to sclerotinia wilt caused by *S. sclerotiorum* varies among genotypes of sunflower. The line HA61 was considered to exhibit partial resistance that was transferable to F_1 progeny in some crosses (Dueck and Campbell, 1978). In a more thorough study, Fick and Gulya (1980) evaluated the incidence of wilt in 20 hybrids derived from 11 female parents with 6 male parents. The regression coefficient (b=0.31) calculated from the female parent-offspring regression suggested that resistance was heritable but only at a low level. The performance (disease incidence) of some hybrid combinations was better than expected from the performance of the parental lines. Large Specific Combining Ability (SCA) effects occurred compared to General Combining Ability (GCA) effects. Significant SCA effects for resistance to sclerotinia wilt were also detected in the breeding lines and hybrids evaluated in other studies (Fick, Gulya and Auwater, 1983; Vranceanu, Pirvu, Stoenescu and Iliescu, 1984; Pirvu, Vranceanu and Stoenescu, 1985). In contrast, Tourvieille de Labrouhe and Vear(1990) examined 36 hybrids derived from six female and six male parents and concluded that GCA effects were greater than SCA effects. Furthermore, the regression coefficient (R=0.58) for the regression between parental lines and hybrids indicated a relatively high level of heritability for the germplasm examined. In general, inheritance of resistance is considered complex and under the control of several genes. Additive gene effects are considered more important than dominance and epistatic gene effects (Fick *et al.*, 1983; Pirvu *et al.*, 1985).

Few studies have been made into the inheritance of resistance to leaf infection and middle stem rot. Cuk(1976) suggested that the line HA61 was immune and that resistance was dominantly inherited in F_1 hybrids. In a later study, SCA effects for resistance were demonstrated in a comparison of 6 F_1 hybrids based on a resistant female (cms-9) and 6 different restorers. Susceptibility to leaf infection is related to the presence of sugary exudations from extrafloral nectaries on the leaf margins and petioles which provide a nutrient source for ascospores to initiate infection (Sedun and Brown, 1987). Occurrence of extrafloral nectaries differs between species of *Helianthus* (Rogers, 1985). It is likely that differences also exist between cultivated lines of sunflower and these differences are related to the variations observed in susceptibility to leaf infection by *Sclerotinia sclerotiorum*. Pirvu *et al.* (1985) reported that the resistance of two lines, CS-77-999-1 and

CS-77-1081, to infection of the stem was controlled by a single recessive gene s_1. This resistance is conferred by formation of a thicker cuticle preventing penetration of the fungus (Dr. Florin Stoenescu, *pers. comm.*).

The inheritance of resistance to head rot is also generally considered to be under polygenic control with additive effects more important than dominance (Tourvieille de Labrouhe and Vear, 1984; Robert, Vear and Tourvieille de Labrouhe, 1987; Vear and Tourvieille de Labrouhe, 1988*a*, Dedio, 1992). Inheritance studies have been conducted using two artificial inoculation techniques or natural infection. Robert *et al.* (1987) applied mycelial inoculum of *S. sclerotiorum* to the dorsal surface of sunflower heads and measured the rate at which the subsequent lesions developed. Thirty five hybrids derived from 6 female lines crossed by 6 male lines were compared to the parental lines. Strong correlations were found between the hybrid and mid-parent reactions indicating the additive nature of gene action. Vear and Tourvieille de Labrouhe (1988*a*) inoculated the same set of lines by applying ascospores to the floral face and also found that additive effects were more important than dominance. However, some hybrids showed significant SCA effects. Dedio (1992) assessed disease incidence in naturally infected trials and also found that resistance to head rot appeared to be conditioned by additive genes.

No associations between reaction of sunflower genotypes to sclerotinia diseases and other phenotypic traits have been confirmed. Fick and Gulya (1980) found that the relative susceptibility to sclerotinia wilt was not closely correlated with either time to anthesis, plant height, reaction to downy mildew or reaction to Verticillium wilt. Studies found that there was no absolute correlation between reaction to sclerotinia head rot of genotypes producing grain differing in oil content (Vear and Tourvieille de Labrouhe, 1988). Nectar and pollen production did not seem to be significantly correlated with reaction to head rot (Vear, Pham-Delegue, Tourvieille de Labrouhe, Marilleau, Loublier, le Métayer, Douault and Philippon, 1990). Reduced plant height was suggested as being closely related to higher incidence of head rot by Leclerq (1973). However, Dedio(1992) found that correlations between plant height and disease were not consistent between experiments for the same genotypes. Skoric (1992) suggested that a head inclination of 45^{o} was less favourable for development of head rots caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum* when compared to those with a horizontal orientation. The lack of close associations means that selection for independent traits is possible without concern for associations that would either aid or impede breeding.

