Chapter 3

Development of a Greenhouse Assay to Screen Sunflower for Resistance to Alternaria helianthi

Summary

A greenhouse assay to screen sunflower for resistance to *Alternaria helianthi* is described. A comparison of experimental conditions led to the following standard conditions being recommended. The first or second pair of leaves of seedling plants at the V6-V8 growth stage are inoculated using inoculum grown on sunflower leaf extract agar for 5-10 days. Approximately 30 spores are applied to each leaf. A 48h dew period should be applied to plants covered by a plastic tent. A dew period temperature of 26/26°C night/day and a post-dew period temperature relative to that experienced under local growing conditions should be applied. Lesions are measured 7 days after inoculation, and mean lesion size per plant is calculated. Mean lesion size of lines being tested is expressed as a proportion of the mean lesion size of a susceptible standard included in each screening experiment.

3.1 Introduction

Screening and development of germplasm in the greenhouse has generally been avoided for hosts that express quantitative resistance (QR). This is because the expression of QR is often influenced by environmental factors, making it difficult to correlate disease phenoyypes obtained in the greenhouse with those obtained under variable field conditions. In addition, the greenhouse screening techniques required to overcome these difficulties are tedious and time consuming and require special facilities for environment control.

Similarly, the evaluation of germplasm under field conditions is laborious, time-consuming and costly. By screening lines in a greenhouse using a seedling plant assay, the number of lines required for preliminary field testing could be reduced and the speed of identifying resistant germplasm could be increased. Moreover,

greenhouse screening is not restricted by season and can continue year-round, nor is it hindered by the vagaries of weather or the presence of other diseases.

Since the sunflower gene pool appears to contain low levels of resistance to *A. helianthi*, resistant germplasm will have to be developed by using recurrent selection procedures. Initially, this will require the selection of plants that have only a small increase in resistance compared to the base population. Screening techniques must therefore be sensitive enough to detect small differences in resistance. In order to achieve this, variation arising from experimental procedures and conditions must be minimised.

To date, researchers have not adopted a standard screening procedure to screen sunflower lines for their resistance to *A. helianthi*. The production of inoculum, methods of inoculation, the environmental conditions for inoculation and incubation, the age of test plants and plant parts, and the method of disease assessment vary among researchers (Acimovic, 1976; 1979; Islam and Maric, 1980; Morris *et al.*, 1983; Jeffrey *et al.*, 1984; Lipps and Herr, 1986; Carson, 1985a). With the exception of Lipps and Herr (1986), quantitative measurements such as those described by Parlevliet (1976) have not been used. Estimates of disease levels were made without reference to the amount of inoculum applied to each plant. Researchers generally assumed that their inoculation technique was uniform. If their assumption was incorrect then their assessments of resistance to *A. helianthi* may have been inaccurate. Furthermore, the expression of quantitative resistance can be masked when high levels of inoculum are used (Simons, 1972). Therefore, the quantity of inoculum applied to plants should not only be controlled but should also be kept at a moderate level.

In any disease screening programme the infectivity of the inoculum used must be maintained to ensure the reproducibility of inoculation procedures and subsequent infection. Hence, the selection of a culture medium and culturing conditions that fulfil these requirements is essential to the success of the screening procedure. For qualitative assessments the quality of inoculum is less critical because high spore concentrations are commonly applied to plants until run-off to ensure a high level of infection. However, any quantitative procedure will require that inoculum delivery is not only better defined

but also that inoculum quality is high, because low inoculum concentrations will be required in order to detect small differences between plants and accessions. In addition, the loss of pathogenicity of some pathogens grown continuously under axenic conditions is well known (Dhingra and Sinclair, 1985). The use of host substrates and/or the alternation of nutrient `rich' and nutrient `poor' substrates may serve to sustain inoculum quality (Dhingra and Sinclair, 1985).

A. helianthi is known to produce host-specific toxins (Robeson and Strobel, 1985) which may be important in pathogenesis. Rudolf (1976) reported that the nutritive status of artificial media can greatly influence the level of toxin produced by certain fungi. Furthermore, some fungi only produce phytotoxic compounds after a certain period of growth on artificial media (Rudolf, 1976). Thus, the type of media used to produce inoculum and the age of the culture may influence the quantity of toxins produced by the pathogen.

Allen *et al.* (1983a,c) found that the optimum temperature for growth and sporulation, spore germination and infection by *A. helianthi* was in the range of 25-28°C. Abbas *et al.* (1995) also found that conidial infectivity decreased as conidia production temperature increased above 28°C. Allen *et al.* (1983a) further found that exposure to white light increased the number of germ tubes produced per conidium. Media tested by Allen *et al.* (1983c) included potato-dextrose agar (PDA), Richards' solution + 20 grams of agar per litre, PDA + Richards' solution, PDA + sunflower seed extract, and Richards' solution + sunflower seed extract. Each media was assessed according to the mean colony diameter after seven days incubation at 23°C under continuous light. The abundance of mycelial growth and sporulation were assessed by visual observation. PDA + sunflower seed extract was found to give the best combination of growth and sporulation.

Islam and Maric (1978) conducted an extensive study of the influence of temperature, various micronutrients, different sources of carbon, nitrogen and sulphur, and pH on the growth and sporulation of *A. helianthi*. They found that oat, malt extract and potato-dextrose agar were the best media for the growth and sporulation of *A. helianthi*. A pH of 5.3-5.9, the carbon sources, glucose and maltose, and nitrogen supplied as sodium nitrate, asparagine or potassium nitrate gave abundant sporulation. The degree of sporulation was based on visual observation. In their studies, Allen *et al.* (1983a,c) and Islam and Maric (1978) did not compare the infectivity of inoculum produced on the various media tested.

Table 3.1. *A summary of published procedures used by researchers for screening sunflower for resistance to Alternaria helianthi in the greenhouse. (— = not stated)*

	Reference			
Parameter	**Acimovic (1979a)**	**Lipps & Herr (1986)**	**Morris *et al.* (1983)**	**Jeffrey *et al.* (1984)**
Host Genotype Used as a Standard	VNIIMK 8931	Stauffer S 3101 (Commercial hybrid)	none	RBA 3101
Type of Culture Media Used	—	Fresh PDA and V-8 juice agar	PDA	Fresh PDA
Age of Culture	10 days	3 to 4 weeks	2 to 4 weeks	4 weeks
Inoculation Procedure	Spray with spore suspension of unspecified concentration	Spore suspensions of 150, 300, 3000 and 20 000 spores/ml	Spore suspension 10^4 to 10^5 spores/ml	Spore suspension 15, 150, 1500 spores/ml
Inoculation Temperature	—	24°C	16 to 29.4°C	24°C
Length of Dew Period	48 h	48 h	48 h	48 h
Length of Incubation Period	7 days	7 to 9 days	9 days	4 to 5 days
Post-inoculation temp/humidity	—	23 $\pm$ 5°C	7.8 to 41°C 12 to 98% RH	23 $\pm$ 5°C
Age of Plants Used	4 weeks old	V6 to V8 and V12 to V14	V4 to V10	V10
Method of Disease Assessment	Average number lesions on plant parts and % leaf area infected	Average number lesions on plant parts and % leaf area infected	% leaf area infected	% leaf area infected

PDA is commonly used for the production of inoculum by workers screening for resistance to *A. helianthi* (Table 3:1). The age of cultures used for inoculating sunflowers ranged from 9 to 10 days (Acimovic, 1979; Lamarque and Kochman, 1988) to 28 days (Jeffrey *et al.*, 1984). However the environmental conditions under which the inoculum was produced were not stated and in general, no consideration was given to the infectivity of inoculum. Moreover, such high concentrations of inoculum (Table 3:1) were used, that inoculum with a low viability would probably provide adequate infection for most purposes. The lack of control over both the quantity and quality of inoculum used for screening cannot be tolerated in a system where quantitative measurement of resistance is required.

Environmental conditions can affect infection and subsequent disease development and should be strictly controlled. Allen *et al.* (1983d) found that maximum infection of sunflowers occurred at 26°C with a 12h dew period. Furthermore, lesion expansion was promoted by extended periods of leaf wetness. In screening experiments conducted by Morris *et al.* (1983), plants were incubated at temperatures that ranged from 16±29.4°C and 100% RH for 2 days after inoculation. They then incubated these plants for a further 7 days at temperatures that ranged from 7.8 to 41°C with a variation of 12 to 98% relative humidity. Since environmental-genotype interactions are known to alter some types of quantitative resistance (Simmonds, 1991) it is important that a constant and tested environment is used for greenhouse screening experiments. Rotem, (1988a) examined possible sources of environmental variation experienced in controlled environments.

