CHAPTER 3

A COMPARISON OF METHODS USED TO SCREEN SUNFLOWER FOR RESISTANCE TO SCLEROTINIA WILT.

3.1 Summary

A review of the literature revealed that many different methods have been used to screen sunflower for resistance to sclerotinia wilt caused by *Sclerotinia sclerotiorum*. These methods varied from conducting disease nurseries at locations where the soil was naturally or artificially infested to more indirect laboratory methods where sunflower tissue was exposed to the fungus *in vitro* or the roots of seedlings were immersed in culture filtrates. A review of these methods showed that sunflower lines can readily be divided into groups on the basis of susceptibility but no single screening method has gained general acceptance as being the most efficient and effective means of selecting for resistance to sclerotinia wilt. Moreover, there were no reports of sunflower lines shown in repeated studies to be immune to *Sclerotinia sclerotiorum*.

The screening method of Sedun and Brown (1989) was chosen to screen a number of sunflower lines for resistance to *Sclerotinia minor*. The germplasm screened included lines derived from interspecific crosses between *H. annuus* and wild annual species, lines inbred from Argentinian populations and European hybrids, and inbred lines from North America. The linear rate of lesion extension was used to assess varietal reaction. Fifty-six different sunflower lines were screened in total and, of these, only two, PacA1 and HA292, had mean rates of lesion extension less than the resistant check line RHA801 although for several others the rates were not significantly greater (P>0.05).

Another inoculation method was developed in an attempt to increase the amount of useful data that could be collected from each inoculation. The inoculum was placed in the base of the pot after careful removal of the root ball which was then replaced. This method produced less variable data than the method of Sedun and Brown (1989) for assessing the delay from inoculation and appearance of basal stem lesions. Grains of pearl millet colonised by the fungus were used as inoculum. The optimum temperature for disease development by *S. minor* was 20°C as determined by the greater number of plants killed, the faster rate of lesion extension and the shortest delay from inoculation and appearance of the basal stem lesions. Use of 5 to 1000 colonised grains did not consistently affect the

proportion of plants killed, the rate of lesion extension or the time taken to appearance of the basal stem lesions. Inoculum from actively growing fungal colonies gave the greatest amount of disease. Inoculum efficiency decreased as sclerotia formed.

The pot base inoculation method was then used to screen a further selection of sunflower germplasm for resistance to *S. minor*. The germplasm consisted of selections from the survivors of earlier screenings, samples from roadside wild sunflower populations and a number of commercial and experimental hybrids. Among the hybrids the F_1 hybrid from the cross PacA1/RHA801 had lower than average mortality, the longest delay from inoculation to appearance of the basal stem lesions and the slowest rate of lesion extension.

3.2 Introduction

The breeding of crops for resistance to any disease or pest requires an appropriate method of screening the available germplasm for individuals exhibiting superior levels of resistance. The method used must be efficient and effective. That is, a large number of plants must, if necessary, be screened with little possibility of escapes occurring and the screening method must also distinguish between plants possessing different levels of resistance.

Screening for qualitative resistance (*sensu* Robinson, 1976) is relatively easy. The host:pathogen interaction often produces a small number of distinct reactions depending on the degree of pathogen colonisation. For example, the reaction phenotypes of sunflower to infection by sunflower rust (*Puccinia helianthi* Schw.) can be divided into a small number of arbitrary classes ranging from no macroscopic evidence of infection to unhindered growth and sporulation of the fungus (Kochman and Goulter, 1986). Screening for quantitative resistance in contrast often produces symptoms that vary over a continuous range of reactions.

Plants can be evaluated for their resistance to disease either in the field or under controlled conditions. An advantage of field evaluations is that large numbers of individuals can be tested under the growing conditions when the disease is most prevalent. However, the value of field trials is reduced because only a limited number of generations are possible per year, they are costly to conduct and are subject to environmental variability that cannot be effectively controlled. The level and distribution of soil-borne inoculum may be low or uneven and development of disease may be influenced by the activity of interacting soil biota. All of these factors can affect the number of individuals that escape infection and result in the expression of 'pseudo-resistance'.

31

Screening under controlled conditions can be used to facilitate breeding for disease resistance. The greater control of inoculum application and environment provided in glasshouse and laboratory assays can be offset by lack of available space which limits the number of plants that can be screened. This is especially true when plants must be grown to maturity for accurate expression of reaction to disease. Moreover, methods for the artificial inoculation of plants with any given pathogen must be chosen carefully so that the host can express any defence mechanisms that it has.

Another approach to screening germ plasm is to use indirect methods where the host is exposed to some determinant of pathogenesis rather than the pathogen itself. Often this determinant is a toxin released by the pathogen that is responsible for development of disease symptoms. Alternatively, chemical analogues of the toxin that are available commercially can be used. Furusawa (1988) demonstrated that tobacco calli selected *in vitro* for resistance to the herbicide paraquat produced plants that were resistant also to the toxin cercosporin and the pathogenic fungus *Cercospora nicotianae* Ell. & Ev. that produced the toxin. Pollen of *Brassica napus* L. cultivars exposed to toxic extracts from *Alternaria brassicola* (Schw.)Wilts was used in pollination experiments to show that selection for resistance to the fungus had occurred (Hodgkin, 1990). These biochemical approaches provide a further degree of refinement and control over glasshouse screenings but the results of any test must still be shown to be relevant to the level of resistance shown under field conditions.

Many techniques for screening sunflower for resistance to wilt caused by *Sclerotinia sclerotiorum* have been published. The very fact that many methods have been used reflects the difficulty that exists in obtaining accurate information on the reaction of sunflower genotypes to this fungus. Since the reaction of sunflower genotypes to *S. minor* may be correlated to their reaction to *S. sclerotiorum* (Sedun and Brown, 1989) it was considered worthwhile to review the various methods and draw conclusions on the applicability of each. As well, the review indicated a number of sunflower lines that might possess resistance to *Sclerotinia sclerotiorum* (Figure 3.1a) and hence *S. minor* (Figure 3.1b) and, where seed was available these were screened using the method of Sedun and Brown (1989).

From Table 3.1 it can be seen that published screening methods can be divided into groups depending on whether natural or artificial inoculation was conducted in the field or whether a glasshouse test was used or whether some form of indirect screening was applied. Inoculum preparation, inoculation method, incubation conditions and assessment parameters used are also given in Table 3.1 when they were reported by the author.

Tables 3.2-3.4 are compilations of the pedigrees, where known, of sunflower inbred lines, open-pollinated cultivars and hybrids for which the reaction to sunflower wilt caused by *Sclerotinia sclerotiorum* has been reported. The results presented by the various workers have been grouped based on percentage mortality since this is the parameter most often reported in the literature . The arbitrary classes used were:

Highly resistant   - 0-10% mortality
Resistant   - 11-25% mortality
Susceptible   - 26-50% mortality
Highly susceptible   - >50% mortality.

Only those tests where the presence of lines in the highly susceptible class indicated the effectiveness of the test are included. It must be stressed that the data can only to be used as a guide to possible sources of resistance since the data is compiled from different experiments and direct comparisons between many of the genotypes under similar experimental conditions has not been conducted.

Figure 3.1 Root rot of sunflower caused by *Sclerotinia sclerotiorum* showing external sclerotia.

Table 3.1 Summary of published methods to screen sunflower for resistance to Sclerotinia wilt

Inoculum	Inoculation Method	Plant Age/ Growth Stage	Assessment	Remarks	References
Field Screening- Natural Inoculation					
Soil-borne sclerotia present after previous diseased crops	Natural infection		% wilt, % mortality	Reliability of results may be reduced by non-uniform inoculum distribution, drought, high temperatures and poor plant stands	Clarke (1982), Gulya (1981), Gulya (1985), Rashid and Dedio (1992)
Field Screening- Artificial Inoculation					
Whole or ground sclerotia of *Sclerotinia* produced in culture or collected from previously infected crops and stored	i) Sown with seed		% mortality	i) Differences observed between the susceptibility of host lines and importance of position of sclerotia in relation to seed determined .	i) Cuk (1976), Grezes-Besset *et al* (1994). Dorrell and Huang (1978, Huang and Hoes (1980), Huang (1981), Pirvu, Vranceanu and Stoenescu (1985)
	ii) Incorporated into soil			ii) Linear relationship found between inoculum density and disease incidence.	ii) Fu, Quan, Mean and Gun (1992)
	iii) Buried close to stem base			iii) Good repeatability reported	iii) Tourvieille de Labrouhe and Vear (1984a)
Cereal Grain colonised by *Sclerotinia*	i) Buried at base of stem		% mortality	Mortality differed between host lines.	i) Dueck and Campbell (1978), Bazzalo, Dimarco, Martinez and Dalco (1992)
	ii) Buried at a distance from stem base	42 day-old	% mortality, % incidence of basal stem lesions	Provided good differentiation between host genotypes.	ii) Dueck and Campbell (1978), Mancl and Schein (1982), Sedun and Brown (1989)
Toothpicks or petiole pieces colonised by *S. sclerotiorum*	Inserted into stems at ground level or 5 or 60cm above soil level		% mortality	Showed that susceptibility of stems changed with plant maturity. Inoculation at stem base differentiated between host lines while severe infections at 60cm did not differentiate between host lines	Auger and Nome (1970), Cuk (1976), Gulyás and Mesterházy (1992)

