CHAPTER THREE

GROWTH AND SPORULATION OF *PYRENOPHORA SEMENIPERDA* CULTURED *IN VITRO*

3.1 Introduction

Daniel, *et al.* (1973), outlined three major criteria that a potential bioherbicidal candidate must fulfil. The first criterion was that the pathogen must produce abundant and durable inoculum in artificial culture. Fungi that sporulate in liquid culture may be favoured for the commercial development of mycoherbicides and indeed, this is often seen as an essential requirement (Boyette *et al.,* 1991). However, some fungi that do not readily sporulate in submerged culture have also shown mycoherbicidal promise (Boyette *et al.,* 1991). Furthermore, a mycoherbicide may be produced by a combination of submerged and solid substrate culture. For example Walker & Riley (1982) produced spores of *Alternaria cassiae* Jurair & Khan for use as a mycoherbicide against sicklepod (*Cassia obtusifolia* L.) by producing mycelium in submerged culture which was then collected and dried to produce large amounts of inoculum. Mycoherbicides may also be developed using mycelial fragments as suitable propagules; an extremely attractive prospect given that mycelium is generally easy to produce in submerged culture. Conway (1976) used mycelial fragments of *C. rodmanii* as infective propagules for the biocontrol of water hyacinth in Florida USA.

To produce abundant inoculum in artificial culture the optimal conditions under which a pathogen will grow and sporulate must be determined. Very little research has been carried out on the optimal conditions required for the growth and sporulation of *P. semeniperda.* Brittlebank & Adam (1924) reported growth on a wide range of common media. Some workers have reported sporulation of *P. semeniperda* on artificial media (Kreitlow & Bleak, 1964; Paul, 1969) although optimal conditions for sporulation have not been reported. In contrast, work on optimal conditions for the growth and sporulation of other species of *Pyrenophora* has been more widely reported, particularly species such as *P. teres,* and *Pyrenophora tritici-repentis* Drechsler which are pathogenic to the winter cereals (Onesirosan & Bantarri, 1969; Platt, Morrall & Gruen, 1977). Although preliminary experiments showed

that *P. semeniperda* will grow readily on a number of common artificial media, the production of conidia tended to be sparse and the large numbers of conidia required to prepare spore suspensions for field and glasshouse experiments were not easily be produced. Many procedures by which *Pyrenophora* spp. have been induced to sporulate appear in the literature (Lukens, 1960; Srinivasan, *et al.*, 1971; Gulati & Mathur, 1979; Grbavac, 1981, Deadman & Cooke, 1985; Speakman & Pommer, 1986). However, the use of these methods for the induction of sporulation by *P. semeniperda* either did not result in consistent sporulation or were too laborious and inefficient for producing large quantities of conidia.

The experiments reported in this chapter were designed to establish the optimal conditions for growth and sporulation of *P. semeniperda* under artificial conditions. This information was required so that abundant quantities of both conidial and mycelial inoculum could be produced for subsequent laboratory and field studies reported in this thesis. The parameters investigated were: culture medium, temperature, pH, photoperiod, light quality and methods of inducing sporulation.

3.2 Materials and Methods

The growth of isolates cultured on solid media was assessed in the following manner. Prior to inoculation with fungal cores, the underside of Petri plates were marked with eight points equidistant from one another around the circumference of each plate, this made four colony diameter transects. At each assessment, measurements of colony diam. were made on each plate using a clear plastic ruler (mm) along each of the four marked transects.

Sporulation was assessed by placing ten 3 mm diam. cores of fungal mass and agar medium sampled from each culture plate into 5 ml of refrigerated distilled water contained in a screw top vial. Ten cores were removed in a set pattern from each plate. Five of the cores were obtained at equal distances along one transect of the plate and five from a second perpendicular transect. Thus, sporulation was assessed from mycelia of different ages on each colony. Vials were shaken vigorously by hand for 30 sec. to detach conidia and a 1 ml aliquot was dispensed into a Sedgewick Rafter counting cell. Sporulation was expressed in two ways: the number of conidia cm^{-2} of colony and number of conidia $colony^{-1}$.

3.2.1 Effect of culture medium on growth and sporulation.

Growth. Eleven media were tested for their ability to promote growth of isolates 580681 and 580534 of *P. semeniperda*. Experiments were conducted in darkness in a controlled forced-air incubator (Laboratory Equipment, Sydney Australia) at 20 ± 1 °C. The 11 media tested were potato dextrose agar (PDA); 1/4 strength PDA (1/4PDA); Czapek Dox agar (CDA); tap water agar (TWA); malt extract agar (MEA); V8 juice agar (V8A); V8 plus benomyl agar (V8+b); modified alphacel medium (MAM); oatmeal agar (OMA); bromus leaf extract agar (BLEA) and wheat leaf extract agar (WLEA) (Appendix 1). All inoculated Petri plates were sealed with Parafilm® to reduce water loss and prevent contamination. Fifteen replicates were used in the experiments.