The age of test plants and plant parts can also affect the expression of resistance to *A. helianthi*. Allen *et al.* (1983b) found that plants were more susceptible to *A. helianthi* at anthesis and the seed filling stages of growth. Using a single genotype and isolate of *A. helianthi*, they also found that older leaves were more susceptible than younger and expanding leaves when plants were at the vegetative and budding stages of growth. Lesions that formed on the apical region of young expanding leaves were larger than lesions that formed on the petiolar region of the same leaves (Allen, 1981). These differences may have been due to differences in tissue age or resulted from higher levels of leaf wetness in the

apical region due to leaf orientation, or both. Nonetheless, these factors should be considered to ensure that all plants receive an equal assessment when disease ratings are made.

The environmental conditions applied during the inoculation and incubation periods, the age of test plants and plant parts and the method of inoculation have been given insufficient attention by most workers (Acimovic, 1976; Islam and Maric, 1980; Morris *et al.*, 1983) who have screened sunflowers for resistance to *A. helianthi*. There is a clear need for researchers to adopt a set of standard screening procedures to a) ensure the accuracy and reproducability of their own results and b) allow researchers to compare results.

The benefits of using a greenhouse assay to speed the development of resistant germplasm cannot be overstated and the effort expended in developing such an assay may be worthwhile, providing the accuracy of selection can be verified under field conditions. This chapter describes the development of techniques for screening sunflower germplasm for resistance to *A. helianthi* in the greenhouse. Chapters 5, 6 and 7 deal with the verification of the method derived from this research.

3.2 Materials and Methods

3.2.1 Statistical Analyses of Data

Infection frequency data were fitted to a binomial model with the computer program Genstat (Payne *et al.*, 1987) and an analysis of deviance was performed to determine differences between treatments. Data for lesion size and number were screened for normality and homogeniety of variance (Bartlett's test) using the computer program Systat (Wilkinson, Hill & Vang, 1992) and log-transformed where necessary. Treatment differences were determined using analysis of variance, and pairwise comparisons of treatment means were performed using Scheffe's Significant Difference test (SSD)

3.2.2 Experiments to Standardise the Production of Inoculum

(i) Determining the effect of culture media on mycelial growth, sporulation and conidial infectivity

Sixteen different isolates of *A.helianthi* were grown on eight different media to compare conidial production. The isolates used were obtained from a range of locations throughout Queensland. The media tested included sunflower leaf extract agar (SLEA) prepared from a decoction of 100g sunflower leaves (cultivar B89) per litre of tap water, potato carrot agar (PCA), potato dextrose agar (PDA), malt extract agar (MEA), synthetic medium (SM; Islam & Maric, 1978), cornmeal agar (CMA), oatmeal agar (OA) and V8 juice agar (V8A). The pH of each medium was adjusted to 6.0 using 1M NaOH where necessary. The centre of each of three plates (replicates) of each medium was inoculated with a 6-mm-diameter plug taken from the edge of a colony of each of the sixteen isolates of *A. helianthi*. The cultures were placed in a light box and incubated for 14 days at 25± 2°C under a near-UV light with a 12h photoperiod. The amount of sporulation and the density of mycelial growth were assessed by visual observation using an arbitrary 0-3 scale where 0 = absent, 1 =low, 2 = moderate and 3 = high. Mean colony diameter was also determined. Data for all isolates were pooled to give a single value for each parameter measured on each of the eight different media.

To test whether the infectivity of spores was influenced by the medium on which they were produced, plants were inoculated with spores derived from cultures grown on each of the eight media (9-day-old cultures). Single-spore inoculum was prepared by removing a single spore from each culture with a micro-needle and placing it onto a filter-paper pad (10 mm diameter, 2 mm thick). The first pair of true leaves of 20 plants was inoculated by placing pads, spore surface against the leaf, so that each leaf received four pads (Figure 3.1). Plants were placed in a humidity tent in a controlled environment cabinet and incubated as previously described. Lesion number and size were recorded for each treatment 7 days after inoculation.

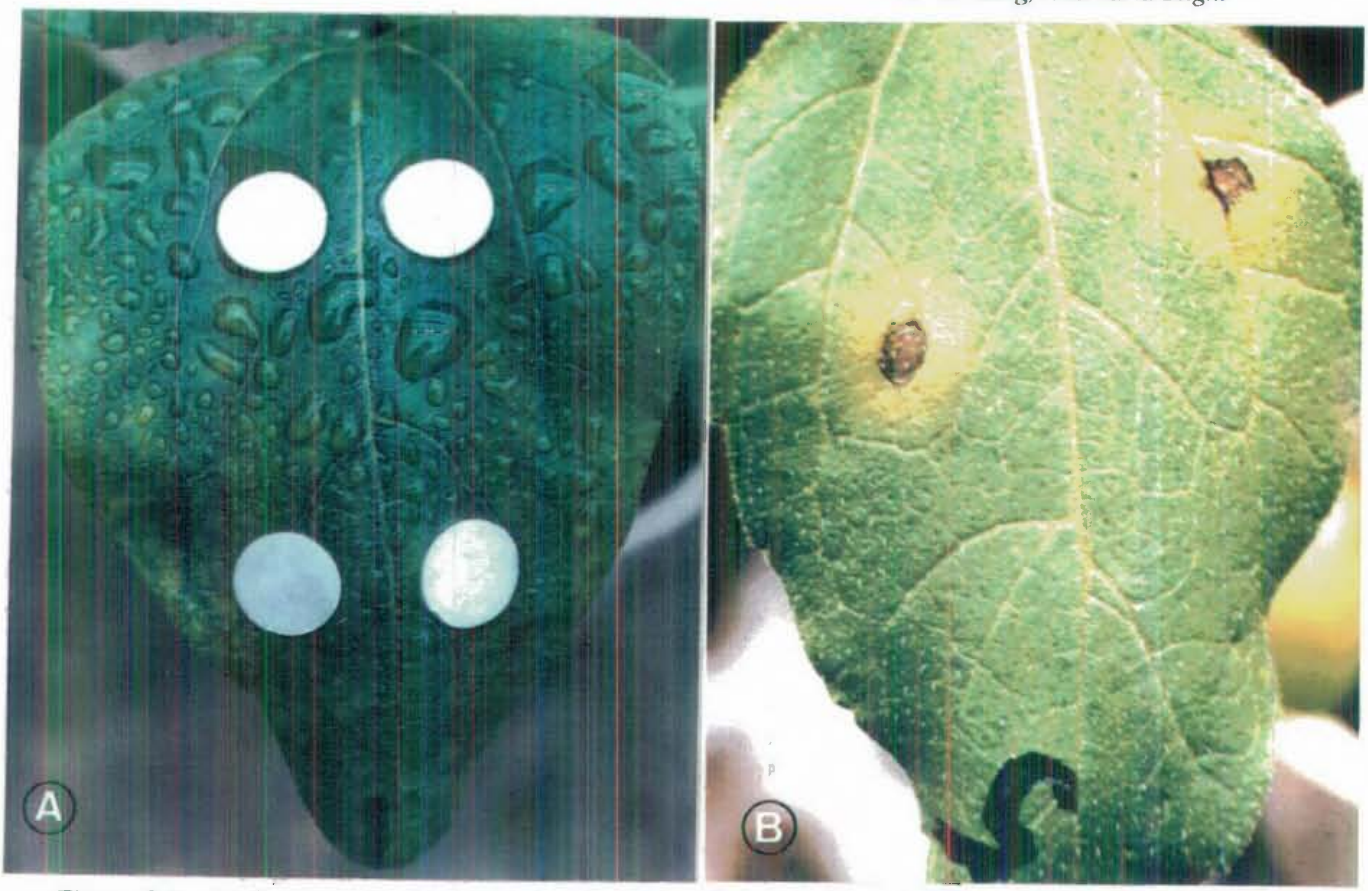

Figure 3.1. **A)** *Sunflower leaf inoculated with moistened pads each containing a single spore of* A. helianthi and **B)** lesions resulting from pad inoculations.

(ii) Determining the germination and infectivity of conidia obtained from sporulation bands of different age

The centres of plates containing SLEA and PCA were inoculated with conidia of *A.helianthi* and incubated at 25± 2°C under a 12h photoperiod of near-UV light. A distinct band of sporulation was produced during exposure of the cultures to the near-UV light. Each sporulation band was separated from the next by a band of very sparse sporulation that corresponded with the 12h dark period. After several weeks, the bands of sporulation formed a pattern of concentric rings that showed the sporulation history of each culture (Figure 3.2). The age of each sporulation band could be determined from its position relative to the centre of the colony, with age decreasing from the centre outwards. Conidial suspensions were made from sporulation bands corresponding to 1-, 4-, 9-, 14-, 20-, and 28- day-old positions in the colony. A drop of each suspension was placed on a 10-mm-diameter 1.5% water agar block (five replicates per treatment) which

was mounted on a microscope slide in a humidity cabinet and incubated under continuous fluorescent lighting at 25± 2°C for 5 h. Conidia were then stained with trypan blue and 100 spores per treatment were examined using a light microscope, to determine the percentage germination for each treatment.