1.4 Experimental Objectives

Sclerotinia diseases are a continuing and increasing problem to sunflower production in many areas of the world. In Australia, head or capitulum rot caused by *Sclerotinia sclerotiorum* occurs throughout the northern New South Wales and Queensland production areas. Sunflower wilt caused by *Sclerotinia minor* occurs frequently in the northern Victorian irrigation areas and occasionally in crops in northern New South Wales (Figure 1.1). Wilt caused by *S. minor* has recently been reported from California (Gulya, Woods, Bell and Mancl, 1991). Yield losses of over 50% can occur with sclerotinia wilt diseases (Dorrell and Huang, 1978; Clarke, 1982). These diseases must be controlled before they threaten the viability of the sunflower industry in Australia. This is especially true of *S. minor* which has been a contributing factor in the reduction of the area sown to sunflower in Victoria. Effective control of the disease may allow the return of sunflower culture to this high yielding area.

Any control methods used must be suitable for a broad-acre crop that returns relatively low economic margins. Present control options have severe disadvantages;

- chemical control- effective compounds are known but their use is uneconomical.
- biological control- a number of organisms have demonstrated efficacy in pot and small plot trials but are of questionable value in reducing sclerotial numbers over large areas.
- cultural control- soil flooding and soil-solarization may not be effectively applied to large areas.
- genetic control- variation among sunflower genotypes for susceptibility to wilt exists but immunity is not known.

Hybrids with enhanced levels of resistance must be an integral part of any control strategy. The studies reported in this thesis therefore focused on developing an understanding of how the resistance of sunflower to sclerotinia wilt caused by *S. minor* could be improved. *Sclerotinia minor* was chosen for study because it is important in Australia and poses an international threat to sunflower culture and a number of studies have shown that this species is a more aggressive pathogen on common hosts than *S. sclerotiorum* (Held and Haenseler, 1953; Phipps and Porter, 1982; Sedun and Brown, 1989).

The objectives of the research were to:

1. Review the methods used to screen sunflower for resistance to sclerotinia wilt and, if necessary, develop a new method applicable to the selection of sunflower germplasm resistant to *Sclerotinia minor*.
2. Try and correlate the results of the glasshouse inoculation method and any other screening methods of interest with results from field sites infested with *S. minor*.

3. Use the screening procedure in a recurrent phenotypic selection plant breeding method to demonstrate its utility in the development of sunflower germplasm with enhanced resistance to *S. minor*.
4. Examine a number of sunflower lines differing in susceptibility to *S. minor* for characteristics (ideotypes) that might contribute towards resistance to *S. minor*. Firstly, test for correlation between root characteristics and reaction of sunflower to *S. minor*.
5. Examine the effect of calcium nutrition on reaction of sunflower to *S. minor* and whether there is a correlation between calcium content of a range of genotypes and susceptibility.
6. Investigate whether correlations exist between susceptibility to damage by oxalate and disease susceptibility.
7. Investigate whether phenolic compounds with putative phytoalexin activity against *S. minor* exist in sunflower.

Figure 1.1. Decayed stem base of a sunflower plant bearing external sclerotia of *Sclerotinia minor*.

CHAPTER 2

GENERAL MATERIALS AND METHODS

A number of procedures and sunflower germplasm were used routinely in the experiments described in this thesis. These are described here to avoid repetition.

2.1 Description of Sunflower Lines Used.

Sixty-nine inbred sunflower lines, thirty-five sunflower hybrids and twenty-three wild sunflower accessions with diverse derivation were used in the following study. These lines have been encoded since many of these lines were provided by Pacific Seeds Pty Ltd (Toowoomba, Australia) and include lines produced by pedigree selection within public and proprietary germplasm (Appendix 1). In general, description of pedigrees follows the nomenclature used in the release notices or other publications. Pedigree symbolism consistent with System II as recommended by Fehr (1987). The terms adapted / unadapted are rather subjective and refer as to whether the line could be used 'as is' in the formulation of hybrids satisfying the commercial ideals of morphological uniformity and stature.