Assessment of the effects of the 11 culture media on growth was made every 48 h by measuring four colony diam. plate^{-1}. Observations on morphological characteristics of vegetative stromata were recorded at each assessment.

Sporulation. All tests were carried out in a laboratory under uncontrolled temperature with a range of 15 to 25 °C. Illumination was supplied by 2 x 40 W cool white and 2 x 40 W "black light blue" tubes positioned 40 cm above the Petri plates (34.1 μmol m^{-2} s^{-1}). The culture media tested were: MAM; WLEA; BLEA; V8A; clarified V8 juice agar (CV8) (as for V8A but using 200 ml of V8 juice centrifuged for 30 min at 2800 rpm (rotor size 30 cm)); 1/4PDA; wild oat leaf extract agar (WOA) (as for BLEA but using *A. fatua* leaf pieces); OMA; CDA and TWA. Petri plates were not sealed with Parafilm®. Sporulation was assessed on 10 replicate Petri plates at 14 days after inoculation.

3.2.2 Effect of temperature on growth, sporulation, conidial germination and conidial germinability.

Growth and conidial germination. The effect of temperature on growth and conidial germination of *P. semeniperda* was studied by placing 10 replicate inoculated 1/4PDA Petri plates into seven separate forced-air incubators in darkness at 5, 10, 15, 20, 25, 30 and 35 ± 1 °C. Growth was assessed every 24 h by measuring four colony diam. plate^{-1}.

Conidial germination was assessed by spraying a conidial suspension (2 x 10^4 conidia ml^{-1}) onto Petri plates containing TWA using a hand held atomiser, and incubating the plates for 16 h at the seven temperatures Each treatment was replicated five times. After incubation, the percentage germination of conidia was scored by assessing the first 200

conidia encountered when focussing along two perpendicular transects. A conidium was considered to have germinated if the germ tube was longer than the conidium from which it had arisen. Petri plates were sealed with Parafilm®.

Sporulation and conidial germinability. The effect of temperature on sporulation of isolate 580681 was tested using a thermo-gradient plate (Larsen, 1971). Ten ml of MAM was dispensed in 70 mm diam. Petri plates. Tests were conducted over a range of 10 to 35 °C (5 °C increments) with a 12 h photoperiod (36.7 µmol m^{-2} s^{-1}) provided by 2 x 40 W cool white and 1 x 40 W black light tubes positioned 60 cm above the Petri plates. Seven replicate Petri plates were not sealed. Sporulation was assessed at 7 days after inoculation. Germinability of conidia produced over the range of 10 to 30 °C was assessed using the methods described above for conidial germination except that plates (five replicates) were incubated only at 20 °C. Conidia were obtained from culture plates used to assess the effects of temperature on sporulation.

3.2.3 Effect of pH on growth and sporulation.

Growth. Growth of *P. semeniperda* was assessed over the range pH 3.7 to 10.1. Experiments were performed four times. Liquid cultures of isolate 580681 were grown in a modification of the medium used by Tomás & Bockus (1987). The culture medium consisted of 9 g sucrose, 5 g ammonium tartrate, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.13 g $CaCl_2$, 0.1 g NaCl and 1 g of yeast extract l^{-1}. The pH of the media were adjusted by substituting various buffers for distilled water to give initial values of : 3.7, 4.8, 5.6, 6.6, 7.3, using citrate-phosphate buffer (0.1 M citric acid and 0.2 M Na_2HPO_4); 8.4, using phosphate buffer (0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4); and 9.5, 9.9, 10.1, using carbonate-bicarbonate buffer (0.2 M Na_2CO_3 and 0.2 M $NaHCO_3$) (Dhingra & Sinclair, 1987). Unbuffered medium (initial pH 7.0) was tested as a control. Media (100 ml) were decanted into 250 ml capacity bottles, inoculated and incubated for 7 days under laboratory conditions (17 to 25 °C, 9.8 µmol $m^{-2}s^{-1}$) on an orbital shaker revolving at 130 rpm. For each pH treatment, there were three replicates and a fourth bottle used as an uninoculated control. After incubation, the final pH of the culture medium was determined. To calculate fungal biomass, cultures were filtered through pre-weighed, oven dried Whatman No. 1 filter papers, oven dried (80 °C) overnight and re-weighed.

Sporulation. To investigate the effect of pH on sporulation the pH was adjusted by substituting citrate-phosphate buffer (0.1 M citric acid and 0.2 M Na_2HPO_4 (Dhingra & Sinclair, 1987)) to MAM for distilled H_2O. The initial pH values of the media tested were 4.75, 5.34, 6.03, 6.58, 7.05 and 7.46. In addition, unbuffered MAM (pH approx. 5.0) was used as a control. Media with pH values of below 4.2 and above 7.8 could not be tested as these would not gel. Ten replicates of inoculated (isolate 580681) culture plates were incubated at 17 to 28 °C with a 12 h photoperiod under near ultra violet light (35.8 µmol m^{-2} s^{-1}) for 14 days. Petri plates were not sealed.