Single-spore inoculum was prepared from sporulation bands corresponding to 5-, 10-, 14- and 28- day-old positions on each medium. The first pair of leaves of 20 plants were inoculated and incubated as previously described. Lesion size and number were recorded 7 days after inoculation.

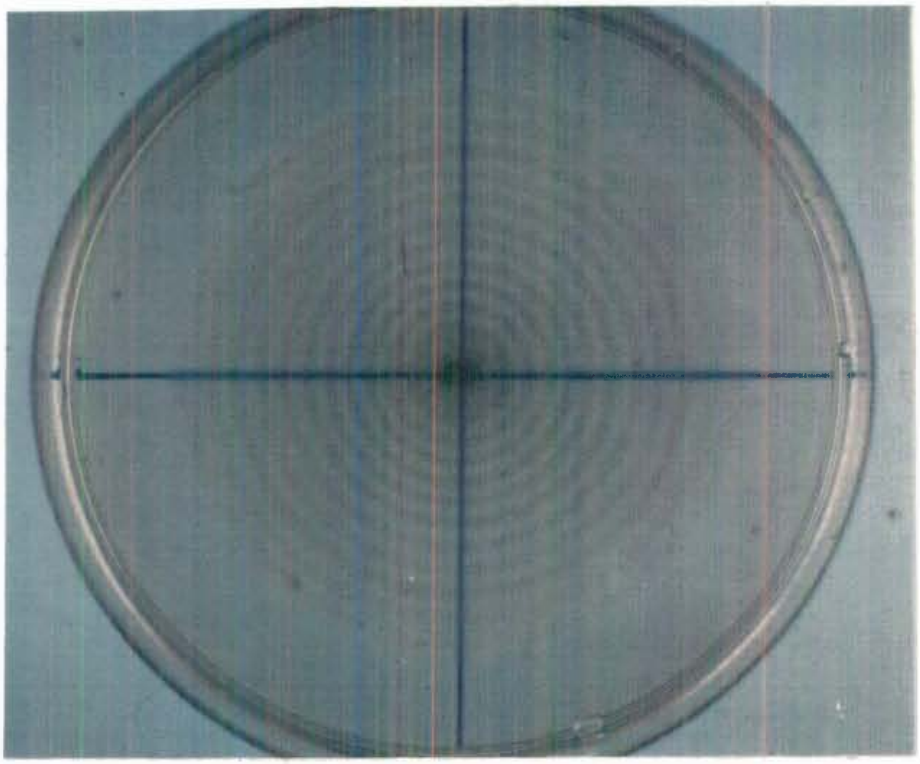

Figure 3.2. *A colony of* A. helianthi *showing the distinct bands of sporulation that formed following exposure to alternate 12h periods of near UV light and darkness.*

(iii) Determining the effect of spore morphology and germination characteristics on infectivity of conidia

Colonies of *A. helianthi* were grown on SLEA and PCA for 9 days at 25± 2°C under a near-UV light with a 12h photoperiod. Conidial suspensions were prepared from sporulation bands corresponding to 9-, 4-, and 1-day-old positions on each medium. A drop of spore suspension from each treatment was placed on a 10-mm-diameter water

agar block (five replicates per treatment) and incubated as previously described. Conidia were then stained with trypan blue and the length of conidia, the number of septa, and the number and length of germ tubes of each of 100 spores (20 from each replicate) were recorded. The second pair of true leaves of 20 plants was inoculated (pad technique) with single spores derived from sporulation bands corresponding to 9-, 4-, and 1-day-old positions on each medium. Plants were incubated as previously described and the number of lesions that developed was recorded 7 days after inoculation.

(iv) Determining the effect of successive subculturing on the infectivity of conidia

Single conidia of an isolate from Capella in central Queensland were removed from leaf lesions and placed on PCA, SLEA and PDA and incubated at 25± 2°C under near-UV light with a 12h photoperiod. Each of these cultures was subcultured every 21 days onto the same type of medium. Leaf tissue infected by the Capella isolate was placed on 1.5% water agar and incubated in darkness for 2 days at 25 ±2°C, before being transferred to the light box with the other cultures for a further 5 days. Spore suspensions were prepared from cultures grown on PCA and SLEA 7 days after the fourth subculture and from the sporulating leaf tissue. A drop of each suspension was placed onto a water agar block (10mm diameter), which was mounted on a glass slide, and incubated at 25 ±2°C in a humid cabinet for 16h. Percentage germination was determined by examining 100 spores from each medium.

Single spore inoculum was prepared from each culture 7 days after the fourth subculture and from the sporulating leaf lesions. The second pair of true leaves of 20 plants was inoculated (pad technique) so that each leaf carried a spore from the PCA and SLEA media. Plants were incubated as previously described. The number and size of lesions produced were recorded. An infection potential for the inoculum was determined by multiplying the number of spores applied by the proportion of spores that germinated *in vitro*. The infection frequency was calculated by dividing the number of infections that developed by the infection potential of spores placed on the leaf.

***(v) Examination of bacteria contaminating cultures of* A. helianthi**

Cultures of *A. helianthi* were found to contain bacteria that appeared to have an inhibitory effect on growth and sporulation. These contaminants were not visually obvious and did not grow beyond the margins of the *A. helianthi* colony and could therefore be easily overlooked. Bacteria were not eliminated from cultures after using single spore or hyphal tipping culture techniques, nor did the application of a range of antibiotics and antibacterial procedures completely eliminate bacteria from cultures (Kong, Kochman and Brown, 1997). Since it was thought that the presence of bacteria could have a detrimental effect on inoculum production and quality, bacteria were isolated so that their effect on *A. helianthi* could be studied more closely.

Bacteria were isolated from healthy sunflower leaves collected from the field, from lesions of *A. helianthi* and *S. helianthi* collected from the field and from cultures of *A. helianthi* isolated from infected field-grown plants. Bacterial isolates were obtained by macerating leaf tissue in sterile distilled water and streaking a 10 µL loopful of the suspension onto nutrient agar media (Becton Dickinson, Cockeysville, MD 21030, USA). A contaminated culture of *A. helianthi* was flooded with sterile distilled water, allowed to stand for 30 minutes, then a 10 µL loopful of the water was streaked onto nutrient agar media. The plates were incubated for 24 h in darkness at 25±2°C.

The plates were then examined and 61 single bacterial colonies were removed and subcultured on nutrient agar and incubated as before. A bioassay was used to observe growth inhibition of *A. helianthi*. Each bacterial isolate was streaked onto 2 nutrient agar plates (replicates) so that the resulting colony developed towards the edge of the plate, as shown in Figure 3.7. These cultures were incubated for two days and were then seeded with spores of *A. helianthi* by inverting 7-day-old cultures of *A. helianthi* over the plates containing the bacteria and lightly tapping to dislodge the *A. helianthi* spores. The plates were then incubated for 5 days in darkness at 25±2°C. Assay plates were examined using a stereo microscope (x100 magnificaton) and light microscope (x400 and x1000 magnification). Antagonism was assessed by measuring the width of the zone of

inhibition around each bacterial streak. Conidia were also removed from 10-day-old cultures containig bacterial contaminants and observed with a light microscope (x400 and x1000 magnification).

Five bacterial isolates (K90/21, K90/23, K90/17, K91/1, K92/2) that showed *in vitro* inhibition of germination of *A. helianthi* conidia were sent to the International Mycological Institute (IMI), Bakeham Lane, Egham, Surrey TW20 9TY, UK for identification. Isolates K90/21 and K92/2 were derived from *A. helianthi* cultures, isolate K90/23 was derived from *A. helianthi* lesions and isolates K90/17 and K91/1 were derived from healthy sunflower leaves.

(vi) Determining the variation in pathogenicity among isolates

Eight isolates were collected from a range of locations throughout Queensland: Capella site 1 (Ah4), Capella site 2 (Ah47) and Arcturus (Ah27) in the Central Highlands; Clifton (Ah20) and Roma (Ah31) on the eastern and western Darling Downs respectively; Gatton (Ah7) in the Lockyer Valley; Monto (Ah51) located in the hinterland midway between the Darling Downs and Central Highlands; and Rosslyn Bay (RbAh7l) on the central coast. A further isolate from the Ord River (Ah59) in Western Australia was also selected for this study. The second pair of leaves of plants of the sunflower line B89 were spray-inoculated with spores of each isolate using an airbrush, 25 to 30 plants per isolate. The inoculated plants were divided into two groups of 12 to 15 plants per group and incubated in separate growth cabinets for 48h. Lesion number and size were measured 7 days after inoculation.