34

Inoculum	Inoculation Method	Plant Age/ Growth Stage	Assessment	Remarks	References
Field Screening- Artificial Inoculation Cont'd					
Agar discs colonised by *Sclerotinia*	Applied to stem base and covered to reduce desiccation		% mortality	Differences observed in the rate at which different genotypes wilted and died	Cuk (1974)
Agar discs colonised by *Sclerotinia*	Applied to tips of leaves and covered to avoid desiccation		Length of lesion along main leaf vein	Technique is non-destructive to plant if infected leaf is removed. Strong correlation reported between lesion length and reaction to wilt.	Castano, Hemery-Tardin, Tourvieille de Labrouhe and Vear (1992)
Glasshouse and Laboratory Screening					
Fungal sclerotia	Placed under seed at sowing		% mortality	Results considered too variable	Rashid (1992)
Cereal Grain colonised by *Sclerotinia*	i) Placed at soil surface against base of hypocotyl and covered to reduce desiccation		% mortality	Method used to demonstrate effect of plant age on susceptibility	i) Bazzalo, Heber, del Pero Martinez and Caso (1985), Baltensperger, Kerr and Steadman (1993)
	ii) Seedlings transplanted into potting mix incorporating inoculum		% survival	Transplanting seedlings into the mixture may cause mechanical injuries and pre-dispose plants to infection.	ii) Thompson, Rogers, Zimmerman, Huang, Whelan and Miller (1978)
	iii) Inoculum inserted into soil to a depth of 5cm at the edge of the pot.	35-40 day old	Linear rate of subsequent lesion extension up the stem.	Rates differed between the genotypes tested. Used for both *Sclerotinia sclerotiorum* and *S. minor*.	iii) Sedun and Brown (1989)
	iv) Seedlings removed from pots and placed onto inoculum	21 day old	% mortality	Correlated with disease incidence in the field	iv) Grezes-Beset, Tournade, Arnauld, Urs, George, Castellanet and Toppan (1994)

Glasshouse and Laboratory Screening cont'd

Inoculum	Inoculation Method	Plant Age/ Growth Stage	Assessment	Remarks	References
Mycelial suspensions prepared from macerated agar cultures	i) Cotton wad saturated with mycelial suspension wrapped around hypocotyl	4-6 leaf stage	Rate of mortality	Rate at which plants died differed between sunflower varieties.	i) Grauert, Schlosser and Schuster (1980)
	ii) Seed sown into a mixture of suspension and sand	4-6 leaf stage	% Survival	Method provided a ranking of the susceptibility of 3 hybrids comparable to field observations	ii) Rashid (1992)
	iii) Suspension used to drench potting mix around base of growing seedlings	4-6 leaf stage	% Survival	Results considered too variable and severe.	iii) Rashid (1992)
Agar discs colonised by *Sclerotinia*	i) Inoculum placed in slit at top of young stems decapitated at a uniform height		Rate of lesion extension down the stem	Significant differences found between host genotypes.	Robert, Vear and Tourvieille de Labrouhe (1987)
	ii) Disc placed below seed at sowing or against stem base		% survival	Results considered too variable to be useful.	ii) Rashid (1992)
Agar in test tubes colonised by *Sclerotinia*	i) etiolated seedling hypocotyls excised and placed in test tubes with one end in contact with culture		Rate of lesion extension along the hypocotyl	Significant differences found between the sunflower hybrids tested but correlation with field reactions was poor.	i)Tourvieille de Labrouhe and Vear (1984)
	ii) sunflower petioles used. Incubated 23-24°C for 24h, transfer to 31°C for 24h then return to 24°C for 24h.		Length of lesion that develops in the 24h after the high temperature incubation	The method is non-destructive to the whole plant and is based on the observation that some sunflower genotypes produce anti-fungal compounds while the fungus is quiescent during the high temperature incubation. Preliminary report that lines with reduced length of lesion growth were less susceptible in the field.	ii) Martinson (1992)

Inoculum	Inoculation Method	Plant Age/ Growth Stage	Assessment	Remarks	References
Indirect Screening Methods					
Culture filtrate (autoclaved to denature thermolabile enzymes)	i) Bare roots of whole seedlings immersed in filtrate		Wilt index calculated based on observed wilting after 24h.	A range of wilt indices were found for the sunflower lines tested. Correlations with field data, were determined, was poor.	i) Huang and Dorrell (1978) Tourvielle de Labrouhe and Vear (1984)
	ii) Sunflower grown transferred to media containing culture filtrate	4-week-old calli	Fresh weight of calli after a further 4 weeks incubation	Good correlation found between fresh weight of calli and field reaction of the 3 hybrids tested.	ii) Hartman (1991)
Oxalic Acid / Oxalate	i) Leaf cells isolated and incubated in the presence of different concentrations of oxalic acid		LD$_{50}$ calculated	Tolerance of leaf cells to oxalic acid differed between host genotypes and was influenced by the age of the source leaf.	i) Noyes and Hancock (1981)
	ii) Bare roots of whole seedlings immersed in oxalic acid solution		Wilt index calculated	Correlation of host genotype wilt indices with field reactions poor.	ii) Tourvielle de Labrouhe and Vear (1984)
	iii) 2nd pair of true leaves excised and fed 8.88mM oxalic acid solution	26 day-old seedlings	Northern blots of mRNA transcripts for hydroxyproline - rich glycoproteins prepared	Induction of transcripts occurred earlier in the host line considered tolerant than in the susceptible line. The tolerant line was a propriety line with no published field data. The technique also requires the availability of facilities for RNA extraction and analysis.	iii) Mouly, Rumeau and Esqerré-Tugayè (19920

37

The pedigrees of sunflower lines for which reaction to sclerotinia wilt have been published show the historical emphasis that was placed on germplasm of Russian origin for establishing sunflower as an oilseed crop around the world. Unfortunately information on the pedigrees of the Russian-derived germplasm was not readily available. However, one trend exhibited in the following tables is that there are more sunflower inbreds that have been classified as 'highly resistant' than hybrids or open pollinated cultivars

Table 3.2. Pedigrees and reaction of sunflower inbred lines to wilt caused by *Sclerotinia sclerotiorum*.

Inbred	Pedigree[1]	Class HS	S	R	HR
CM10	?			14[2]	
CM15	?	14			
CM303	Selection from VNIIMK 893		10,13		
CM338	S37-388/Smenna/Smenna		10		
CM361	Selection from VNI MK 893			6,11	12
CM379	?	10,12			
CM392	?	12	7,10		
CM400	S37-388/Peredovik/Peredovik	2,4	10,11,12	6	
CM447	Selection from Gene Pool 1 (50 Russian lines)	10			
CM467	Selection from Gene Pool 1 (50 Russian lines)		10		
CM497	Selection from Gene Pool 1 (50 Russian lines)			10,11	
CM502	?	4			
CM506	?	4			
CM526	Selection from Gene Pool 1 (Russian lines)			10,11	2
CM527	?			10	
CM533	?	10			
CM557	?		10		
CM558	?	10			
CM566	?		10		
CM575	?			10	
HA13	Selection from VNI MK 1646		10		
HA61	953-88-3/Armavirski 3497	18	5	5,8,13	4,9,10,17
HA89	Selection from CM303		2,16,17	8	6
HA124	Selection from VNIIMK 8883	2	8	6, 16	9,17
HA232	Smenna[2]//HA6/HA8			3	
HA290	P-21VR1[4]/HA60			6	

Table 3.2 *Cont'd*		HS	S	R	HR
HA292	Commander[3]/Mennonite RR			2,9	
HA299	cms P-21VR1/(Chernianka 66/HA11)//HA62-4-5/T66006-2)	8		6	
HA303	Selection from Voshod			6	
HA304	Commander/Mennonite RR			2	9
HA305	Commander/Mennonite RR				9
HA308	?				17
PK 104/75	?				7
RHA265	Peredovik[2]/953-102-1-1-41			3	
RHA266	Peredovik[2]/953-102-1-1-41	18		3	
RHA273	Chernianka 66/HA119//HA62-4-5/2/T66006-2			17	10,11
RHA274	Chernianka 66/HA119//HA62-4-5/2/T66006-2		16		6
RHA276	Chernianka 66/HA119//HA62-4-5/2/T66006-2				2,9
RHA278	Chernianka 66/HA119//HA62-4-5/2/T66006-2				6
RHA297	Reselection from RHA274			6	
RHA298	Selection from cmsHA89/RHA274			6,9	
RHA801	Selection from a R line composite				9,16,17
S37-388	Selection from Mennonite		14		
953-88-3	Sunrise/unknown wild sunflowers		10		12

1. Pedigree symbolism as used in release notices 2. Numbers refer to references after Table 3.3.

Table 3.3. Pedigrees and reaction of sunflower cultivars and hybrids to wilt caused by *Sclerotinia sclerotiorum*.

Cultivar/		Class			
Hybrid	Pedigree	HS	S	R	HR
Advance	S37-388/Sunrise			1	
Airelle	French hybrid	7,15			
Armavirskij 50	Russian open pollinated cultivar			16	
Armavirskij 3497	Russian open pollinated cultivar	1			
Chernianka 66	Russian open pollinated cultivar	1			
Commander	Selection from Mennonite	13		1	
Cordobes INTA	Argentinian open pollinated cultivar		1		
Corona	Russian open pollinated cultivar	5			
Guayacan INTA	Argentinian open pollinated cultivar			1	
Harkovskij 100	Russian open pollinated cultivar			19	
Impira INTA	Argentinian open pollinated cultivar	1			

Table 3.3 cont'd		HS	S	R	HR
INRA 4701	Frencl hybrid	5,15			
INRA 7702	Frencl hybrid	5,7			
Issanka	Russian open pollinated cultivar			1	
Klein INTA	Argentinian open pollinated cultivar			1	
Krasnodaretz	Russian open pollinated cultivar	5		1	
Mennonite	Russian open pollinated cultivar		14		
Pehuen INTA	Argentinian open pollinated cultivar			1	
Peredovik	Russian open pollinated cultivar	1,7			
Record			1		
Remil	Frencl hybrid		15		
Salyut	Russian open pollinated cultivar			19	
Smenna	Russian open pollinated cultivar		14		
Sputnik	Russian open pollinated cultivar			19	
Start	Russian open pollinated cultivar			19	
Stepniak	Russian open pollinated cultivar	1			
Sunrise	Canadian selection from Russian inbreds			1	
USDA 894	cmsHA89/R 1A274 hybrid	8,9	17		
VNIIMK 6540	Russian open pollinated cultivar	1			
VNIIMK 8883	Russian open pollinated cultivar			1	
VNIIMK 8931	Russian open pollinated cultivar	1,3			

2. Numbers refer to the following references 1. Agrawat, Mathur and Chhipa (1977), 2. Bazzalo, Dimarco, Martinez and Dalco(1992), 3. Cuk(1976), 4 Dueck(1979b), 5. Dueck and Campbell(1978), 6. Fick, Gulya and Auwater(1983), 7. Grauert, Schlossei and Schuster(1980), 8. Gulya(1981), 9. Gulya(1985), 10. Huang(1981), 11. Huang and Dedio(1932), 12. Kolte, Singh and Tewari(1976), 13. Mancl and Schein(1982), 14. Putt(1958), 15. Tourvielle de Labrouhe and Vear(1984), 16. Miller(1992), 17. Mancl and Gulya(1993), 18. Castaño, Vear and Tourvieille de Labrouhe (1993) 19. Miller (1992).