3.2.4 Effect of photoperiod on growth and sporulation.

Growth. Cultures of isolate 580681 were grown on 1/4PDA in four separate incubators set at 25 ± 1°C. Three incubators were fitted with 2 x 30W cool white tubes and 1 x 30W black light tube positioned on the front wall of the incubator. No light was provided in the fourth incubator. The following diurnal light regimes and their respective irradiance measurements (average of 10 readings at top of Petri plates) were tested: 24 hr photoperiod (18.05 µmol m^{-2} s^{-1}), 12 hr photoperiod (17.8 µmol m^{-2} s^{-1}), 6 hr photoperiod (17.3 µmol m^{-2} s^{-1}) and 24 hr darkness (0 µmol m^{-2} s^{-1}). Radial growth was assessed 3, 5, 7 and 14 days after inoculation using the colony diameter method outlined above. Assessment of cultures grown in darkness were made in a laboratory at night with only diffuse light. Ten replicates were used in the experiments. Petri plates were sealed with Parafilm®.

Sporulation. Cultures of isolate 580681 were grown on MAM under the same light regimes described above. However, unsealed Petri plates were placed in the lids of glass plates (9 cm diam.) to reduce water loss and prevent contamination from the air-flow within the incubators. Sporulation was assessed at 14 days after inoculation. Ten replicates were used in the experiments.

3.2.5 Effect of light quality on growth and sporulation.

To investigate the effect of light quality on growth and sporulation of isolate 580681, inoculated Petri plates (1/4PDA for growth and MAM for sporulation) were enclosed in coloured cellophane envelopes. The spectral characteristics of each of six different coloured cellophane sheets was determined using a diode array spectrophotometer (HP4825, Hewlett Packard, CA, USA) in the wavelength range 320 to 820 nm (Table 3.1). Clear plastic Petri

plates were used as controls. The inoculated Petri plates were incubated at 20 ± 1 °C (460.5 μmol m^{-2} s^{-1}) for 14 days. Measurements of colony diameters were made every 48 h and sporulation was assessed after 14 days

Table 3.1. Spectral characteristics of cellophane sheets used to envelope inoculated Petri plates. Wavelengths transmitted in the visible spectrum (400-700 nm). All cellophane transmitted near-ultra violet light (320-400 nm) but not wavelengths shorter than 300 nm.

Cellophane colour	Transmittance (nm)
Purple	400-440
Blue	400-500
Green	480-540
Yellow	500-700
Orange	600-700
Red	600-700
Clear	400-700

3.2.6 Growth and sporulation in liquid culture.

Nine liquid culture media were tested for their ability to promote growth and sporulation of *P. semeniperda.* The nine media tested were: Czapek Dox broth (CDB), Fries medium (Fries), V8 juice broth (V8), Bromus leaf extract broth (LEB), medium used by Tomás & Bockus (1987) (T&B), sucrose asparagine broth (SAB), sucrose and water broth (SWB), modified alphacel broth (MAB), and malt extract broth (MEB) (Appendix 1).

Inoculated culture bottles were incubated in a laboratory with a temperature range of 17 to 25 °C on an orbital shaker set at 130 rpm and with a 12 h photoperiod (35.8 μmol $m^{-2}s^{-1}$) supplied by 2 x 40W cool white and 2 x 40W black light fluorescent tubes. Treatments were replicated four times with uninoculated media serving as controls.

After 14 days incubation evidence of sporulation was investigated by pouring the mycelial balls into a Petri plate and examining under a dissecting microscope (x 80). Fungal biomass was determined by filtering cultures through pre-weighed oven dried Whatman No. 1 filter papers, oven drying (80 °C) overnight and re-weighing.

3.2.7 The effect of wounding on sporulation.

Different degrees of wounding were examined for their effect on sporulation. Inoculated, unsealed Petri plates (isolate 580681, MAM) were incubated for 7 days under NUV light (320 - 420 nm) with illumination supplied by 2 x 40W cool white and 2 x 40W black light blue tubes positioned 40cm above the Petri plates (36.5 µmol m^{-2} s^{-1}, 12 hour photoperiod). The temperature ranged between 17 and 25 °C. Seven days after inoculation, colonies were either left unwounded or wounded with one of the following sized cork borers: 3; 5; 7; 9 mm to cut through the mycelium and agar medium. This procedure was repeated until the whole colony had been wounded. Wounds were made in such a way as to minimise overlapping. Cultures were returned to incubation conditions and sporulation was assessed 7 days later. Ten replicates were used in duplicate experiments.