3.2.3 Experiments to Establish Optimal Host and Environmental Conditions for Infection

(i) Determining the effect of leaf position on lesion size

The first, second and third pair of true leaves of 12 plants (V8-V10 growth stage) were spray inoculated with an airbrush as previously described (Chapter 2, section 2.5.2). Four

plants were inoculated with each of the three isolates collected from Clifton (isolate Ah20), Monto (isolate Ah21) and Capella (isolate Ah24) in Queensland. Plants were incubated as previously described (Chapter 2, section 2.5.3). Forty lesions on each leaf pair were measured 3 days after inoculation.

(ii) Determining the effect of the method used to maintain leaf wetness on lesion size

Fifteen plants at the V8 growth stage were used to determine the effect of three methods of maintaining leaf wetness, on infection by *A. helianthi*. The second pair of true leaves of ten plants were inoculated using an airbrush. Five of these plants were placed in a greenhouse mist cabinet (Defensor R mister) and kept at 28± 4°C and five plants were placed in a humidity tent in a controlled environment cabinet and kept at 26±1°C. A further five plants were inoculated using the pad technique (four pads per leaf) and placed in the same humidity tent (26±1°C) as the 5 spray-inoculated plants. After 48h all fifteen plants were transferred to a growth cabinet and incubated for a further 24h at 20/26± 1°C night/day temperatures. Lesions on all plants were measured at 72h after inoculation.

(iii) Determining the effect of dew period temperature regimes and duration of dew period on infection

Two sunflower lines, B89 which is susceptible to *A. helianthi* and 10020 which has some resistance to *A.helianthi* (see Chapter 4), were used for this experiment. Thirty plants of each line (three plants in each of 10 pots) were inoculated with an airbrush and placed in humidity tents in each of three growth cabinets set at 20/26, 26/26 and 26/30 ± 1°C night/day temperatures respectively. Five pots (15 plants) of each line were removed from the humidity tents after 24h and transferred to another growth cabinet set at 20/26 ± 1°C night/day temperatures. The remaining five pots in each cabinet were removed after 48h and placed in the second cabinet with the other plants. Lesions on all plants were counted and measured at 7 days after inoculation.

(iv) Determining the effect of post-dew period temperature regimes on lesion size

Fifteen pots of each of the sunflower lines B89 and 10020 were inoculated with an airbrush and incubated for 48h. Five pots of each line were then placed in each of three growth cabinets set at either 20/26, 26/26 or 26/30 ± 1°C night/day temperatures respectively and incubated for a further 5 days. Lesions were measured at 7 days after inoculation.

(v) Determining the effect of plant density on infection

Seventy-five plants (3 plants in each of 25 pots) of the sunflower line B89 were grown to the V8 growth stage. Prior to inoculation, two plants were removed from each of eighteen pots by cutting the stem at soil level. Plants in the remaining seven pots were left intact. These two treatments were regarded as low (one plant per pot) and high (three plants per pot) plant densities. Plants in the two density categories were spray-inoculated using an airbrush and incubated on separate sides of the same growth cabinet. Pots were arranged so that each pot was touching its neighbour. Lesion number and size were measured at 7 days after inoculation.

(vi) Determining the effect of inoculum dose on infection of susceptible and resistant lines.

Spore suspensions containing 75, 150, 300 and 600 spores per mL were prepared from a seven-day-old culture of *A. helianthi*. The second pair of leaves of plants of B89 (susceptible) and 10020 (resistant) were spray inoculated with the different spore concentrations, so that each leaf received 0.2 mL of a particular spore suspension. Leaves therefore approximately received either 15, 30, 60 or 120 spores. Each pot of three plants represented a treatment. Pots were arranged in a randomised complete block design of four replicates and incubated in a controlled environment cabinet as previously described. All lesions were measured at 7 days after inoculation.

3.3 Results

3.3.1 Experiments to Standardise the Production of Inoculum

(i) Effect of culture media on mycelial growth, sporulation and conidial infectivity

Mean values for sporulation and colony diameter were greatest on SLEA, PCA and V8A media (Table 3.2). The density of mycelial growth was least on SLEA. Moderate to high densities of mycelium occurred on PDA and SM, resulting in thick felty colonies, but radial growth was slow.

Table 3.2. *Effect of culture media on growth and sporulation of* A. helianthi *after 14 days at 25 ±2° C+. Mycelium density and sporulation were rated on an arbitrary scale of 0 to 3 where 0 = absent, 1 = low, 2 = moderate and 3 =high. Colony diameter means followed by the same letter are not significantly different.*

Type of Media	Mycelium Density	Sporulation	Mean Colony Diameter(mm)
Malt extract agar (MEA)	1.7 b	2.0 bc	12.6 e
Potato dextrose agar (PDA)	2.3 a	2.3 b	18.7 c
Oatmeal agar (OA)	2.0 ab	1.9 bc	15.2 d
Cornmeal agar (CMA)	1.7 b	1.6c	16.3 c
Potato carrot agar (PCA)	1.5 b	2.8 a	35.5 b
Sunflower-leaf extract agar(SLEA)	1.0 c	2.8 a	34.0 b
V8 juice agar (V8A)	2.0 ab	2.7 a	45.5 a
Synthetic media (SM)	2.3 a	2.0 bc	15.5 d

+ under near UV light with a 12h photoperiod

Table 3.3 shows the percentage infection caused by conidia produced on the eight different media. Infectivity of conidia collected from SLEA was significantly greater than that caused by conidia from all the other media tested. Lesions resulting from conidia grown on the various media were not significantly different in size.

Table 3.3. *Effect of culture medium on the infectivity of conidia of* Alternaria helianthi. *Means followed by the same letter are not significantly different.*

Medium	% Infection	Mean Lesion Size (mm^2)
SLEA	68 a	4.15 a
PCA	50 b	4.02 a
PDA	50 b	3.73 a
MEA	45 b	3.65 a
SM	40 b	3.60 a
CMA	30 c	3.90 a
OMA	20 c	3.62 a
V8A	20 c	4.12 a

(ii) Germination and infectivity of conidia obtained from sporulation bands of different age

The percentage germination of conidia decreased steadily when taken from sporulation bands more than 4 days old (Figure 3.3). There was no significant difference in the percentage germination of conidia obtained from sporulation bands of the same age on SLEA and PCA.

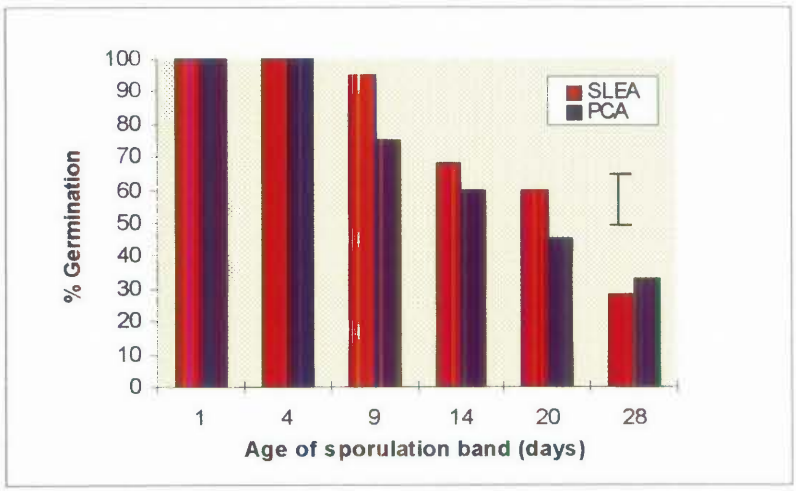

Figure 3.3. *Germination of conidia of* Alternaria helianthi *obtained from sporulation bands of different age on PCA and SLEA. Bar = Standard error.*

Conidia taken from the 5-day-old sporulation band on SLEA had the greatest infectivity (70%). The infectivity of conidia produced on SLEA decreased steadily as the age of the sporulation band from which conidia were taken increased (Figure 3.4). There was no significant difference in the number of infections caused by conidia obtained from 5-, 10- and 14-day-old sporulation bands on PCA. Lesions resulting from conidia produced on 5-, 10-, 14- and 28-day-old sporulation bands were not significantly different in size (Figure 3.4).