2.2 Potting Medium Used for Culture of Sunflower Plants

Unless otherwise described, sunflower seedlings were raised in a mixture of peat moss:vermiculite:sand (1:1:1 v/v/v). To 40L of this mixture was added 180g Dolomite, 160g Osmocote Plus™ slow release (3-4 month) fertiliser containing micronutients and 50g D.A.P. (diammonium phosphate). The final pH was normally around 6. Since the potting medium was loam free it was not sterilised or pasteurised.

2.3 Culture of Sunflower Plants and Assessment of Growth Stages.

Unless otherwise stated in the body of the thesis, sunflower plants were raised in 10cm diameter plastic pots. These were filled with the medium described above and sown by placing two sunflower seeds at a depth of 2cm in the centre of the pot. Pots were thinned to one plant as soon as it was obvious that two seedlings were emerging. Pots were watered

once daily until seedlings were at about the six true leaf stage (Growth Stage V6) when twice daily watering commenced.

Plant Growth Stages where described are based on the scheme of Schneiter and Miller (1981). This method uses the number of leaves longer than 4cm to describe the vegetative stages of growth and development of the floral bud to describe reproductive stages. Hence, the growth stage V12 describes a plant with 12 leaves longer than 4cm and no bud visible while the stages from R1 (bud visible) to R6 (post-anthesis seed fill) describe plants in the reproductive phase of growth.

2.4 Production of Inoculum of Sclerotinia minor.

Kreitlow (1951) first described the use of grain colonised by *Sclerotinia* as inoculum. Colonised seed of a pearl millet (*Pennisetum typhoides* (Burm.)(Staph. and C.E. Hubb)) hybrid provided by Pacific Seeds Pty Ltd (Toowoomba, Australia) was used as inoculum in all experiments described in the present study because the grain was uniform in size, relatively large for this species and spherical. This shape allowed colonised grain to be easily manipulated during inoculation.

Uniform inoculum was produced by placing 1kg pearl millet grain in 600mL distilled water, microwaving at 650W for 15 minutes to kill any viable grain, allowing this to cool, returning to initial weight by addition of distilled water and allowing to stand overnight in the refrigerator. The mixture was then stirred thoroughly and 125g aliquot's dispensed into 250 ml Erlenmeyer flasks which were sealed with non-absorbent cotton wool plugs and an additional aluminium foil cover placed over the cotton wool. Flasks were autoclaved once for 30min at 15 psi and 121°C. The flasks were shaken as they cooled to prevent clumping of the millet grains. Three discs (5mm diameter) taken from cultures of *Sclerotinia minor* growing on Potato Dextrose Agar were introduced into each flask after it had cooled. Flasks were incubated in darkness at 20°C. After an initial 48h undisturbed incubation the flasks were shaken once daily in an attempt to achieve even fungal colonisation of the grain. The inoculum was used after 10 days incubation when the grain was well colonised and before many sclerotial initials became visible.

2.5 Choice and Maintenance of an Isolate of Sclerotinia minor *for Use in Screening Experiments*

Six isolates of *Sclerotinia minor* were screened for pathogenicity on the sunflower inbred lines RHA801 and CM497 using the method of Sedun and Brown(1989). These isolates

were obtained from single sclerotia that were collected by the author from infected sunflower plants growing in Victoria or northern New South Wales or from cultures stored in the Botany Department, University of New England. One isolate designated as UNE#Sm3 which was originally isolated from a plant of the sunflower hybrid Dekalb Dk610 growing near Premer, New South Wales was chosen for further studies because it was the most pathogenic as determined by the number of inoculated plants of both inbred lines that were killed and the rapid rate of lesion extension.