3.2.8 Integrated optima for mass production of conidia.

Having established the optima for temperature, pH, light quality/period required for sporulation of *P. semeniperda*, this knowledge was combined to validate a technique for mass production of conidia. The relative abilities of NUV, white fluorescent light, wounding and sealing culture plates with Parafilm® to promote sporulation were investigated.

Inoculated Petri plates (isolate 580681, MAM) were placed either under NUV light (320 to 420 nm) with illumination supplied by 2 x 40W cool white and 2 x 40W black light blue tubes positioned 40cm above the Petri plates (36.5 µmol m^{-2} s^{-1}) or cool white fluorescent light with illumination supplied by 3 x 40W Osram cool white fluorescent tubes positioned 40 cm above the Petri plates (37.7 µmol m^{-2} s^{-1}). A 12 hour photoperiod was used for each light source and the temperature ranged between 17 and 25 °C.

Wounding of colonies was either undertaken at seven days after inoculation using a 3 mm diam. cork borer as described above, or colonies were left undisturbed. Petri plates were either sealed with Parafilm® strips (10 cm x 1.5 cm) by wrapping a double layer around the edge of the plate, or left unsealed.

Ten replicates of each treatment were used as outlined in Table 3.2. Data were subjected to a logarithmic transformation prior to analysis of variance.

Table 3.2. Twelve treatments used to assess the effect of NUV light, white fluorescent light, wounding and sealing culture plates with Parafilm® to enhance sporulation by *P. semeniperda*.

NUV	White fluorescent
Wounded, Parafilm® +	Wounded, Parafilm® +
Wounded, Parafilm® ±	Wounded, Parafilm® ±
Wounded, Parafilm® -	Wounded, Parafilm® -
Unwounded, Parafilm® +	Unwounded, Parafilm® +
Unwounded, Parafilm® ±	Unwounded, Parafilm® ±
Unwounded, Parafilm® -	Unwounded, Parafilm® -

Plates were either wounded at seven days after inoculation or left undisturbed.
Plates were sealed with Parafilm® for 14 days (Parafilm® +)
Plates were sealed for the first 7 days, and then the seal was removed (Parafilm® ±)
Plates were unsealed for 14 days (Parafilm® -)

3.3 Results

In those experiments where isolates 580681 and 580534 were compared, radial growth and sporulation did not differ significantly (P>0.05) so data were pooled for statistical analyses.

3.3.1 Effect of culture medium on growth and sporulation.

Growth. *P. semeniperda* grew equally well (P>0.05) on the 2 media containing oatmeal (OMA and MAM) and radial growth was faster on these than on any of the other media tested. Radial growth was linear on all media (Table 3.3). Growth on WLEA was characterised by a slight reduction in the growth rate between days 10 and 12 (P>0.05). Growth on the 2 media containing leaf extracts was similar (P=0.045). Faster radial growth, indicated by the slope co-efficient in the linear regression equations, occurred on 1/4PDA than the other media tested, excepting MAM and OMA.

Colonies cultured on MAM and OMA were characterised by dense mycelia and black stromata. Stromata were characteristically 3 to 5 mm in length. Colonies cultured on other media had less dense mycelia and stromata formation was not visible at 14 days after

inoculation although stromatal initials were embedded in all the other media tested except CDA and TWA. Colonies cultured on PDA and to a lesser extent 1/4PDA were characterised by the formation of large chains of thick-walled hyphal cells which presumably functioned as chlamydospores.

Sporulation. The MAM supported significantly greater conidial production than any other medium tested ($P<0.01$) with respect to conidia colony^{-1} (Table 3.3). Although the CV8 and V8A supported sporulation not significantly different from that of MAM when expressed as conidia cm^{-2}, relatively few conidia colony^{-1} were produced due to the small colony size on these media. No conidia were produced on CDA or TWA. Although many stromata were formed on the MAM, conidiophores and conidia were primarily produced on mycelia.

Table 3.3. Effect of culture medium on colony growth and sporulation of *P. semeniperda*. In the regression equations Y = colony diameter and X = days after inoculation. Data within the same column followed by a common letter were not significantly different according to Tukey's HSD test ($P>0.01$). NT=not tested. *Data pooled for isolates 580534 and 580681.