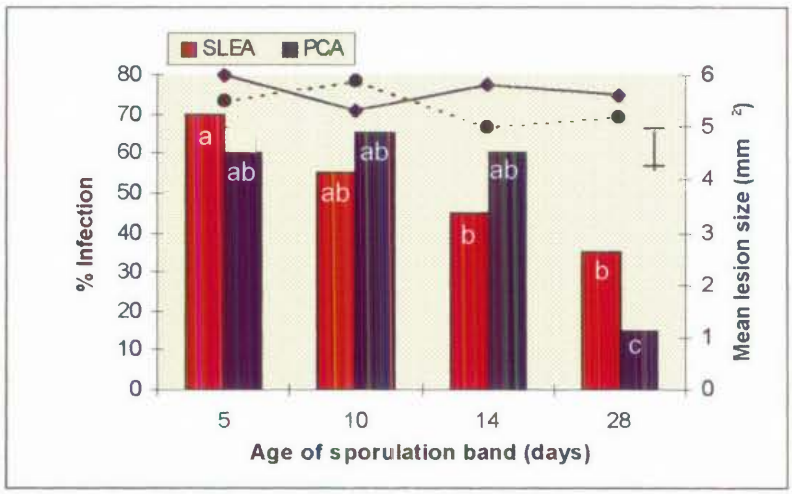

Figure 3.4. *Infectivity of conidia of* Alternaria helianthi *obtained from sporulation bands of different age on PCA and SLEA. For lesion number,* ◆ *= line B89 and* ● *= line 10020. Bar = Standard error.*

(iii) Effect of spore morphology and germination characteristics on infectivity of conidia

Conidia from 4- and 9-day-old sporulation bands were larger and possessed more septa than those from the 1-day-old sporulation band (Table 3.4). The percentage germination of conidia from the 9-day-old sporulation band on PCA was significantly lower than that of conidia from all other sporulation bands. The mean number of septa per conidium increased with the age of the sporulation band on which the conidia were produced. The mean number of germ tubes produced by germinating conidia was greatest for conidia from 4-day-old sporulation bands. Germ tubes produced by conidia from the 9-day-old sporulation bands on PCA and SLEA were significantly shorter than those produced by conidia from 4- and 1-day-old sporulation

bands on PCA and SLEA. Conidia from the 4-day-old sporulation band on SLEA had the greatest infectivity.

Table 3.4. *Morphological, germination and infection characteristics of conidia of* Alternaria helianthi *obtained from sporulation bands of different age on* [a]*PCA and* [b]SLEA. *Means followed by the same letter are not significantly different.*

Age of sporulation band	Type of medium	Spore length (mm)	Mean no. of septa/ conidium	Germination (%)	Mean no. of germ tubes/ conidium	Mean length of germ tubes (mm)	Infection (%)
1-day-old	PCA	79.7 c	6.7 d	100 a	4.7 c	141.0 a	5.5 c
	SLEA	64.9 d	4.4 e	97 a	3.5 d	123.5 b	5.5 c
4-day-old	PCA	92.5 a	7.5 bc	100 a	6.5 b	123.8 b	60 b
	SLEA	92.0 a	7.7 b	100 a	8.4 a	81.7 c	85 a
9-day-old	PCA	88.9 a	7.3 c	70 b	2.3 e	65.0 e	65 b
	SLEA	93.0 a	8.0 a	93 a	5.1 c	51.2 d	65 b

[a]PCA = potato carrot agar
[b]SLEA = sunflower leaf extract agar

(iv) Effect of successive subculturing on infectivity of conidia

The *A. helianthi* isolate grown on PDA failed to sporulate after the fourth subculture and therefore could not be used for the infectivity experiment. The infection frequencies of conidia grown on PCA, SLEA and leaf tissue were 22.7%, 30.1% and 76%, respectively. Conidia produced on leaf tissue were significantly more infective than conidia obtained from the subcultured isolate. Lesions caused by conidia produced on leaf tissue were significantly larger (3.1mm^2) than lesions produced by conidia subcultured on PCA (1.3mm^2) and SLEA (1.8mm^2)

(v) Inhibition of* A. helianthi *caused by bacteria isolated from sunflower leaves and* A. helianthi *cultures.

Thirty-eight of the 61 bacterial isolates tested induced some degree of germination and growth inhibition of *A. helianthi*. Table 3.5 shows the number of bacterial isolates that caused inhibition zones in each of seven size categories. Isolates obtained from both the *S. helianthi* lesions and the *A. helianthi* cultures caused inhibition zones of between 20

and 30 mm. Isolates obtained from *A. helianthi* lesions varied from causing no inhibition (two isolates) to causing an inhibition zone of 22 mm (one isolate).

Table 3.5. *Isolates screened for antagonism to* A. helianthi in vitro *grouped according to the size of inhibition zones caused by each.*

Number of isolates	Mean size of inhibition zone (mm)
23	0
13	5-10
7	11-15
5	16-20
5	21-25
7	26-30
1	35-40

A variety of unusual features was observed in the zones of inhibition. These included reduced conidial germination, germ-tube swelling causing vesicle formation (Figure3.5) excessive germ-tube branching, lysis of germ-tubes, absence of sporulation and a reduced rate of hyphal growth.

Bacteria were observed inside conidia removed from 10-day-old cultures of *A. helianthi*. Cells of conidia occupied by bacteria were translucent in appearance. The vigorous movement of particles within these cells indicated that they contained large numbers of bacteria. Some of these cells burst open, presumably from the pressure exerted by the glass cover slip on the weakened cell walls, allowing a stream of bacteria to issue from the rupture in the cell wall (Figure 3.6). After some minutes the conidial cell was emptied of its contents and appeared deflated. The conidium wall appeared thin and almost clear.

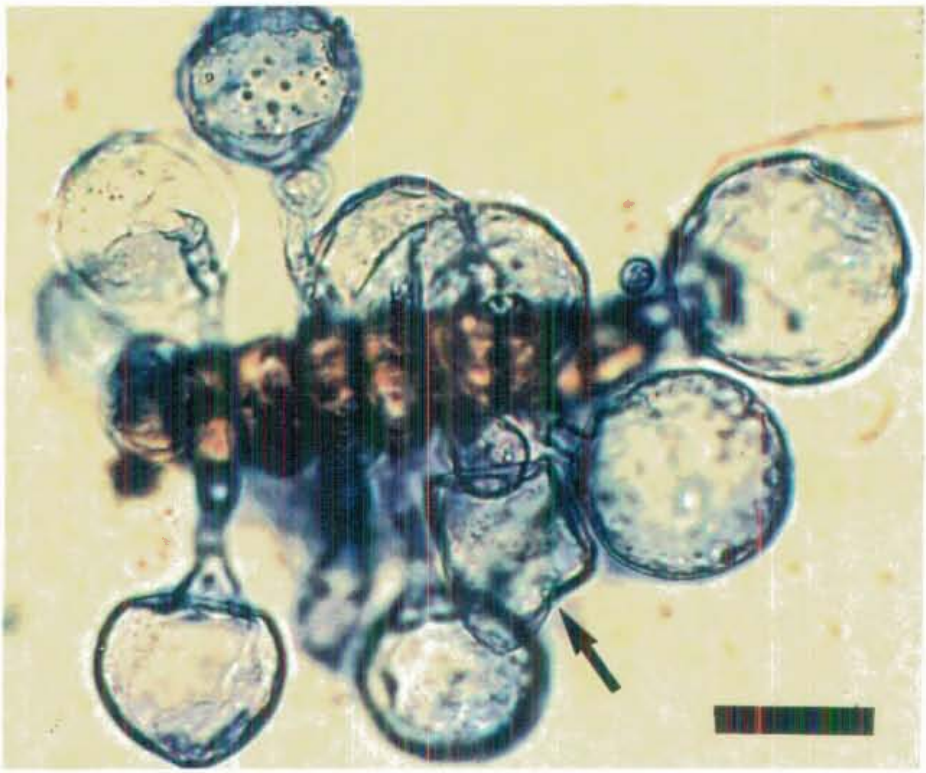

Figure 3.5. *Vesicles caused by* B. subtilis *(K90/23); a ruptured vesicle (arrowed). scale bar = ca 20μm.*