S. minor UNE#3 was stored either at 4°C as air-dried sclerotia or at 15°C under water. Water storage consisted of taking 6mm diameter discs from the growing margin of the fungus on potato dextrose agar and placing these in sterile distilled water in McCartney bottles (Boesewinkel, 1976). Sclerotia were taken from 21 day old Potato Dextrose Agar cultures and allowed to air dry in the air flow of a laminar flow cabinet. The sclerotia were then placed in sterile McCartney bottles and refrigerated. Good viability was maintained over the 2.5 years of this study. As well, re-isolations were made from inoculated sunflower plants during the course of the study. Only re-isolations made from diseased plants of the resistant check RHA801 were used in the production of inoculum for subsequent inoculations.

2.5 Parameters Used to Assess Reaction of Sunflower Lines to Infection by Sclerotinia minor.

A number of symptoms are produced on sunflower plants whose roots are infected with *Sclerotinia minor*. Plants show various degrees of wilting that normally precedes the appearance of a lesion at the base of the stem. Plants may recover turgor overnight and only wilt during the middle of the day when demands for water are high. Permanent wilting occurs as the infection progresses.

Daily observations were made of plants for either 21 or 28 days after inoculation for the first appearance of a lesion at the base of the stem. The time from inoculation to appearance of a lesion was recorded as the assessment parameter 'Delay' in the later experiments. Length of lesions from soil surface to lesion margin were measured for ten days after the first observations or until the lesions were at least 100mm long. The rate of lesion extension was calculated as the slope of the linear regression of lesion length against time (days).

2.7 Calculation of Root Index

While developing the pot base inoculation method described in later experiments differences in the amount of root growth visible on the root ball were observed between the sunflower lines. These differences seemed to be consistent between different trials. Consequently a root index measurement was developed as an estimate of the proportion of the bottom surface area of the root ball occupied by roots. The bottom surface of the root ball of plants were photographed for further analysis. The area of the surface occupied by roots was estimated by digital imaging of photocopies of the photographs using a ΔT Area meter (Delta-T Devices, Burwell, Cambridge, England). The photocopies were 'touched-up' to darken the vermiculite grains evident. A range of photographs representing various root areas were chosen to provide the range expected. For expediency, the classes chosen were

0 no roots evident,
1 1-25% root ball bottom surface occupied by roots
2 26-50% "
3 51-75% "
4 >76% ".

Root index (RI) for each line was calculated as $RI = (\sum (nr))/T$ where n = number of plants in each class, r = root rating and T = total number of plants assessed.

Representative photographs were then used for comparative purposes to delimit the classes when rating roots during experiments

2.8 Experimental Designs and Statistical Analyses

Various forms of experimental designs and statistical analyses were used. Randomised complete block designs were used in the glasshouse and growth cabinet experiments. One way analyses of variance (ANOVA) were performed on the data since unequal replicate numbers are tolerated. Unequal replicate numbers were frequently generated when various proportions of inoculated plants developed symptoms. Symptomless plants (i.e. absence of visible lesion) were not treated as null effects as the possibility of disease escape could not be eliminated. Means were separated by least significant difference (L.S.D.) or by Duncan's Multiple Range Test. Bartlett's Test was used to test for homogeneity of data before the results of replicated experiments were combined for analysis. The computer statistical package MSTAT (Michigan State University) was used for many of these analyses. AGROBASE/4 (agronomix software inc., Manitoba, Canada) was used to design and analyse the modified augmented design field screenings.

The procedure used for comparing the number of plants killed in each treatment in the pot screening trials was as suggested by Mr Joel Sleigh (Biometrician) Zeneca Seeds, Jeallot's Hill, United Kingdom);

- The overall proportion (p) of plants killed by *S. minor* in the experiment was determined. This was used as an estimate of the probability that any individual plant from the test population would die
- The data is considered to be binomially distributed as each plant was assessed as either 'alive' or 'dead'
- Estimated treatment variance was p(1-p)/n where p is the overall proportion of the population that died and n is the number of plants in each treatment
- the test statistic $z = (p_i-p_j) / \sqrt{V(p_i-p_j)}$ where p_i and p_j are the proportions of plants killed for the two test lines under comparison and $V(p_i-p_j) = V(p_i) + V(p_j) = 2(p(1-p)/n)$
- the test statistic z was compared to tabular value for two tailed tests at the 5% significance level for determination of statistical significance
- By reversing the equations the number of plants considered significant could be estimated. That is, tabular value from normal distribution table x 2 x (p(1-p)/n).

A spreadsheet (Microsoft Works™) was designed for these analyses.