Medium	**Regression equation**	**Multiple correlation (r^2)**	**Sporulation (x 10^3)***	
			Conidia cm^{-2}	**Conidia colony^{-1}**
MAM	Y = -0.5(± 0.8) + 3.3(± 0.[illegible])X	0.996	16.4 ± 1**a**	1376.0 ± 85**a**
OMA	Y = -0.2(± 0.9) + 3.2(± 0.[illegible])X	0.995	3.6 ± 0.38**de**	303.1 ± 32**b**
BLEA	Y = 1.4(± 1) + 2.2(± 0.1)X	0.987	6.6 ± 1.2**cd**	148.6 ± 26**c**
WLEA	Y = 4.6(± 1) + 2.1(± 0.1)X	0.983	0.2 ± 0.04**e**	9.6 ± 1.7**e**
WOA	NT		1.5 ± 0.15**e**	47 ± 4.3**d**
PDA	Y = 2.8(± 0.7) + 1.6(± 0.1)X	0.987	NT	NT
1/4PDA	Y = 0.9(± 0.2) + 2.4(± 0.05)X	0.999	10.2 ± 0.8**bc**	513 ± 46.4**b**
V8A	Y = 2.1(± 0.3) + 1.7(± 0.04)X	0.997	13.7 ± 1.2**ab**	356.5 ± 33**b**
CV8	NT		12.7 ± 1.4**ab**	144.8 ± 16**c**
V8+b	Y = 2.6(± 0.7) + 1.9(± 0.1)X	0.99	NT	NT
CDA	Y = 1.2(± 0.8) + 1.9(± 0.1)X	0.987	0	0
TWA	Y = 1.6(± 0.7) + 1.5(± 0.1)X	0.985	0	0

3.3.2 Effect of temperature on growth, sporulation, conidial germination and conidial germinability.

Growth. *P. semeniperda* grew at a linear rate for all temperatures tested except 35 °C and significantly faster at 25 °C ($P<0.01$) than any other temperature tested (Table 3.4). Growth rates at 15 °C and 20 °C did not significantly differ ($P>0.05$) and no growth occurred at 35 °C. Mycelium was considered to be dead at 35 °C since moving mycelium to more favourable temperatures of 20 to 25 °C did not result in a resumption of growth. Only slight growth was observed at 5 °C. The optimum temperature for growth calculated from the polynomial regression equation (Fig. 3.1) was 23.2 ± 0.5 °C.

Table 3.4. The effect of temperature on growth of *P. semeniperda.* Linear equations were generated after pooling data for both isolates tested In the regression equations Y = colony diameter and X = days after inoculation.

Temperature (°C)	Regression equation	Multiple correlation (r^2)
5	Y = 0.2(± 0.1) + 0.7(± 0.03)X	0.903
10	Y = 1.2(± 0.2) + 1.7(± 0.05)X	0.945
15	Y = 1.1(± 0.1) + 3.6(± 0.03)X	0.994
20	Y = 2.0(± 0.2) + 3.8(± 0.05)X	0.99
25	Y = 3.0(± 0.2) + 4.8(± 0.04)X	0.994
30	Y = 4.7(± 0.[illegible]) + 3.0(± 0.1)X	0.945

Sporulation, conidial germinability and conidial germination. Production of conidia was greater at 15 and 20 °C than at the other temperatures tested, whether expressed as conidia cm^{-2} or conidia $colony^{-1}$. No conidia were produced at 35 °C and the mycelium was considered dead. The optimal temperatures calculated from the polynomial regression equations for conidial production were 18.3 ± 0.3 °C and 19.2 ± 0.05 °C for conidia cm^{-2} (Fig. 3.2) and conidia $colony^{-1}$ (Fig. 3.3) respectively. Germinability exceeded 90 % for conidia produced at temperatures between 0 and 25 °C, but conidia produced at 30 °C had significantly ($P<0.01$) lower germinability (Table 3.5). Conidia germinated at all temperatures tested but a significantly greater ($P<0.01$) proportion germinated at 20, 25 and 30°C.

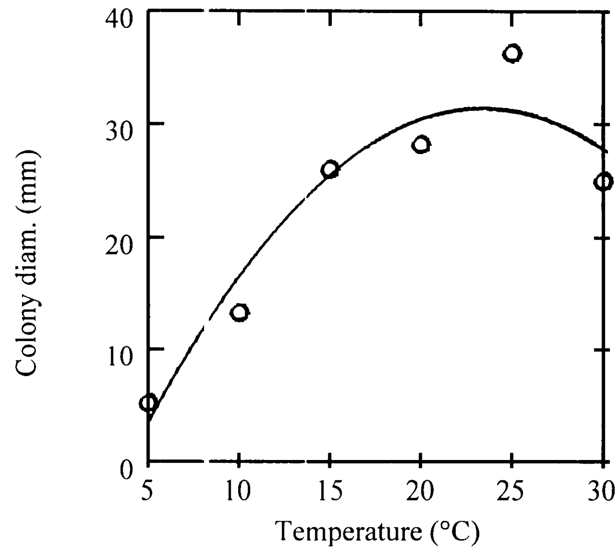

Fig. 3.1. Effect of temperature on colony diameter of *P. semeniperda* at 7 days after inoculation. Data points represent means of 10 replicates over duplicate experiments. The regression equation is Y = -13.7(±1.9) + 3.9(±0.2)X - 0.08(±0.01)X^2 where Y = colony diameter and X = incubation temperature ($r^2 = 0.902$).