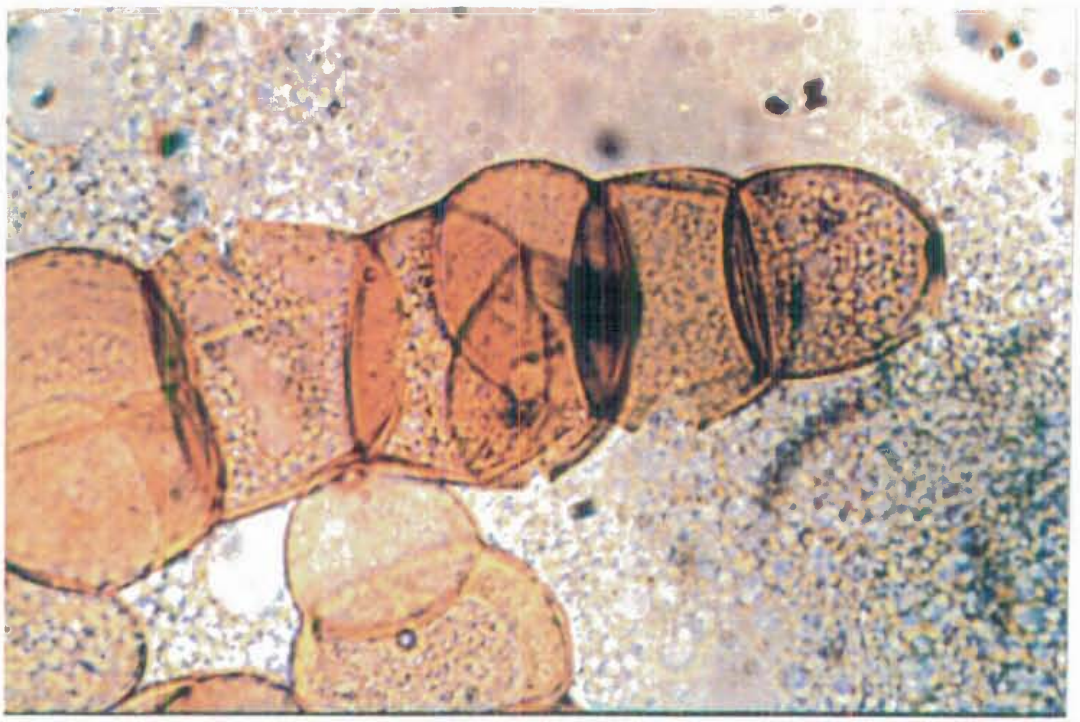

Figure 3.6. *Bacteria oozing from the lumen of a ruptured conidium of* A. helianthi.

Table 3.6 gives the identities assigned to the five isolates by Dr G.S. Saddler (IMI). All five isolates were members of the genus *Bacillus*. The extent of inhibition of growth of *A. helianthi* caused by bacterial isolate *B.cereus* (K90/17) is shown in Figure 3.7.

Table 3.6. *Identifications of five isolates of bacteria that inhibited germination and germ-tube growth of conidia of* A. helianthi *in vitro.*

Identification	Accession number
Bacillus subtilis (Ehrenberg,1835) Cohn, 1872, 174 AL	K90/21
Bacillus subtilis (Ehrenberg,1835)Cohn, 1872 174 AL	K90/23
Bacillus cereus (Frankland & Frankland1887 AL.	K90/17
*Bacillus sp.*Cohn, 1872	K91/1
Bacillus mycoides Flügge 1886 AL	K92/2

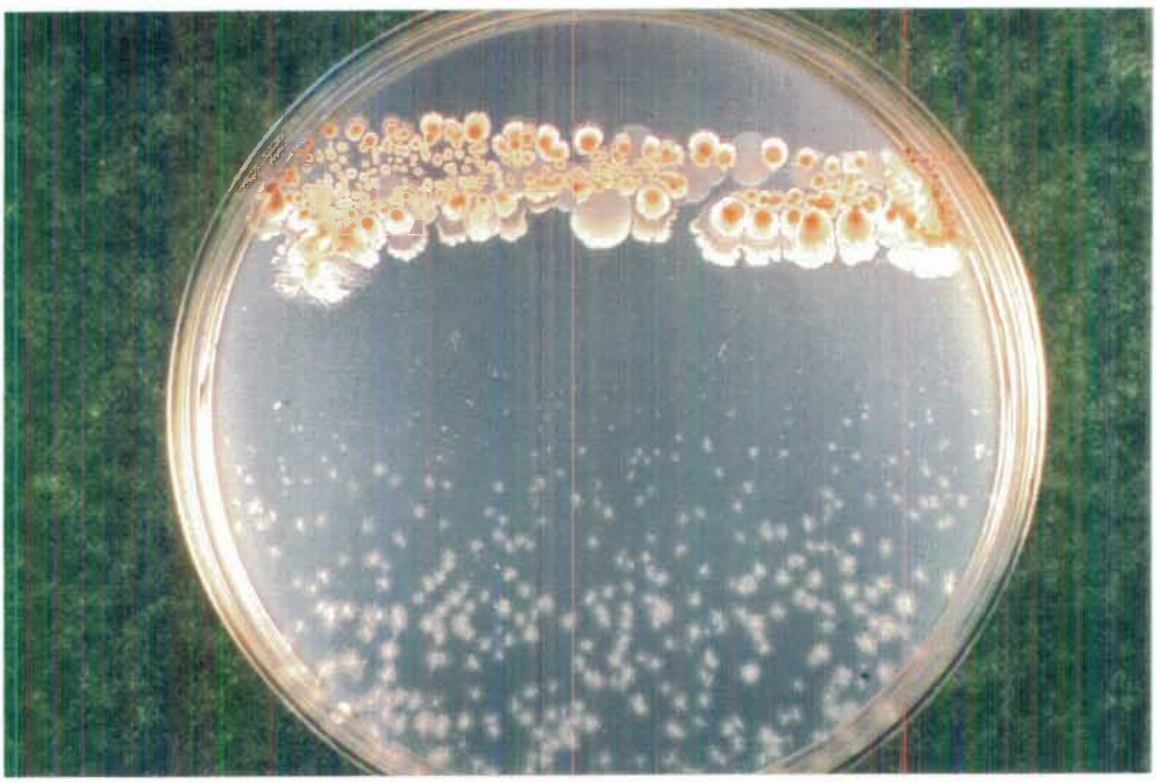

Figure 3.7. *Bioassay plate showing zone of inhibition of germination and growth of* A. helianthi *caused by* B.cereus *isolate K90/17.*

(vi) Variation in pathogenicity among isolates

The mean size and number of lesions were not significantly different between the two inoculated groups of plants so the data from each group were pooled (Table 3.7). For lesion size, two groups of isolates were detected, the first consisting of isolates Ah7, Ah20, Ah27, Ah31, Ah51 and the second consisting of isolates AH4, RBAh7l, Ah47, Ah59. Although these two groups were significantly different, the difference in mean lesion size from the smallest (RBAh7l) to the largest (Ah51) was only 0.94mm^2. For lesion number, three groups of isolates were detected as indicated by the letters assigned to individual isolate means in Table 3.7. Overall, isolates Ah20 and Ah50 were more pathogenic in lesion size and number, while Ah4, Ah47 and Ah59 were less pathogenic in both lesion size and number.

Table 3.7. *Mean number and size of lesions of nine isolates of* Alternaria helianthi *on the sunflower line B89. Means followed by the same letter are not significantly different.*

	Lesion size (mm^2)			Lesion number		
Isolate	LS Mean	Standard Error	DF	LS Mean	Standard Error	DF
Ah4	3.40 b	0.164	27	11.82 c	1.374	27
Ah7	3.66 a	0.174	24	14.79 b	1.457	24
Ah20	3.96 a	0.164	27	23.67 a	1.374	27
RbAh7l	3.11 b	0.174	24	16.54 b	1.457	24
Ah27	3.63 a	0.174	24	17.25 b	1.457	24
Ah31	3.71 a	0.178	23	16.22 b	1.488	23
Ah47	3.27 b	0.174	24	12.50 c	1.457	24
Ah51	4.05 a	0.178	23	23.96 a	1.488	23
Ah59	3.45 b	0.182	22	12.96 c	1.522	22

3.3.2 Experiments to Establish Host and Environmental Conditions

(i) Effect of leaf position on lesion size

The lesions produced on the first, second and third pair of leaves by isolates Ah21 and Ah24 were significantly different in size (Figure 3.8). However, there was no difference in the size of lesions produced by these two isolates on the same leaf pair. Lesions produced by isolate Ah20 on the first and second leaf pairs were of the same size, but

lesions produced on the third pair of leaves were significantly smaller than those on all other leaf pairs.