Vranceanu, Stoenescu and Soare (1937) reported that the sunflower hybrids listed in Table 3.4 had demonstrated 'field resistance' to *S. sclerotiorum* in F.A.O. (United Nations Food and Agriculture Organisation) European sunflower trials conducted over the period from 1976 to 1985. The authors were not specific as to whether their observations were made on the incidence of wilt, head rot or both. These results suggest that the Romanian sunflower breeding programs had accessed a number of sources providing superior resistance to *S. sclerotiorum* or, alternatively, the several hybrids had a common source of resistance (eg. an inbred parental line) in common.

Table 3.4. Sunflower hybrids observed to have field resistance to *S. sclerotiorum* in F.A.O. European Sunflower Trials 1976-1985 (from Vranceanu *et al.*, 1987).

Country of Origin	Sunflower Hybrid			
France	Remil	Relax	H9xPac1	
Germany	H-219/79			
Spain	Halcon			
Romania	Romsun 22	Romsun 25	Romsun 40	Romsun 44
	Romsun 134	Select		
Yugoslavia	NS-H-4	NS-Shine		

Other sunflower germplasm that has been reported to possess a degree of resistance to sclerotinia wilt includes a number of wild species such as *Helianthus debilis* T. & G., *H. petiolaris* Nutt. and a number of perennial species (Pustovoit and Krasnokutskaya, 1976).

Experimental 3.3

3.3.1. Preliminary screening of sunflower genotypes for resistance to sclerotinia wilt caused by Sclerotinia minor.

The preceding section showed tha there are many ways of screening sunflower for resistance to *Sclerotinia sclerotiorum*. These methods have confirmed that there is considerable variation among sunflower genotypes for susceptibility to *S. sclerotiorum*. The following experiment examined the reaction of a number of sunflower genotypes to *Sclerotinia minor* following inoculation with the method described by Sedun and Brown (1989). Initially genotypes regarded as possessing resistance to *S. sclerotiorum* were sought but few of the lines were available in Australia. All sunflower sowing seed coming into Australia undergoes Post-entry Quarantine to prevent the introduction of downy mildew (*Plasmopara halstedii*) that includes hot water treatment, fungicide application and growth in a enclosed glasshouse for one growth cycle before seed is released. This restricted the introduction of desirab e lines from overseas that could be used in this study. It is also impossible to introduce seed of F_1 hybrids. Consequently, selfed progeny taken from hybrids were used.

Materials and method

Seed of 58 diverse sunflower genotypes was supplied by Pacific Seeds Pty Ltd

(Toowoomba, Australia). Included was material derived from the wild species breeding projects of Dr Gerald Seiler (United States Department of Agriculture, Fargo, North Dakota), United States Department of Agriculture and Agriculture Canada inbreds, Argentinian lines and lines derived from selfed hybrids of European origin.

Seedlings were grown singly in standard potting mix contained in 10cm diameter plastic pots in a glasshouse. Plants were inoculated 35-40 days after sowing at the time when the majority of seedlings were at Growth Stage R1 (early bud). Inoculation was performed by using a 16mm ∅ (i.d.) cork borer to remove a core of potting mix and roots adjacent to the edge of the pot and filling the hole with 8g of colonised pearl millet seed before sealing the hole with the core that was initially removed. The interval between removal of the core and re-sealing was always less than 30sec. This method was based on that published by Sedun and Brown(1989). Glasshouse air temperatures were 12 - 27°C during the post-inoculation period.

The sunflower lines were screened in three separate groups; one containing entries derived from pedigree crosses involving wild species of *Helianthus*, one with entries thought to be fertility restorers (for the PET1 cytoplasmic male sterility system), and the last group consisted of male sterile and male sterility maintainer lines. The United States Department of Agriculture sunflower inbred lines RHA801 and *cms*HA89 were included as 'resistant' and 'susceptible' checks respectively in each screening. A completely randomised design was used with 10 replicate pots of each entry.

Results

The first symptom noticed at 3 days after inoculation was a progressive wilting of the leaves. Following lesion appearance the plants wilted completely and were considered dead. The mortalities recorded for the host genotypes varied considerably (Tables 3.5-3.7) with fewer plants of RHA801 being killed in each experiment. The rate of lesion extension varied significantly (P<0.05) among the host genotypes with the rate on RHA801 being the slowest or not significantly different from the slowest in each experiment.

Among the lines derived from crosses with wild species the rate of lesion extension ranged from 5.0 to 15.0 mm.d^{-1}. Lines derived from single crosses between *cms*HA89 and *Helianthus paradoxus* Heiser (UNE#H22, H42, H41) had significantly higher (P<0.05) rates of lesion extension than *cms*HA89. Lines derived from three-way crosses between HA89, *H. paradoxus* and *H. argophyllus* T. & G. were either more susceptible, as measured by the rate of lesion extension, than *cms*HA89 (UNE#H38) or more resistant (UNE#H44).

Table 3.5. Reaction of sunflower lines involving crosses with wild species to inoculation with *Sclerotinia minor* using the screening method of Sedun and Brown (1989) (ranked by increasing rate of lesion extension).

UNE Accession #	Source	Mortality (/10)	Rate of Lesion Extension (mm.d^{-1})
1	RHA801*	5	4.7
23	*cmsHA89/H.exilis*	8	5.0
24	*H.paradoxus*/HA89//*H. argophyllus*	8	6.5
8	HA89//CM5/*H.argophyllus*	4	6.9
25	*cmsHA89*/H. *annuus*-21	7	7.4
3	*cmsHA89**	7	7.4
43	HA89//CM5/*H. argophyllus*	6	7.4
37	*cmsHA89/ H. argophyllus*	8	7.8
27	*cmsHA89/ H. debilis sylvestris*	6	8.2
39	*cmsHA89/H. annuus*-21	7	8.4
29	*cmsHA89/H. exilis*	9	8.6
30	*cmsHA89/H. debilis sylvestris*	7	8.7
31	*cmsHA89/H. deserticola*	7	8.7
45	HA89//CM5/*H.argophyllus*	10	8.8
32	*cmsHA89/H. petiolaris*	6	8.9
33	*cmsHA89/H. annuus*-21	8	9.1
34	*cmsHA89//cmsHA89/H. paradoxus*	7	9.1
35	*cmsHA89/H. exilis*	7	10.2
28	*cmsHA89/H.annuus*-21	9	10.3
26	*cmsHA89/H.argophyllus*	6	11.4
38	*H. paradoxus// cmsHA89/H. argophyllus*	4	12.9
40	*cmsHA89//cmsHA89/H. petiolaris fallax*	9	13.4
22	*cmsHA89/H. paradoxus*	9	13.6
42	*cmsHA89/H. paradoxus*	6	13.6
41	*cmsHA89/H. paradoxus*	5	15.0
*Check Lines	L.S.D.(P=0.05)	5	4.421

The time from inoculation to lesion appearance was also recorded in the later experiments and presented as the assessment parameter 'Delay'. This parameter was expected to provide a measure of the resistance of roots to lesion progression. Regression analysis of the data showed that the rate of lesion extension and time from inoculation to lesion appearance were not significantly correlated although the trend was apparent that delay was inversely

proportional to rate of lesion extension. The coefficient of correlation for the fertility restorer lines (Table 3.6) was r = -0 318 (P=0.184) and for the sterility maintainer lines (Table 3.7) was r = -0.283 (P=0.271).

The rate of lesion extension for the restorer lines ranged from 7.3 to 12.8 mm.d^{-1} (RHA801=7.3mm.d^{-1}, *cms*HA89= 8.9mm.d^{-1}). Only the test entries 953-88-3, UNE#H55, UNE#H21, UNE#H59 and PacR1 had slower rates than the susceptible check *cms*HA89 (Table 3.6).

Table 3.6. Reaction of sunflower lines with probable fertility restoration genes to inoculation with *Sclerotinia minor* using the screening method of Sedun and Brown (1989) (ranked by increasing rate of lesion extension).

UNE Accession #	Source	Mortality (/10)	Rate of Lesion Extension (mm.d^{-1})	Delay (d)
1	RHA801*	2	7.3	8.0
46	953-88-3	5	7.4	12.8
55	Saenz Pena 74-2-1	6	7.4	11.5
21	SFM#3	8	8.6	8.4
59	Saenz Pena 74-2-1	9	8.6	8.7
47	Pac R1	9	8.6	12.4
3	*cms*HA89*	8	8.9	10.0
17	77-5-67-8A	5	9.4	12.6
61	Felix	9	9.6	8.5
13	HA-R5	8	9.6	9.7
60	Felix	7	10.4	12.8
59	Saenz Pena 74-1-2	9	10.7	7.0
12	HA-R4	9	10.8	8.9
76	Felix	9	11.3	7.6
20	SFM#2	6	11.9	7.5
58	Saenz Pena 74-2-1	8	12.7	10.5
19	SFM#1	9	12.8	6.8
* Check lines L.S.D. (P=0.05)		4	3.741	3.729

The rate of lesion extension in UNE#H58 and UNE#H19 was significantly greater (P<0.05) than in *cms*HA89. The mean delay between inoculation and lesion appearance ranged from 7.0 to 12.8 days among the restorer lines (RHA801= 8.0d, *cms*HA89= 10.0d).