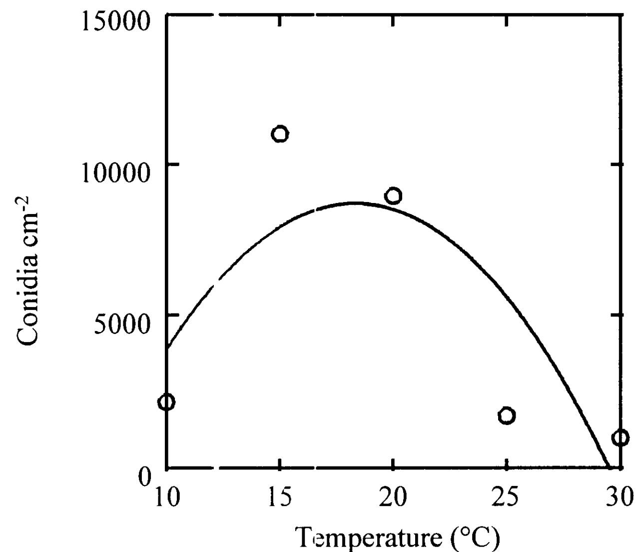

Fig. 3.2. Effect of temperature on conidial production of *P. semeniperda* at 14 days after inoculation. Data points represent means of 10 replicates over duplicate experiments. The regression equation is Y = -14.6(±4.1) (10^3) + 2.5(±0.5) (10^3)X - 69.5(±11.2)X^2, where Y = number of conidia cm^{-2} of colony area and X = incubation temperature ($r^2 = 0.855$).

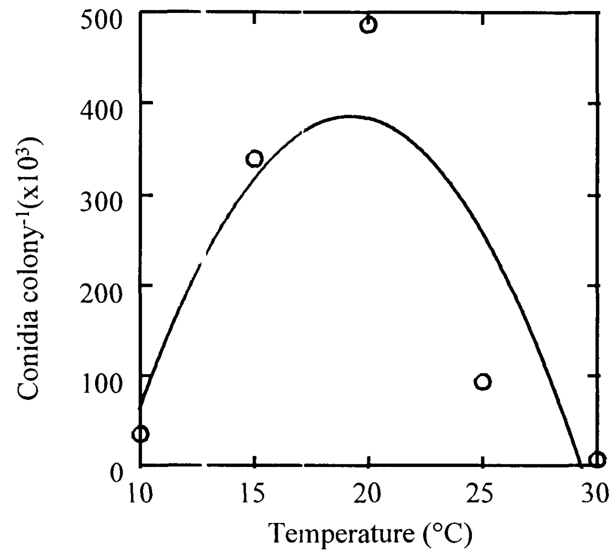

Fig. 3.3. Effect of temperature on conidial production of *P. semeniperda* at 14 days after inoculation. Data points represent means of 10 replicates over duplicate experiments. The regression equation is $Y = -1(\pm 0.2)(10^6) + 1.5(\pm 0.2)(10^5)X - 3.8(0.4)(10^3)X^2$, where Y = number of conidia colony^{-1} and X = incubation temperature ($r^2 = 0.882$).

Table 3.5. The effect of temperature on conidial germination and germinability of conidia of *P. semeniperda* produced at different temperatures. Data within the same column followed by a common letter were not significantly different according to Tukey's HSD test ($P>0.01$). NT= Not tested. *Temperatures on the thermogradient plate had ranges of ±2.5 °C.

Temperature (°C)	Germination (%)	Germinability * (%)
5	0.7 ± 0.4**e**	NT
10	65.6 ± 0.7**c**	94 ± 1**a**
15	75.4 ± 1.9**b**	98.5 ± 1.2**a**
20	89.2 ± 0.6**a**	97 ± 0.9**a**
25	91.2 ± 1**a**	98 ± 1**a**
30	90.6 ±0.9**a**	78.5 ± 2.2**b**
35	11.2 ± 3.5**d**	NT

3.3.3 Effect of pH on growth and sporulation.

Growth. Isolate 580681 produced greatest biomass at pH 4.8 and the predicted optimum calculated from the polynomial regression equation was 4.7±0.5 (Fig. 3.4). At pH 8.4 growth was negligible and inconsistent over all trials. No growth was observed in the 3 most alkaline solutions tested (pH 9.1, 9.9, 10.1). The medium of Tomás & Bockus (1987) without the addition of buffer solution (pH 7.0) resulted in slightly greater biomass than either of the media with pH adjustments to 6 6 or 7.3.

Sporulation. Isolate 580681 sporulated at all the pH levels tested. Sporulation whether measured as conidia cm^{-2} or conidia $colony^{-1}$ was maximal at pH 5.34 and on unbuffered MAM (pH 5.0). The pH optima calculated from the polynomial regression equations for conidial production were 5.7 ± 0.1 and 5.4 ± 0.2 for conidia cm^{-2} (Fig. 3.5) and conidia $colony^{-1}$ (Fig. 3.6) respectively. Good sporulation was also evident at pH 6.03. Radial growth was retarded at all pH levels tested compared to that on MAM without the addition of buffer solution.