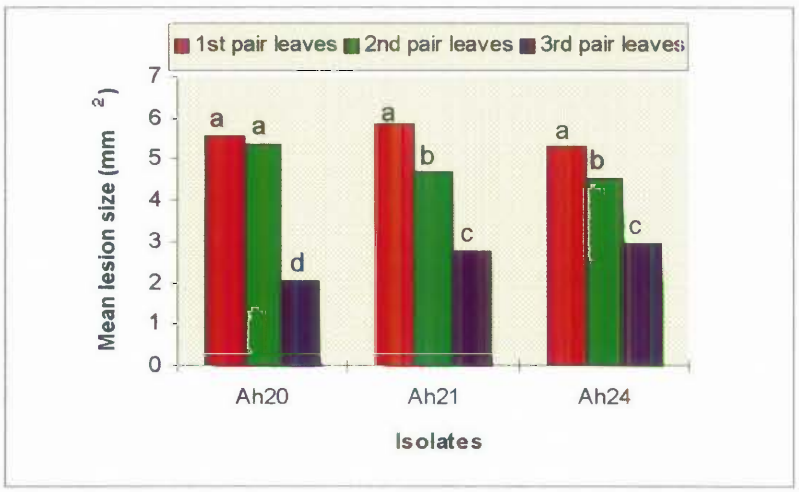

Figure 3.8. *Effect of leaf position on lesion size on sunflower plants inoculated with three isolates of* Alternaria helianthi. *Mean lesion size followed by the same letter are not significantly different.*

(ii) Effect of the method used to maintain leaf wetness on lesion size

The mean sizes of lesions resulting from pad, mist cabinet and humidity tent inoculations were significantly different from each other, being 28.6, 11.8, and 6.2mm^2 respectively.

(iii) Effect of dew period temperature regimes and duration of dew period on infection

Lesions were significantly smaller on the line 10020 than on B89 at all temperatures and at both dew periods (Figures 3.9 and 3.10). There was no significant difference in the size of lesions formed on B89 at the three different temperature regimes when a 24h dew period was applied (Figure 3.9). The largest lesions were formed on B89 when a 48h dew period and temperature regimes of 20/26 and 26/26°C were applied (Figure 3.10). Lesions on 10020 were largest at the 26/26°C temperature regime when a 48h dew period was applied (Figure 3.10).

The number of lesions formed on B89 continued to increase with temperature for both dew periods. However, there was no significant difference in the number of lesions

(iv) Effect of post dew period temperature on lesion size

Lesions were significantly larger on the sunflower line B89 than on 10020 at all temperatures. On both lines, lesions were significantly larger at 26/26°C than at the other temperatures tested (Figure 3.11). Some blighting was evident on both lines at the 26/30°C temperature regime.

(v) Effect of plant density on infection

Lesions produced at the high plant density were significantly larger (6.0mm^2) than lesions produced at the low plant density (4.8mm^2). The mean number of lesions per plant resulting from the high (46.3) and low (47.5) plant densities were not significantly different.

(vi) Effect of inoculum dose on infection of susceptible and resistant lines

Lesions were larger and more numerous on B89 than on 10020 at all inoculum dose rates. Lesion number and size were constant at inoculum dose rates of 15 and 30 spores/leaf for both B89 and 10020 (Figure 3.12). Lesion number was also constant at inoculum dose rates of 60 and 120 spores/leaf for both lines. The largest lesions were formed on B89 inoculated with 120 spores/leaf.

Lesions on 10020 were less than half the size of those on B89 at inoculum dose rates of 15 and 30 spores/leaf, but were 68% and 93% of the size of lesions on B89 at 60 and 120 spores/leaf respectively (Table 3.8). The number of lesions on 10020, expressed as a proportion of the number on B89, was reasonably constant at all inoculum dose rates.

The decline in the germination frequency of conidia from sporulation bands of different ages was followed by a similar decline in the ability of conidia to initiate infection. However, a high germination percentage did not ensure a high level of infection since conidia from 1-day-old sporulation bands caused a low level of infection despite showing 100% germination and longer germ tubes than conidia from older sporulation bands. There appears to be no clear relationship between morphological and germination characteristics of conidia and their infectivity. Infectivity is probably a function of the physiological state of conidia. The substrate on which the fungus was grown influenced the age at which conidia reached peak infectivity. This peak occurred in conidia at around 4 and 10 days of age when *A. helianthi* was grown on SLEA and PCA respectively. Acimovic (1979a;b) and Lamarque & Kochman (1988) found that conidia collected from 10-day-old and 7-9-day-old cultures, respectively, gave a high percentage infection. The age of cultures used by other researchers (Morris *et al.*, 1983; Jeffrey, Lipps & Herr, 1984; Lipps & Herr, 1986) varied from 14 to 28 days old.

The attenuation and decline in pathogenicity of cultures successively subcultured on artificial media suggests that inoculum used in screening experiments should be obtained from cultures in their first generation on artificial media. Similar observations were made in relation to *A. dauci* (Kühn) that was repeatedly cultured on V8 or PDA media (Strandberg, 1987). The build up of bacteria in older cultures of *A. helianthi* may be responsible for this decline in growth, sporulation and pathogenicity.

Certain bacteria that were isolated from healthy sunflower leaves and from lesions of both *A. helianthi* and *S. helianthi,* were antagonistic to *A. helianthi* in culture. A range of unusual morphological characteristics were observed among conidia exposed to antagonistic bacteria. These included excessive germ-tube swelling and branching, vesicle formation and germtube lysis. Swinburne Barr and Brown (1975) found that antifungal compounds produced *in vitro* by *B. subtilis* caused germ-tubes of *Nectria galligena* Bres. to swell and burst. Germ-tube malformation and/or swelling due to

bacterial metabolites has also been observed in *C. sativus* (Fradkin and Patrick, 1985), three cereal rusts (Morgan, 1963) and *B. maydis* (Sleesman and Leben, 1976). Many of the bacterial isolates antagonistic to *A. helianthi* also caused germ-tube malformation, excessive branching and swelling resulting in the formation of vesicles that sometimes burst. Germinating conidia exhibited some or all of these characterisics depending on their proximity to the bacterial colony. This gradual transition was probably due to a concentration gradient of the antagonistic metabolites produced by the bacteria. Several possible causes of the antagonisms described here, were investigated by Kong, Kochman and Brown, (1997).

As well as antagonism caused by bacterial metabolites, bacteria may also have been endoparasites of *A. helianthi.* The presence of bacteria within the lumen of ungerminated, intact conidia, suggests that bacteria either entered conidia through small perforations in the conidium wall, or were incorporated in conidial cells during conidiogenesis. Since the literature contains few reports of parasitism of fungi by bacteria, these observations are unusual. Levine, Bramberg and Atkinson (1936) reported that a species of *Bacillus* was parasitic on the urediniospores of *Puccinia* sp. on cereals. Pon *et al.* (1954) found a species of *Xanthomonas* that was parasitic on urediniospores of *Puccinia graminis* Pers. f.sp. *tritici* Eriks & Henn. The relationships between antagonistic/endoparasitic bacteria and *A. helianthi* were more fully investigated by Kong, Kochman and Brown, (1997). The work presented here clearly indicates that bacteria are difficult to exclude from cultures of *A. helianthi* and can reduce the viability of inoculum used for screening experiments.

The greater susceptibilty of older leaves reported by Allen *et al.* (1983b) was confirmed in this study using three isolates of *A. helianthi*. Age-conditioned susceptability has been reported in many *Alternaria*-host systems (Rotem, 1994) and many hosts show a pattern where seedling and mature plants are highly susceptible, but young to middle-aged plants exhibit a level of resistance. These factors of leaf and plant age have not been given due consideration by researchers using greenhouse screening assays. It is suggested therefore that the assessment of resistance should be confined to particular leaf pairs, preferably to

the lower and fully expanded leaves, of plants at the same growth stage. Genotype comparisons should only be made when there is uniformity for these factors.

Lesion size was found to be related to the method used to maintain leaf wetness during incubation. Leaf wetness maintained by continuous misting or by the application of a moistened pad containing inoculum resulted in larger lesions than when leaf wetness was maintained by ensuring high relative humidity with a plastic tent. Furthermore, the increase in the dew period from 24h to 48h at temperatures of 20/26°C and 26/26°C (night/day) caused an increase in the size of lesions on the susceptible line B89. Using a single genotype, Allen, Brown & Kochman (1983d) found that a dew period of 18h at 22°C was required to cause the same number of infections as that achieved in 12h at 25°C. Islam & Maric (1980) and Acimovic (1979b) found that the number of infections increased with increasing dew period up to 72h. The partially resistant line (10020) used in this study, showed little response in terms of lesion size and number to the different temperature regimes and dew periods applied. For both B89 and 10020, the combination of high temperature (26/30°C night/day) and long dew period (48h) resulted in smaller lesions than those formed at the lower temperatures and the same dew period. However, blighting was promoted by these conditions, and was characterised by the sudden collapse of large areas of tissue surrounding a lesion.

A constant temperature of 26°C during the dew and post-dew period was optimum for lesion size on both the susceptible and partially resistant sunflower lines tested. Dew period temperature had a greater effect on the number of lesions formed on the susceptible line than on the partially resistant line. These factors indicate the importance of environmental conditions to the infection and development of *A. helianthi* on sunflower leaves. The duration of the dew period, the dew period temperature and post-dew period temperature should be standardised and strictly controlled when screening for resistance.