The mean delays for PacR1, 77-5-67-8A, 953-88-3-2-3 and UNE#H60 were significantly longer than the delay for RHA801   No entries had delays significantly longer than *cms*HA89 although several were arithmetically longer. The sunflower lines PAC R1 and 953-88-3 had both delays longer and rates of lesion extension lower than the more susceptible check line for each parameter.

Among the sterility maintainer sunflower lines the rate of lesion extension ranged from 10.0 to 23.7 mm.d^{-1} and delay from 5.1 to 9.0d (RHA801= 11.6mm.d^{-1}, 6.4d; *cms*HA89= 15.7mm.d^{-1}, 5.8d)(Table 3.6). The rate of lesion extension was lower for PacA1 and HA292 than for RHA801 and greater for HA304, HA124, HA335, HA305, HA61-1 and HA-R1 than for *cms*HA89. The delay for 953-88-3 was significantly longer (P<0.05) than for either of the check lines while the delay for PacA1, HA292, HA340, HA304 and HA335 was arithmetically greater than for RHA801 (Table 3.7).

Table 3.7. Reaction of sunflower sterility maintainer lines and cytoplasmic male sterile lines to inoculation with *Sclerotinia minor* using the screening method of Sedun and Brown (1989) (ranked by increasing rate of lesion extension).

UNE Accession #	Source	Mortality (/10)	Rate of Lesion Extension (mm.d^{-1})	Delay (d)
49	Pac A1	10	10.0	7.5
5	HA292	9	11.5	7.1
1	RHA801*	8	11.6	6.4
16	CM526	10	12.7	6.2
11	HA-R2	10	12.9	5.7
14	CM361	7	13.8	6.2
9	HA340	10	14.6	7.2
50	Pac A2	10	14.8	5.1
18	953-88-3	8	15.3	9.0
52	Pac A4	10	15.5	5.3
3	*cms*HA89*	10	15.7	5.8
6	HA304	9	16.1	6.8
4	HA124	6	16.2	6.2
8	HA335	9	18.9	7.5
7	HA305	10	19.2	6.3
2	HA61-1	10	19.6	5.5
10	HA-R1	10	23.7	5.3
*Check Lines L.S.D.(P=0.05)		5	4.21	2.25

3.3.2. The pot base inoculation method: Effect of temperature on the in vitro growth of Sclerotinia minor, rate of lesion extension on excised sunflower hypocotyls and production of disease on inoculated sunflower plants.

The inoculation method used by Sedun and Brown (1989) and used above was modified in an attempt to reduce the amount of root damage inflicted on plants during inoculation and also to minimise variability in the delay from inoculation to appearance of the stem base lesion. This was done by placing the inoculum at a set distance from the stem base, in this case, in the pot base. A number of experiments were performed to optimise what will be referred to as the pot base inoculation method.

Temperature is one of the major environmental parameters affecting the epidemiology of plant diseases. While in many cases it is relatively easy to find the *in vitro* temperature most favoured by the pathogens this temperature may not accurately reflect the optimum temperature for disease development. The following experiments were conducted to determine the optimum temperature for colonisation of sunflower by *Sclerotinia minor* so that the inoculation conditions most favourable for disease development could be defined.

Materials and method

The effect of temperature on the rate of fungal growth in juvenile sunflower tissue was measured on excised etiolated hypocotyls. Seed of the sunflower inbred lines RHA801 and *cms*HA89 were sown in seedling flats containing normal potting medium. A single seed was placed at the intersections of a 2cm x 2cm grid. Trays were incubated in a darkened incubator at 25°C until hypocotyls were approximately 10cm long. The trays were then transferred to a controlled environment cabinet providing 25°C, photon flux densities of 350 μM.m^{-2}.sec^{-1} in 12h photoperiods for 4 days during which time the seedlings became green. Hypocotyls were then excised at soil level and below the cotyledons, were surface sterilised in 1% sodium hypochlorite for 2min, rinsed in sterile distilled water, blotted dry on sterile paper towel, and transferred to 15x160mm test tubes containing 1.5ml V-8 juice agar that had been inoculated with *S. minor* two days previously and incubated at 20°C. Each test tube was sealed with an aluminium cap. Ten test tubes of each sunflower line were transferred to incubators operating at 5, 10, 15, 20, 25, 30 and 35°C. All incubations were conducted in darkness since some incubators had no provision for lights. The length of water-soaked lesions on the hypocotyls was measured daily. It was often easier to invert the test tube so that the hypocotyls hung down since lesion development caused many to collapse.

The effect of temperature on disease development was also assessed on whole plants. Thirty seedlings of the sunflower inbred lines RHA801 and *cms*HA89 were raised in a

controlled environment cabinet at a temperature of 20±1ºC. Ten seedlings of each line were transferred to other controlled environment cabinets operating at either 15±1ºC or 25 ±1ºC when plants were 35 days old (Growth Stage V10-12). Photosynthetic photon flux densities at seedling height were adjusted to $300\pm25\mu M.m^{-2}.sec^{-1}$ in each cabinet by adjusting the height of the supporting platform relative to the light banks. Plants were grown under the new conditions for 48 hours then inoculated by removing the pot, placing 5g of moist inoculum in the base of the pot and replacing the root ball.

Plastic Petri plates (10cm ∅) containing 15ml potato dextrose agar were inoculated by placing 3mm ∅ plugs taken from the margins of *S. minor* cultures growing on potato dextrose agar in the centre of the plates. The inoculated plates were sealed with Parafilm™ and wrapped in aluminium foil to exclude light. Six plates were placed in each controlled environment cabinet with the inoculated plants. Colony diameters were measured daily.

The delay from inoculation to appearance of a lesion at the stem base was determined by examining plants each day. The length of lesions that formed was measured daily and the linear rate of lesion extension calculated. Mortality was determined as the proportion of inoculated plants that had been killed by stem lesions at 28 days after inoculation. The experiment was repeated once with different controlled environment cabinets used to provide the temperature regimes.

Results

The rate of lesion growth on excised sunflower hypocotyls of both inbred sunflower lines was most rapid at 20ºC and rapidly decreased at higher and lower temperatures (Figure 3.1). The water-soaked lesions were easily visible through the test tube and often these were associated with tufts of aerial hyphae just behind the lesion front.

The optimum temperature for disease development on the two sunflower lines inoculated by the pot base method was 20ºC. A greater proportion of plants were killed, basal stem lesions appeared sooner and the rate of lesion extension was quickest at this temperature (Table 3.8). The rate of fungal growth *in vitro* was also greater at 20ºC than at either 15 or 25ºC.
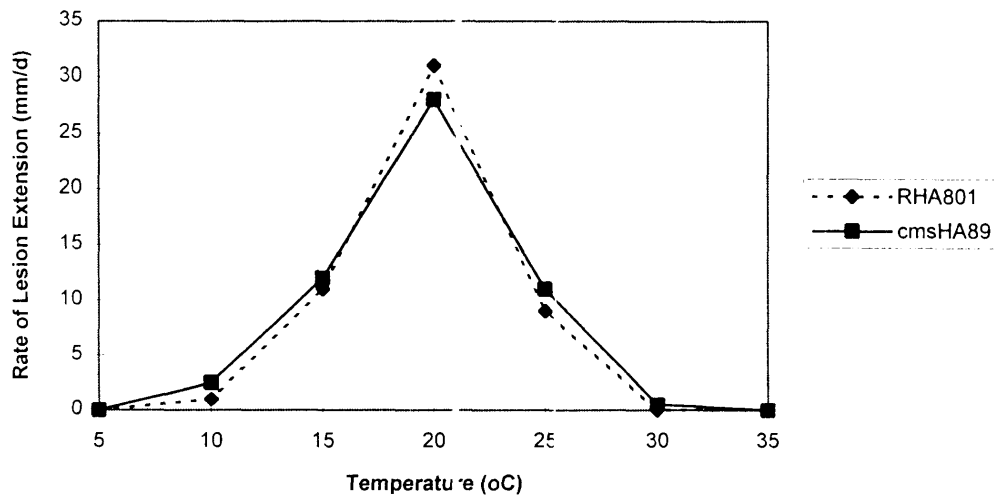
Figure 3.1. Effect of incubation temperature on the rate of lesion extension on elongated hypocotyls of the sunflower inbred lines RHA801 and *cms*HA89 inoculated with *Sclerotinia minor*.

Table 3.8. Effect of incubation temperature on disease development in two inbred sunflower lines inoculated with *Sclerotinia minor*.

Line	Assessment[1]	Incubation Temperature		
		15°C	20°C	25°C
RHA801	Mortality (/10)[2]	9 *a*	10 *a*	1 *b*
	Delay (d)	20.8 *a*	12.0 *b*	10.0 *b*
	Rate of lesion extension (mm.d⁻¹)	7.30 *a*	8.43 *a*	7.20 *a*
*cms*HA89	Mortality (/10)	9 *a*	10 *a*	6 *a*
	Delay (d)	16.3 *a*	14.2 *a*	18.0 *a*
	Rate of lesion extension (mm.d⁻¹)	9.5 *a*	10.3 *a*	10.9 *a*
In vitro	Rate of colony growth (mm.d⁻¹)	26.9 *b*	36.3 *a*	6.5 *c*

1. See text. 2. Means were calculated from the combination of two experiments. Means in each row followed by at least one least one lower case letter in common are not significantly different (P<0.05) according to Duncans multiple range test or the procedure described in Section 2.8 in the case of mortality.