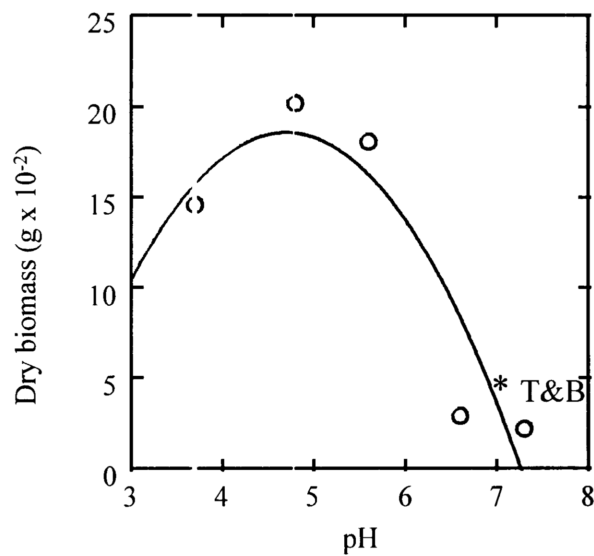

Fig. 3.4. Effect of pH on biomass production of *P. semeniperda* at 7 days after inoculation. Data points represent means of 3 replicates over 4 duplicate experiments. The regression equation is $Y = -0.44(\pm 0.5) + 0.27(\pm 0.2)X - 0.03(\pm 0.01)X^2$, where Y = dry biomass of colony and X = pH of culture medium ($r^2 = 0.954$). *T&B is unbuffered medium.

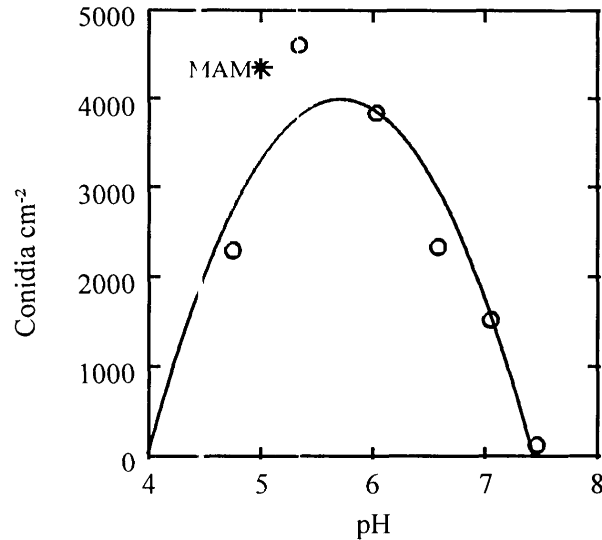

Fig. 3.5. Effect of pH on conidial production of *P. semeniperda* at 14 days after inoculation. Data points represent means of 10 replicates over duplicate experiments. The regression equation is $Y = -4.0(\pm 1.3)\ (10^4) + 1.5(\pm 0.5)\ (10^4)X - 1.3(\pm 0.4)\ (10^3)X^2$, where Y = number of conidia cm^{-2} of colony area and X = pH of culture medium ($r^2 = 0.973$). *MAM is unbuffered medium.

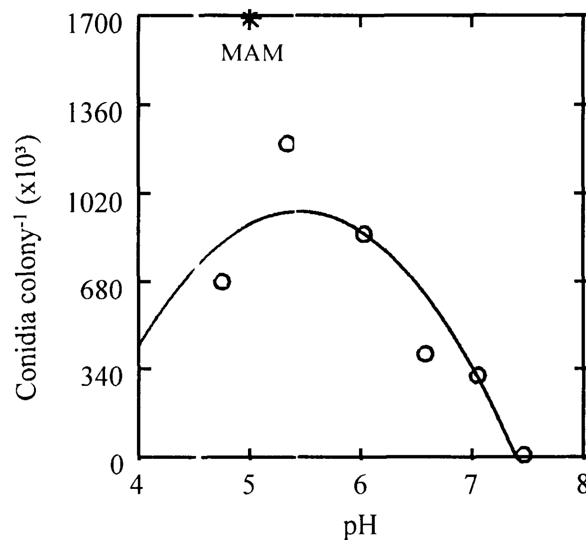

Fig. 3.6. Effect of pH on conidial production of *P. semeniperda* at 14 days after inoculation. Data points represent means of 10 replicates over duplicate experiments. The regression equation is $Y = -6.5(\pm 4.7)\ (10^6) + 2.7(\pm 1.6)\ (10^6)X - 2.5(\pm 1.3)\ (10^5)X^2$, where Y = number of conidia $colony^{-1}$ and X = pH of culture medium ($r^2 = 0.949$). *MAM is unbuffered medium.