The conditions used by workers screening sunflowers for resistance to *A. helianthi* vary considerably, as is shown in Table 3:1. The wide and uncontrolled ranges in temperature and relative humidity that occur in the greenhouse are highlighted in the screening conducted by Morris *et al.* (1983).

Carson (1986) showed that under field conditions disease severity increased with planting density. In the growth cabinet studies reported here, the density of plants influenced lesion size. Lesion number did not differ between the two plant densities, probably because conditions for infection were optimum. The density of foliage probably modifies the microenvironment resulting in higher temperature and humidity and longer periods of leaf wetness, thus favouring disease development. Differences in plant architecture among genotypes might therefore cause different environmental modifications which in turn may cause different levels of disease. Care should therefore be taken not to unduly alter plant density between successive screening experiments.

In any quantitative assay, it is important that the number of spores applied to leaves is suitable for revealing differences in resistance. Few, if any, researchers have given adequate attention to the effects of inoculum dose on infection. Jeffrey *et al.* found that a high spore concentration (1500 spores/mL) caused lesions to coalesce which prevented an accurate measurement of infection. However, they did not state the amount of spore suspension and hence, inoculum dose, actually applied to each leaf. Using a microdrop technique, Carson and Medhi (1983) found that the infectivity of conidia decreased with increasing concentrations of spores applied. However neither the spore concentrations nor the volume of the "microdrop that they used" were stated. Hence, their observations may have been due to the technique that they used, where large numbers of spores in a "microdrop" of water may have induced self-inhibition of germination resulting in low levels of infection. Germination inhibitors have been reported from *A. brassicicola* (Schw.) Wiltshire (Mukadam, 1982), *A. solani* Sorauer and *A. macrospora* Zimm. (Rotem, 1994).

The effects of inoculum dose and lesion development on a susceptible (B89) and resistant (10020) host were studied to determine an appropriate inoculum load for resistance screening. Results showed that the number of infections and the size of lesions were constant at low spore doses (15 and 30 spores/leaf) on both hosts. At higher spore doses (60 and 120 spores/leaf), lesion number remained constant, but lesions were larger on both hosts inoculated with 120 spores/leaf. At 120 spores/leaf, the difference in lesion size between B89 and 10020 was small relative to the differences at lower spore doses, indicating that resistance was less effective with this inoculum load. The reason why so little resistance to *A. helianthi* has been reported may be because many researchers spray plants until run-off with spore suspensions containing as much as $1x10^6$ spores per mL, thereby causing resistance to be masked. Researchers rarely consider the number of spores that are applied per leaf.

In any disease screening programme, the isolate of the pathogen used to select resistant plants should exhibit a high degree of pathogenicity, so that only the most resistant plants are selected. Among workers screening sunflowers for resistance to *A. helianthi* (Acimovic, 1976; Mehdi *et al.*, 1984; Morris *et al.*, 1983; Lipps & Herr, 1986; Block, 1990), only Lipps & Herr (1986) considered the pathogenicity of the isolates they used. Jeffrey *et al.* (1984) detected considerable variation in the numbers of lesions caused by the ten isolates of *A. helianthi* they tested. Among the *A. helianthi* isolates tested in this study, there was little pathogenic variation for the character of lesion size, but there was considerable variation for lesion number. Isolates that produced the largest lesions (Ah20 and Ah51) also produced more lesions than the other isolates tested.

In this study, isolates were compared on the basis of lesion size and number. If other components of partial resistance had been measured and a range of host genotypes was used, more differences among isolates might have been found. A search of IMI descriptions of fungal pathogens revealed few reports of pathogenic variation among *Alternaria* species. The reason for this may be the insensitivity of assays used to detect pathogenic variation. Since the type of culture medium and the age of inoculum can

affect the infectivity of conidia, care should be taken to standardise culture conditions to ensure that differences in pathogenicity among isolates are independent of differences in culture media and inoculum age. Whether Jeffrey *et al.* (1984) did this is uncertain.

A reduced infection frequency is regarded as one of the components of quantitative resistance (Parlevliet, 1976). However, its use as a measure of resistance is only valid if all plants receive the same amount of inoculum. Although a number of researchers (Shane *et al.*, 1981; Morris *et al.*, 1983; Jeffrey *et al.*, 1984; Block, 1990) quantified the inoculum they used for screening for *A. helianthi* resistance, none of them determined the amount of inoculum that was actually applied to each plant, and only Jeffrey *et al.* gave consideration to which leaves were inoculated or assessed. Under these conditions, differences in the infection frequency and therefore in disease severity between plants could have resulted from the inoculation technique itself, rather than from actual differences in resistance among genotypes. Furthermore, differences could have been due to leaf age effects unless tissue of the same age was used to evaluate plants. Moreover, when percentage severity is used to select resistant plants, genotypes with large leaves appear to be more resistant than those with small leaves even though each may have the same amount of diseased tissue. In this regard, percentage severity does not distinguish lesion size and number, considered to be important factors contributing to quantitative resistance.

Unless a uniform amount of inoculum is applied to leaf surfaces, the infection frequency and consequently disease severity based on percentage infected tissue are not reliable indicators of resistance. Difficulty in achieving a uniform distribution of spores in suspensions impede our ability to consistently deliver the same number of spores to each leaf. Hence, greater emphasis has been placed on mean lesion size as the primary criterion for selecting for resistance to *Alternaria* blight. For necrotrophic pathogens such as *A. helianthi*, this character can be assessed with greater accuracy than other components of partial resistance. Lesion number often has a high coefficient of variation and latent period and spore production are strongly influenced by environmental

conditions (Parlevliet, 1992). Whilst selection for a single component of quantitative resistance is convenient, it is not entirely desirable because other components might be neglected in the selection process. Nonetheless, Parlevliet (1992) showed that, with leaf rust on barley *(Puccinia hordei*), components such as latent period, reduced infection frequency, pustule size and spore production are controlled by the same genes. Many of the resistant sunflower lines studied (see Chapter 7) had fewer, smaller lesions than more susceptible lines. These characters were exhibited by the partially resistant line used in the studies on temperature and dew period. Given that there are only very low levels of resistance to *A. helianthi* in the sunflower gene pool, the character of reduced lesion size has strong epidemiological implications, and serves as a starting point for future development of resistance.

Since the purpose of resistance screening is to detect genetic variation among sunflower lines, it is important to minimise the level of variation arising from other sources, which might otherwise obscure true genetic effects. Variables such as age and inoculum dose rate, host age and density, environmental conditions for inoculation and incubation have been shown to affect the reaction of sunflower plants to infection by *A.helianthi.* Although optimum conditions for infection in the greenhouse are known, care should be taken not to invoke conditions so favourable to infection and disease development that differences in resistance among genotypes are undetectable. The apparent lack of resistance in the sunflower gene pool indicates that higher levels of resistance will have to be developed by recurrent selection methods. The rate at which progress is made will depend firstly on the ability to accurately identify sources of resistance and secondly on the heritability of the selected resistance. In view of these factors, the following standard conditions for screening sunflower in the greenhouse for resistance to *A.helianthi* have been recommended (Kong, Kochman and Brown, 1995).

1. A highly pathogenic isolate of *A. helianthi* should be used.
2. Inoculum should be 5-10 days of age and from the first generation culture on SLEA.

3. Plants should be at growth stage V6-V8 (Schneiter & Miller, 1981), and potting mix and plant nutrition throughout the growing period should be standardised.
4. The first or second leaf pair should be inoculated with about 30 spores of *A. helianthi* per leaf (or not more than 1-2 spores per cm^2) and techniques employed to ensure that all leaves receive the same amount of inoculum.
5. Environmental conditions should consist of a 48h post-inoculation dew period when a humidity tent is used and a 12h photoperiod with a night/day temperature regime similar to that experienced during the cropping season.
6. Lesion size should be measured 7 days after inoculation. Plants should be selected according to their mean lesion size relative to the susceptible line used.
7. Blocking designs may be necessary to reduce the impact of microenvironmental variation in semi-controlled environments (Phelps *et al.*, 1991).
8. Use of an inbred susceptible genotype against which the resistance of other lines can be measured.

These recommendations represent a set of conditions which, based on the biological evidence presented in this chapter, constitute a logical starting point on which to found a quantitative screening assay. In Chapter 7, this assay is used to determine components of resistance to *A. helianthi* and correlation analyses performed to ascertain which, if any, of these components provide a reliable indicator of resistance under field conditions. The success of this will rely heavily on the assay developed from the work reported in this chapter.