3.3.3. The pot base inoculation method: Effect of inoculum quantity.

To optimise inoculation conditions in screening experiments it was necessary to control the level at which inoculum could be applied so that maximum mortality occurred in susceptible individuals but still providing valid discrimination of levels of quantitative resistance among test lines.

Materials and method

The effect that the quantity of applied inoculum had on three disease assessment parameters was investigated on the sunflower inbred lines RHA801, PacR2, PacA1 and *cms*HA89. Plants were raised in 10cm plastic pots containing standard potting medium under glasshouse conditions where for the duration of this experiment temperatures ranged from 16°C to 27°C. Plants were inoculated after 45 days growth when all plants were at early reproductive growth stage (G.S. R1 - R2).

Plants were inoculated after gentle removal of the pot by placing either 5, 25, 125, 500 or 1000 colonised grains in the base of the pot and replacing the root ball. The latter three grain numbers were estimated on the basis of grain weight since it was impractical to count the number of grains used at these inoculum levels. Ten samples of 100 colonised grains were used to determine the average weight per grain after mixing of all the grain. Ten replicate plants of each sunflower line were inoculated with each inoculum level. Plants were examined daily for the first appearance of a basal stem lesion. Lesion lengths were then measured daily so that the mean rate of lesion extension could be calculated. The number of plants that had developed stem lesions in the 28 days following inoculation were recorded.

Results

The parameters used to assess reaction of the sunflower lines to inoculation with *S. minor* were not significantly affected by inoculum dose. Application of five or 1000 colonised grains did not consistently affect the number of plants killed, the time taken for basal stem lesions to appear or the rate of extension of lesions. Contingency table analysis of the effect of inoculum dose on mortality of the four sunflower inbred lines (Table 3.9) demonstrated independence between dose and sunflower line ($\chi^2 = 1.916$, P=0.999).

Differences in the mean time taken for lesions to appear at stem bases were not consistent but there was a general tendency towards a shorter delay with increased inoculum dose (Table 3.10). No significant differences (P>0.05) were found for the effect of inoculum dose on the rate of lesion extension (Table 3.11).

Table 3.9. Effect of inoculum dose on mortality (/10) of four sunflower inbred lines.

Sunflower	Inoculum Dose[1]				
Line	5	25	125	500	1000
	Mortality (/10)				
RHA801	3[2]	4	3	4	5
PacR2	9	8	10	7	10
PacA1	8	10	10	8	10
cmsHA89	9	9	9	10	8
Mean:	7.3	7.8	8.0	7.3	8.3

1. Number of colonised millet grains appliec to the base of the pot. 2. There were no significant effects (P>0.05) of inoculum dose on mortality for a ɲy of the sunflower lines.

Table 3.10. Effect of inoculum dose on time (days) taken for stem lesions to develop on four sunflower inbred lines.

Sunflower	Inoculum Dose[1]				
Line	5	25	125	500	1000
	Time to Appearance of Stem Lesions (d)				
RHA801	24.5 *a*	15.5 *b*	22.3 *ab*	15.5 *b*	16.0 *b*
PacR2	14.5 *ab*	17.3 *a*	13.8 *ab*	13.5 *ab*	12.4 *b*
PacA1	18.1 *a*	19.2 *a*	17.5 *ab*	13.9 *b*	16.7 *ab*
cmsHA89	15.0 *ab*	16.1 *a*	15.4 *a*	12.7 *ab*	11.6 *b*
Mean:	18.0	17.0	17.3	13.9	14.2

1. Number of colonised millet grains appliɜd 2. Means in each row followed by at least one lower case letter in common are not significantly differɛnt (P>0.05) according to Duncans Multiple Range Test.

Table 3.11. Effect of inoculum dose on the rate of lesion extension (mm.d^{-1}) of four sunflower inbred lines.

Sunflower	Inoculum Dose[1]				
Line	5	25	125	500	1000
	Rate of Lesion Extension (mm.d^{-1})				
RHA801	10.6 *a*	14.9 *a*	10.5 *a*	10.4 *a*	9.9 *a*
PacR2	11.2 *a*	9.2 *a*	11.3 *a*	12.3 *a*	10.7 *a*
PacA1	12.2 *a*	12.7 *a*	11.8 *a*	12.7 *a*	10.8 *a*
cmsHA89	12.1 *a*	12.8 *a*	13.8 *a*	11.9 *a*	12.2 *a*
Mean:	11.5	12.4	11.9	11.8	10.9

1. Key as used for Table 3.10.

3.3.4. The pot base inoculation me hod: Effect of inoculum age on three parameters used to assess host resistance.

In this experiment, the effect of inoculum age on the effectiveness of colonised grain as inoculum was investigated.

Materials and method

Millet grain inoculum used in this experiment had been incubated for either 0, 10, 20 or 30 days. Twelve 500ml Erlenmeyer flasks each containing 125g soaked millet grain were autoclaved on Day 0. Three flasks were inoculated with 3 agar discs (5mm ∅) cut from the actively growing colony margins of *S. minor* UNE#3 cultured on potato dextrose agar. All flasks were then incubated at 2 $^{\circ}$C in darkness. Ten and twenty days later another three flasks were inoculated with 3 agar discs (5mm ∅) cut from fresh colonies of *S. minor* UNE#3. Flasks were shaken daily to encourage even colonisation of the grains. Ten days after the final flasks were inoculated the grain were collected and used as inoculum.

Forty plants of the sunflower inbred ines RHA801 and *cms*HA89 were raised in standard potting medium in the glasshouse. Plants were inoculated 35 days after sowing when at Growth Stage V12 - R1 by gently removing the pots, placing approximately 50 colonised millet grains in the base of the pots and replacing the root balls. Ten plants of each line were inoculated with each inoculum age. Plants were examined daily for appearance of stem lesions and the length of subsequent stem lesions measured. The number of plants that had developed stem lesions at 28 days after inoculation was recorded.

Results

The longer incubation periods of inoculated grain resulted in progressive development of sclerotia. No fungal growth was observed in uninoculated flasks. The fungus grew vegetatively with very few sclerotia initials (seen as knots of hyphae) evident on grain incubated for 10 days. Sclerotial initials were present on every grain by 20 days and darkening of initials was occurring. Sclerotia had formed after 30 days incubation (Figure 3.2). Age of inoculum had a drama ic effect on its ability to incite disease (Table 3.12). The number of plants killed decreased and the time taken for basal stem lesions to appear increased with increased inoculum maturity. Inoculum where the fungus was still growing vegetatively was best for induction of disease using this inoculation method.
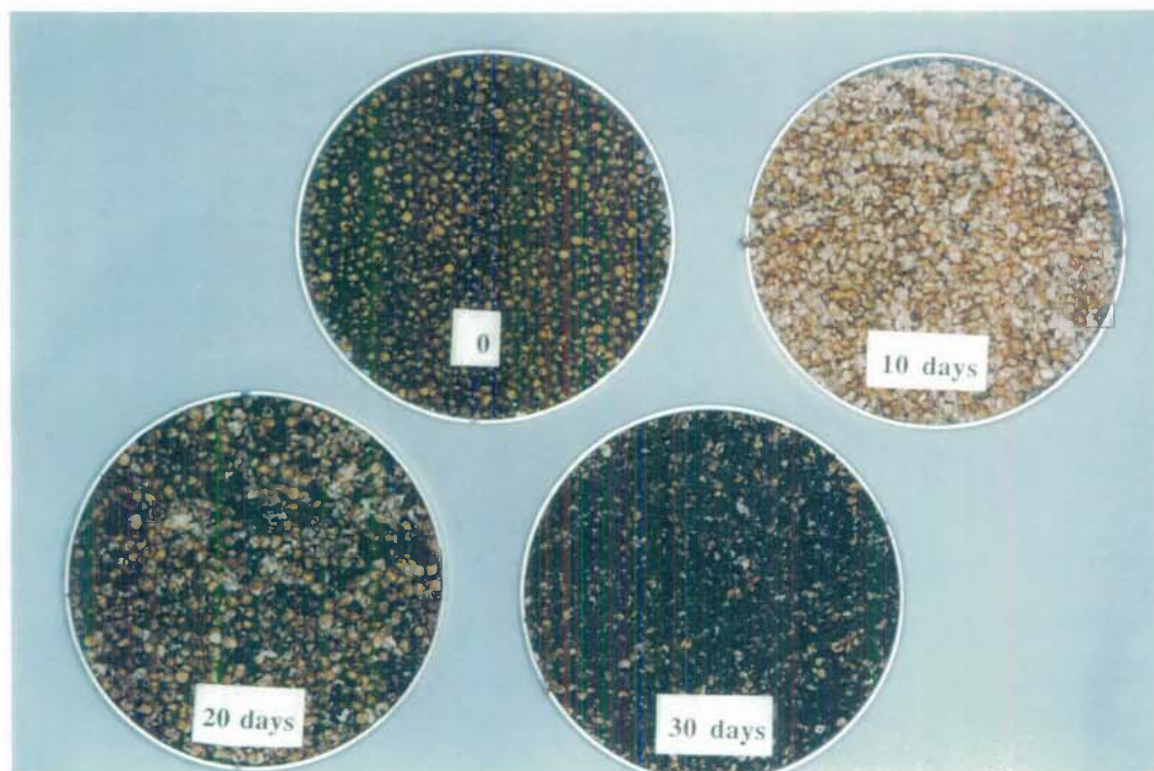
**Figure 3.2** Pearl millet grain colonised with *Sclerotinia minor* showing development of sclerotia.

Table 3.12. Effect of inoculum age on the development of disease on two sunflower lines inoculated with *Sclerotinia minor*.

Line	Assessment Parameter	Inoculum Age (d)			
		0	10	20	30
RHA801	Mortality (/10)[1]	0 *b*	5 *a*	2 *ab*	0 *b*
	Delay (d)	-	18.0 *a*	23.0 *b*	-
	Rate of lesion extension (mm.d^{-1})	-	10.1 *a*	11.0 *a*	-
*cms*HA89	Mortality (/10)	0 *b*	9 *a*	6 *a*	1 *b*
	Delay (d)	-	11.9 *a*	13.0 *a*	11.0 *a*
	Rate of lesion extension (mm.d^{-1})	-	13.8 *a*	12.7 *a*	12.8 *a*

1. Means in each row followed by at least one lower case letter in common are not significantly different ($P>0.05$) according to Duncans Multiple Range Test or by the test described in Section 2.8 for mortality.

52

3.3.5. A comparison of the pot base inoculation method with that of Sedun and Brown (1989).

The pot base inoculation method had a number of potential advantages over the technique used by Sedun and Brown (1989). It was quicker and easier to invert the seedling, remove the pot, place the inoculum in the base of the pot and replace the seedling than it was to cut a core, insert the inoculum and seal the inoculation site. There was comparatively less damage to the roots if the root ball was moist when the pot was removed. The inoculum was also placed at a consistent distance from the stem base and the vigour and growth of the roots could be visualised rather than be assumed. The different placements of inoculum are shown in Figure 3.3.
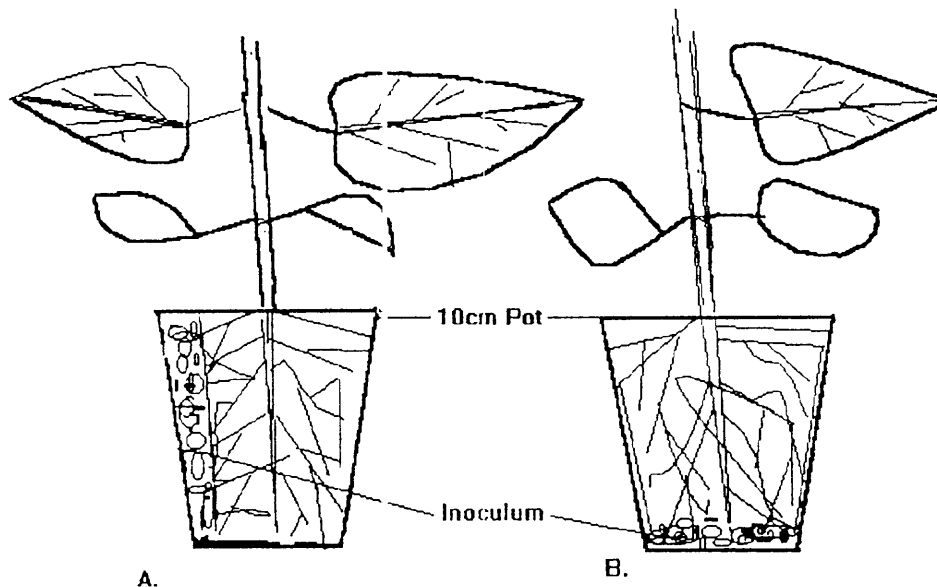
Figure 3.3. Relative placement of inoculum in two methods of inoculating sunflower with Sclerotinia minor A. The method of Sedun and Brown (1989) B. The pot base inoculation method

Materials and method

Three sunflower inbred lines RHA801, cmsHA89 and CM497 were screened using the inoculation method of Sedun and Brown (1989) and the pot base inoculation method. Twenty plants of each line were grown in a controlled environment cabinet supplying a photon flux density of $370\mu M.m^{-2}.sec^{-1}$ in 14 hour photoperiods and a temperature regime of $22/18^{\circ}C$ light/dark. Thirty-five days after sowing, ten plants of each line were

inoculated by the method described by Sedun and Brown (1989) and ten were inoculated by the pot base inoculation method. A completely randomised design was used with both treatments intermingled.

Results

More plants inoculated by the pot base method were killed while the time to lesion appearance was longer on two lines and the rate of lesion extension was also greater (Table 3.13). Greater uniformity in time to lesion appearance on each of the three sunflower lines inoculated by the pot base method was observed when the standard deviation was used as the measure of the spread of observations. Standard deviations of delay for lines inoculated with the method of Sedun and Brown (1989) were close to or exceeded 50% of the mean value. In contrast, the standard deviations of the rates of lesion extension were lower on two lines inoculated with the method of Sedun and Brown (1989). It is necessary therefore to decide which, if any, of the reaction assessment parameters have a correlation with field mortality before deciding which of the inoculation methods is superior. If rate of lesion extension is better correlated then inoculation with the method Sedun and Brown (1989) will provide more uniform results but if the time taken from inoculation to appearance of basal stem lesions is better correlated then the pot base method would be the preferred option.

Table 3.13. Comparison of reactions produced by three sunflower inbred lines inoculated with two methods[1].

Line	Inoculation Method	Mortality (/10)	Delay (days)	Rate of Lesion Extension (mm.d^{-1})
CM497	I	6	10.0 (4.53)[2]	9.53 (1.25)
	II	10	11.2 (2.33)	9.44 (1.71)
cmsHA89	I	8	12.6 (3.46)	9.70 (3.77)
	II	9	11.3 (2.87)	12.10 (3.28)
RHA801	I	6	10.8 (5.63)	7.53 (0.396)
	II	6	14.7 (3.78)	8.87 (1.59)

1. Methods used: I. Sedun and Brown (1989) II. The pot base inoculation method 2. Means with standard deviations in parentheses.

3.3.6. Use of the Pot Base Inoculation Method to screen sunflower germplasm I. Elite Sunflower Inbreds

The pot base inoculation method was used to screen a number of inbred sunflower lines to identify lines for further experimentation.

Materials and method

A number of plants that survived the screening described in Experiments 3.3.2. were allowed to self-fertilise by enclosing the flowering capitula in paper packets to isolate the stigmas from the pollen from other plants. The seed from representative plants of lines was used for re-screening using the pot base inoculation method. A number of other lines derived from the selfing of European hybrids with putative resistance to *Sclerotinia sclerotiorum* were obtained from Pacific Seeds Pty Ltd and were also included. Ten plants of each line were raised in the glasshouse where post-inoculation temperatures ranged from 12°C to 24°C.

Results

RHA801 was the most resistant line based on the time to lesion appearance (delay) and rate of lesion extension (Table 3.14). It was interesting to note that a selection RHA801-3 made from RHA801 showed increased resistance compared to the parental line. Fewer plants were killed, delay was longer and rate of lesion extension was less in the selection. Two selections from the Argentinian line Saenz Peña 74-1-2 (UNE#H55 and UNE#H59) showed low mortality and long delays but lesions progressed rapidly on the plants on which they occurred.

3.3.7. Use of the Pot Base Inoculation Method to screen sunflower germplasm II. Sunflower Experimental Hybrids

Sunflower breeding in Australia has been conducted by a number of private companies and public institutions. Requests were made to these organisations for seed of sunflower hybrids for testing for reaction to *Sclerotinia minor*.

Materials and method

Five commercial sunflower hybrids were obtained (F61, Pioneer Seeds; Hysun 32, Hysun 44, Pacific Seeds; Dk610, Dekalb and Suncross 40+, Agseeds). Experimental hybrids were provided by Pacific Seeds (8 hybrids), Dekalb (3 hybrids), Agseeds (7 hybrids) and the Queensland Department of Primary Industries (Q.D.P.I.) (10 hybrids). No information was provided on the pedigrees of these hybrids and there was no indication of any inter-relatedness. The Q.D.P.I. hybrids however were known to have been produced on

*cms*HA89 (Dr. D. George, Senior Plant Breeder, Hermitage Research Station, via Warwick, Queensland). Three F_1 hybrids were also produced by the author by fertilising the cytoplasmic male sterile lines Pac A2, PacA1 and Pac A4 with pollen of RHA801.

Table 3.14. Reaction of sunflower inbred lines to inoculation with *Sclerotinia minor* (ranked by increasing rate of lesion extension).

UNE Accession #	Source	Mortality (/10)	Delay (days)	Rate of Lesion Extension (mm.d^{-1})
	RHA801-3	3	19.67	6.66
1	RHA801	5	17.50	8.64
21	SFM#3-8-1	7	16.43	8.64
67	'Select'	7	17.14	9.59
53	'Progress'	9	17.88	10.21
63	'Select'	10	14.70	10.59
16	CM526-1-2	10	14.67	10.87
54	'Progress'	9	16.10	12.30
3	*cmsHA89*	9	15.50	12.59
60	'Felix'	9	13.30	12.88
68	'Progress'	9	16.40	13.07
69	'Progress'	9	17.20	13.39
38	Interspecific	8	13.90	13.46
65	Pop. Sint ICRF	8	13.40	13.63
8	HA335-1	7	15.10	14.59
17	77-5-67-8A-1-1	2	18.00	14.90
63	'Select'	7	11.14	15.73
59	Saenz Peña 74-2-1	2	18.00	16.56
CM497-2-1	CM497	9	14.78	16.93
55	Saenz Peña 74-2-1	4	19.00	18.42
L.S.D. (P<0.05)		4	2.967	3.58

Twelve replicate pots of each hybrid were sown in the glasshouse and arranged in a completely randomised design. Plants were inoculated 35 days after sowing with *S. minor* UNE#3. The results obtained for the hybrids was subjected to the Wilcoxson signed rank test to determine whether there were significant differences in the performance of the hybrids originating from different sources.

Results

The sunflower hybrids screened for resistance by inoculating with the pot base inoculation method showed a wide range of reactions to *S. minor* (Table 3.15). No hybrid was immune to the fungus. The mean mortality for all 36 hybrids was 86.91%. Thirteen hybrids had mortalities less than the mean. The mean time for appearance of basal stem lesions ranged from 12.6 days (Dk 610 and Q.D.P.I. 89/3-5) to 20.2 days (PacA1/RHA801) with the total mean of 15.34 being exceeded by 15 hybrids. The linear rate of lesion extension ranged from 9.2 mm.d^{-1} (PacA1/RHA801) to 16.71 mm.d^{-1} (Dk36630) with the rate of 18 hybrids being less than the total mean of 12.33 mm.d^{-1}. Three hybrids (PacA1/RHA801, Pac 9454, Q.D.P.I. 89/11-1) had the combination of mortality lower than average, time to lesion appearance longer than average and rate of lesion extension less than average.

The Wilcoxson signed rank test was used to compare the performance of the hybrids from different sources. Analysis of the rate of lesion extension showed that hybrids from Dekalb performed worse than those from Agseeds (P<0.10) and Pacific Seeds (P<0.10) but were not significantly different to those hybrids from the Queensland Department of Primary Industries, U.N.E. or Pioneer (P>0.10). The performance of hybrids from Agseeds (P<0.05) and Pacific Seeds (P<0.05) were also superior to the hybrids provided by Queensland Department of Primary Industries. These results suggest that Agseeds and Pacific Seeds had available and were using germplasm with some degree of resistance to *Sclerotinia minor* or, at least, they had assessed which germplasm may have resistance.

3.3.8. Use of the Pot Base Inoculation Method to screen sunflower germplasm III. Wild Sunflower Accessions.

A large number of roadside populations of *Helianthus* are found in Australia. Many were introduced as ornamentals and have since become naturalised and adapted to the Australian environment. These populations are of interest because they may possess resistance genes to some of the diseases that affect commercial sunflower crops. They may also act as reservoirs of inoculum of important sunflower diseases such as sunflower rust. In this study samples from a number of these populations were screened for resistance to *Sclerotinia minor* using of the pot base inoculation method.

Materials and method

Seed of 23 roadside *Helianthus* populations collected over a number of years from a number of sites in eastern Australia was provided by Pacific Seeds Pty Ltd (Toowoomba, Australia). Included with *H. annuus* were collections of the silver-leaf sunflower *Helianthus argophyllus* Torrey and Gray and the cucumber-leaf sunflower *Helianthus*

debilis ssp. *cucumerifolius* (T.&G.) Heiser. The seed was placed on wet filter papers in Petri dishes and incubated in the refrigerator (5°C) for 2 weeks to break dormancy before incubating at 25°C. Seedlings were transplanted to potting mix contained in 10cm diameter pots in the glasshouse when the radicles were less than 2cm long. Ten replicate pots of each population were used. The sunflower inbreds RHA801 and *cms*HA89 were split-plant 3 weeks apart in an effort to provide similar growth stages to the wild populations which were expected to be quite variable.

Table 3.15. Reaction of experimental and commercial sunflower hybrids to inoculation with *Sclerotinia minor* (ranked by decreasing rate of lesion extension).

Hybrid	Commercial/ Experimental	Source	Mortality (/12)	Delay (days)	Rate of Lesion Extension $(mm.d^{-1})$
Dk36630	E	Dekalb	12	12.7	16.71
89/1-13	E	C.D.P.I.	12	16.4	14.91
Dk3903	C	Dekalb	9	13.3	14.75
89/3-5	E	C.D.P.I.	10	12.6	14.72
Pac A2/RHA801	E	U.N.E.	10	19.1	14.37
89/4-11	E	C.D.P.I.	8	13.6	13.87
AgX 3740	E	Agseeds	100	15.0	13.80
89/4-1	E	C.D.P.I.	7	16.6	13.60
Suncross 40+	C	Agseeds	10	15.6	13.58
Pac 2592	E	Pacific	12	13.6	13.16
Dk 610	C	Dekalb	12	12.6	13.11
Dk36823	E	Dekalb	12	14.2	12.95
89/2-13	E	C.D.P.I.	9	16.4	12.88
89/QSR1	E/C	C.D.P.I.	12	15.6	12.85
Hysun 44	C	Pacific	12	13.1	12.76
89/7-13	E	C.D.P.I.	11	15.2	12.69
Pac 8619	E	Pacific	12	16.4	12.43
Pac A4/RHA801	E	U.N.E.	9	18.0	12.36
Hysun 32	C	Pacific	12	15.8	12.16
89/11-1	E	C.D.P.I.	8	16.6	12.12
89/6-1	E	C.D.P.I.	9	14.4	12.06
Pac 2515	E	Pacific	12	15.0	11.88
F61	C	Pioneer	12	15.4	11.85
89/6-5	E	C.D.P.I.	11	14.4	11.80
AgX 9391	E	Agseeds	10	14.6	11.68
Pac 2574	E	Pacific	11	17.9	11.62
AgX 9040	E	Agseeds	12	13.8	11.09
Pac 9454	E	Pacific	9	18.9	10.80
AgX 8740	E	Agseeds	11	14.3	10.60
Pac 2576	E	Pacific	9	14.1	10.60
Pac 8699	E	Pacific	12	13.5	10.32
AgX 7240	E	Agseeds	10	16.6	10.27
AgX 9313	E	Agseeds	9	14.8	10.22
AgX 9340	E	Agseeds	11	15.3	10.15
Pac3679	E	Pacific	5	17.0	9.87
PacA1/RHA801	E	U.N.E.	8	20.2	9.17
Means:			8.69	15.34	12.33
L.S.D. (P= 0.05):			5	2.845	3.058

All plants were inoculated by the pot base inoculation method 50 days after transplanting. Glasshouse temperatures during the incubation period ranged from 11-29°C. Plants were examined daily for 28 days after inoculation for the appearance of basal stem lesions.

Results

The sunflower species showed a diversity of morphological characteristics distinct from the cultivated forms. Leaf shape, colour and pubescence differed. The reaction of the lines are shown in Table 3.16 and show that many of the lines could be considered more resistant than the cultivated inbred lines. The rates of lesion extension of split-plantings of the check line *cms*HA89 were significantly different (P<0.05) with slower extension on plants that were the same age as the wild lines but at a reproductive growth stage. This is consistent with the observations from the field inoculations where the rate of lesion extension decreased on older plants (at a later growth stage). The split-plantings of the resistant check line RHA801 showed no significant differences (P>0.05). The rates of lesion extension of the wild lines were less than for RHA801 and the vegetative *cmsHA89* except for one selection of *Helianthus argophyllus* (HS13) which had the most rapid rate of all lines. Other lines of interest include the two *Helianthus debilis* selections (HS09, HS10) and *H. annuus* HS41 which had low numbers of plants killed.

3.4 Discussion

Breeding for resistance to plant diseases often provides the most efficient and economical means of disease control. Genetic control through the use of host resistance is ecologically preferable to the use of fungicides and provides growers with greater flexibility in cropping options. The application of plant breeding strategies in the development of resistant cultivars is itself influenced by the economics of the problem since resources can rarely be justified for a problem in a low value crop where preliminary research indicates that negligible progress can be achieved. Breeding sunflower for resistance to sunflower rust (*Puccinia helianthi*) and downy mildew (*Plasmopara halstedii*), where qualitative monogenic resistance has been readily been identified in germplasm (Goulter, 1990; Miller, Rodriguez and Gulya, 1988), has resulted in many commercial hybrids possessing significant resistance. In contrast, sclerotinia wilts caused by *Sclerotinia sclerotiorum* and *S. minor* are considered to be diseases where absolute resistance or immunity does not exist in cultivated sunflower although quantitative differences do occur (Masirevic and Gulya, 1992).

Table 3.16. Reaction of a number of wild *Helianthus* accessions to inoculation with *Sclerotinia minor* (ranked by increasing rate of lesion extension).

Line	Species	Growth Stage[2]	Mortality (/10)	Delay (days)	Rate of Lesion Extension (mm.d^{-1})
HS25	*H. argophyllus*	V15	10	16.50	7.78
HS59	*H. annuus*	V14	8	13.63	10.74
HS46	*H. annuus*	V15	9	13.75	10.84
HS49	*H. annuus*	V16	10	11.71	11.07
HS39	*H. annuus*	V14	10	14.89	11.44
HS64	*H. annuus*	V14	10	18.63	12.11
HS65	*H. annuus*	V13	7	16.43	12.21
HS48	*H. annuus*	V13	10	14.71	12.31
*cms*HA89*		R2	8	22.25	12.74
HS35	*H. annuus*	V11	7	17.40	13.25
HS47	*H. annuus*	V14	8	11.88	13.27
HS33	*H. annuus*	V13	7	18.33	13.40
HS10	*H. annuus*	V10	2	20.00	13.50
HS55	*H. annuus*	V12	6	17.67	13.60
HS58	*H. annuus*	V14	9	15.44	14.04
HS09	*H. debilis*	V11	4	17.00	14.25
HS40	*H. annuus*	V15	10	13.22	14.44
HS41	*H. annuus*	V14	2	16.00	14.50
HS90	*H. annuus*	V12	10	17.57	15.23
HS57	*H. annuus*	V15	10	13.00	15.23
HS53	*H. annuus*	V12	9	11.86	15.36
HS28	*H. annuus*	V14	8	13.13	15.40
HS32	*H. annuus*	V13	10	11.20	16.22
*cms*HA89*		V9	6	18.17	17.35
RHA801*		V9	6	17.33	18.08
RHA801*		R3	7	18.50	18.60
HS13	*H. argophyllus*	V12	6	14.67	20.37
* Check lines	L.S.D. (P=0.05)		6	4.47	4.62

1. Species identification based on morphological characteristics 2. Growth Stage at inoculation based on scheme of Schneiter and Miller (1981).

Plant breeding for quantitative resistance is technically more difficult than selecting plants expressing qualitative resistance. It is difficult to work without absolute boundaries. Comparison can only be made relative to the performance of standard check lines. These check lines should include examples possessing the best possible level of quantitative resistance available or a minimum level or 'base level' below which the test entries will be automatically discarded. In the screening experiments conducted in this chapter the check lines used were RHA801 and *cms*HA89. RHA801 has consistently been found to have lower incidence of sclerotinia wilt (Gulya, 1985a; Sedun and Brown, 1989; Miller, 1992)

while *cms*HA89 was considered to be susceptible (Bazzalo, Dimarco, Martinez and Dalco, 1992; Miller, 1992) or moderately resistant (Gulya, 1981).

The ultimate test of any screening test is the performance of the germplasm under field conditions where a whole range of interacting factors may influence the assessment of quantitative resistance (Parlevliet, 1992). In controlled studies of foliar pathogens it may be possible to select the components contributing to quantitative resistance such as infection efficiency, latent period, lesion size and sporulation capacity (Parlevliet, 1992). Component analysis of quantitative resistance to soil-borne pathogens is not as well characterised. The very fact that infection and pathogenesis proceed unseen in the soil hinders observational assessments. For this reason disease incidence, as measured by the number of wilted and dead plants, is most often used as the total aggregate of the components contributing to quantitative resistance to sclerotinia wilt in sunflower.

Under field conditions greater uniformity and repeatability of results was reported when 'clean' fields were artificially infested (Pirvu *et al.*, 1985) or inoculum was added at sowing to supplement sclerotia already present (Huang, 1981; Gulya, 1985*a*). Mancl and Schein (1982) buried inoculum beside the stems of field grown plants in an attempt to achieve uniform inoculum application. This technique was later adapted and modified by others (Tourvieille de Labrouhe and Vear, 1984; Sedun and Brown, 1989). The method is laborious and only a relatively small number of plants can be efficiently inoculated unless a plentiful labour force is available. The effort involved increases dramatically when the rate of lesion extension is used as the assessment parameter as suggested by Sedun and Brown (1989) rather than disease incidence.

The reliability of field trials can be reduced by factors beyond the control of the experimenter. Consequently many other methods have been tested in the selection of sunflower for resistance to sclerotinia wilt. The choices seem to be based primarily on convenience and the desire to experiment with new methods. Problems occur were the assessment parameter requires that the stem be infected. It is irrelevant whether a lesion on the stem of one line of young sunflower plants extends at 8mm.d^{-1} while on another the lesion extension is 16mm.d^{-1} because ultimately both plants will be killed before any yield is obtained or the plants can be used for breeding purposes. Destructive selection methods can only be applied on relatively fixed germplasm and is unsuitable for the selection of segregating material early in the breeding program. One interesting exception might be the method used by Robert *et al.*(1987) where seedlings are decapitated and the wound on the stem base is inoculated. The excised stems might be induced to develop roots while the inoculated stem bases are incubated. Rooted upper stems from stem bases exhibiting a low rate of lesion extension could be grown on for breeding purposes.

Most tests indicated that differences in reaction between genotypes could be shown unless the infection pressure was too great. Plant breeders prefer to screen seedlings because a large number can be processed and only those exhibiting a degree of resistance will be grown on and used in breeding. The introduction of petiole pieces, mycelial suspension or toothpicks colonised with *Sclerotinia* into stems successfully differentiated genotypes only when the plants were past early seedl ng stages of growth (Auger and Nome, 1970; Grauert *et al.*, 1980; Cuk, 1976). Such methods can however be criticised because they do not allow expression of any anatomical and biochemical resistance mechanisms active in the stem and may be even less well correlated to the reaction of roots. Inoculation techniques that involve wounding would prevent expression of the cuticular resistance found by Pirvu *et al.* (1985). Similarly, better extrapolations between laboratory and field evaluations were found when soybean and peanus were inoculated without wounding (Chun, Kao and Lockwood, 1987; Melouk, Akem and Bowen, 1992).

Methods that avoid inoculation of sunflower roots would seem to be avoiding the most likely plant organ where resistance would have the greatest effect on disease development. Parlevliet (1992) stressed that a correlation between an assessment parameter and quantitative resistance should be determined before that parameter could be used with confidence to discriminate between breeding lines. In very few cases have the published results of glasshouse screenings been compared to field results.

Indirect methods of selecting sunflower for resistance to sclerotinia wilt have not been extensively tested. Exposure of seedlings, calli and protoplasts to culture filtrates or oxalate also demonstrate genotypic variation and hence the prospect of identifying ideotypes for breeding programs. However, the genotypic differences found have not been shown to be highly correlated to disease incidence in the field. Similarly, the method of using petioles to assess the ability of plants to produce anti-fungal products at high temperatures has been found to be heritable (Martinson, 1992) but how effective such genotypes will be at cooler temperatures where *Sclerotinia* spp are most active remains to be seen. New molecular methodology may make it possible to identify linkages between components of resistance and DNA markers. Changes in mRNA expression levels give an indication of the effect of infection and oxalic acid on host physiology (Mouly and Roby, 1988) but may not be suitable for screening a large number of breeding lines.

In the screening experiments reported in this chapter a number of sunflower inbred lines were screened for resistance to *Sclerotinia minor* using the method of Sedun and Brown (1989). Differences were found between lines for the number of plants killed in the 28 days following inoculation, the time between inoculation and appearance of a lesion at the

soil surface, and the rate of lesion extension along the stem. Of the 56 inbreds tested only three (CM361, 953-88-3 and HA124) had fewer or equal number of plants killed than RHA801 while 28 had a greater mortality than *cms*HA89. Only 2 (Pacific Seeds PacA1 and HA292) had a slower rate of lesion extension than RHA801 while 38 had a rate quicker than *cms*HA89. The longest delay for the checks varied between experiments but overall 13 out of 32 had delays longer than the longest delay for the two check lines. The public lines (CM361, 953-88-3, HA124, HA292) that have been identified here as possible sources of resistance to *Sclerotinia minor* have also previously been found to possess some degree of resistance to *S. sclerotiorum* (Kolte *et al.*, 1976; Fick *et al.*, 1983; Gulya, 1985; Bazzalo, Dimarco, Martinez and Dalco, 1992; Miller, 1992).

Among the sunflower lines derived from interspecific crosses were some with low mortalities and rates of lesion extension not significantly greater (P<0.05) than RHA801. Some lines involving the species *H. exilis* Gray, *H. argophyllus*, *H. annuus* and *H. paradoxus* Heiser were more resistant to *S. minor than* RHA801. Recently, Seiler *et al.* (1993) found interspecific crosses with *H. exilis*, *H. argophyllus*, *H. annuus* and *H. paradoxus* as well as *H. praecox* ssp *runyonii* Heiser, *H. resinosus* Small, *H. petiolaris* ssp *petiolaris* Nuttall, *H. tuberosus* L., *H. anomolous* Blake, and *H. deserticola* Heiser with low levels of mortality to *Sclerotinia sclerotiorum* in field screening nurseries. The roadside wild populations of sunflower in Australia also contain germplasm with resistance to *Sclerotinia minor*. This confirms the observation of Gulya (1985) who included plant introductions from Australia in field nurseries and found that some displayed low levels of mortality.

The failure to find significant correlations between the rate of lesion extension along the stem and time from inoculation to appearance of a lesion at the stem base may be attributed to i) the different organs having different levels of resistance or ii) the variable nature of the data. Support for the first proposition can be found in other studies where poor correlations have been found between the reactions of the different organs of the sunflower plant to *Sclerotinia*. French researchers have tested inoculations of roots, hypocotyls, cotyledons, stems, floral buds and capitula and found very few strong correlations of reaction (Tourvieille de Labrouhe and Vear, 1990). If roots and stems are to considered as separate components then it would seem unreasonable to use stem inoculations or assessment of stem reactions as the sole measure of quantitative resistance to sclerotinia wilt. The parameter 'delay' is assumed to be a measure of the time taken for the fungus to grow along the roots from the site of inoculation to the stem base. Sedun and Brown (1989) preferred to disregard it because it was too variable but this variability may be a product of the inoculation method itself. In their method the inoculum is distributed at depth in a narrow band. The variability might arise because roots at different depths are being

infected. Even in small pots root distribution is not uniform therefore the chance of contacting a suitable root might occu· at different depths. The fungus infecting roots close to the soil surface has to progress a shorter distance than if infecting at depth. The pot base inoculation method introduced in this chapter avoided this problem by placing the inoculum at a common position in relation to the stem base. The pot base inoculation method has all the attributes of the method of Sedun and Brown (1989) but is quicker to perform and allows use of another parameter of assessment.

The inbred line PacA1 was shown in Table 3.7 to have a lower rate of lesion extension and longer delay until lesion appearance than the lines Pac A4 and Pac A2. Hybrids made between these lines and RHA801 were tested in Experiment 3.3.7. The resistance shown by the hybrid PacA1/RHA801 gives an indication that crossing resistant x resistant genotypes may produce resistant progeny. It is impossible to draw many conclusions on the heritability of resistance in the other hybrids since pedigree information was not provided and is, understandably, proprietary sensitive information. Comparison of the performance of hybrids from the different seed companies suggest that Agseeds and Pacific Seeds had available and were using germplasm with some degree of resistance to *Sclerotinia minor* or, at least, they have assessed which germplasm may have resistance. Both companies have participated in the field screenings of sunflower germplasm for resistance to *Sclerotinia minor* conducted by Agriculture Victoria (Dr. I. Porter, *pers. comm.*). The sunflower breeding program of the Queensland Department of Primary Industries (since terminated) focused on incorporating resistance to sunflower rust and alternaria blight (*Alternaria helianthi*) into adapted germplasm (Dr. D. George, *pers. comm.*). Resistance to sclerotinia diseases was not considered a priority.

Experiment 3.3.2 was the first time that the proprietary male sterile line Pacific Seeds PacA1 has been screened in the glasshouse. Following the results of the glasshouse trials showing the resistance of PacA1, RHA801 and the F_1 hybrid between these lines Pacific Seeds Pty Ltd produced the hybrid in larger quantities for experimental purposes. The subsequent hybrid Pac3435 has been trialed in Victoria with good results (Porter and Clarke, 1992) and has been tested in later chapters of this thesis.