3.3.4 Effect of photoperiod on growth and sporulation.

Growth. Growth was linear when cultures were exposed to all different photoperiods (Table 3.6). Radial growth of *P. semeniperda* was fastest when cultures were exposed to either total darkness or a 12 h photoperiod (P>0.05). Radial growth was slowest when cultures were exposed to either a 6 h photoperiod or 24 h constant illumination (P>0.05).

Sporulation. Significantly larger numbers of conidia were formed when cultures were exposed to a 12 h photoperiod (P<0.0) (Table 3.6). When expressed as conidia cm^{-2}, similar numbers of conidia were formed when exposed to either a 12 or 6 h photoperiod (P=0.056). Few conidia were produced on those cultures that were exposed to either constant illumination or darkness.

Table 3.6. Effect of photoperiod on colony growth and sporulation of isolate 580681 In the regression equations Y = colony diameter and X = days after inoculation. Data within the same column followed by a common letter were not significantly different according to Tukey's HSD test (P>0.01). D= darkness, L= light.

Photoperiod	**Regression equation**	r^2	**Sporulation (x 10^3)**	
			Conidia cm^{-2}	**Conidia $colony^{-1}$**
24 h D	Y = 5.2(±1.1) + 3.2(±0.1)X	0.995	0.2 ±0.03**c**	22.3 ±2.1**d**
12 h L/12 h D	Y = 8.6(±1.4) + 3.3(±0.2)X	0.960	12.0 ±1.1**a**	1349 ±77**a**
6 h L/6 h D	Y = 9.6(±2.1) + 2.5(±0.4)X	0.933	8.8 ±0.9**a**	673 ±43**b**
24 h L	Y = 9.6(±1.8) + 2.6(±0.9)X	0.810	1.0 ±0.2**b**	80.3 ±9.2**c**

3.3.5 Effect of light quality on growth and sporulation.

Growth. Radial growth of isolate 580681 was linear, irrespective of the wavelength of the light source tested (Table 3.7). Growth was significantly greater in colonies exposed to light that had a majority of the spectrum with wavelengths longer than 540 nm (P<0.01). Growth of colonies was slowest when exposed to either purple, blue or green light and was not significantly different among these treatments (P>0.1) (Fig. 3.7). Growth of colonies exposed to the longest wavelengths tested (red and orange) was similar and no significant difference was observed (P>0.1). Radial growth was significantly faster when colonies were

exposed to yellow light (500 - 700 nm, $P<0.01$). Colonies exposed to wavelengths of all the visible spectrum (400 - 700 nm) were significantly different from all others tested ($P<0.05$).

Sporulation. Conidial production was greatest when cultures were exposed to purple, blue and green light (400 - 540 nm, $P<0.01$) when expressed as either conidia colony^{-1} or conidia cm^{-2} (Table 3.7). Conidial production was least when exposed to yellow light or all the visible spectrum. Conidial production was not different between colonies exposed to red and orange light ($P>0.1$).

Table 3.7. Effect of light quality on colony growth and sporulation of isolate 580681 In the regression equations Y = colony diameter and X = days after inoculation. Data within the same column followed by a common letter were not significantly different according to Tukey's HSD test ($P>0.01$).

Colour	**Regression equation**	r^2	**Sporulation (x 10^3)**	
			Conidia cm^{-2}	**Conidia colony^{-1}**
Purple	Y = -0.03(±1.2) + 3.6(±0.1)X	0.927	22.6 ±2**a**	4394 ±453**a**
Blue	Y = -0.01(±1.7) + 4.0(±0.2)X	0.883	21.7 ±2**a**	4385 ±329**a**
Green	Y = -1.4(±1.5) + 4.0(±0.2)X	0.892	24.8 ±1.8**a**	4451 ±309**a**
Yellow	Y = -0.6(0.5) + 5.1(±0.1)X	0.990	1.4 ±0.07**c**	346 ±18**c**
Orange	Y = -0.6(±1) + 4.4(±0.1)X	0.957	4.5 ±0.1**b**	1006 ±32**b**
Red	Y = -0.5(1.2) + 4.3(±0.1)X	0.942	3.9 ±0.5**b**	726 ±98**b**
Clear	Y = -0.7(±0.6) + 4.8(±0.1)X	0.988	1.4 ±0.06**c**	392 ±20**c**

3.3.6 Growth and sporulation in liquid culture.

P. semeniperda produced most biomass when cultured in V8 juice medium, MEB and MAB ($P<0.05$) (Table 3.8). Good growth was also apparent when colonies were grown in Fries medium and T&B. The least biomass was produced in CDB and SWB.

No conidia were produced by colonies grown in liquid culture. However, microscopic examination of mycelia grown in T&B revealed conidiophore-like structures. Furthermore, when grown in T&B and LEB, *P. semeniperda* produced characteristic vegetative stromata, which may have functioned as conidiophores.

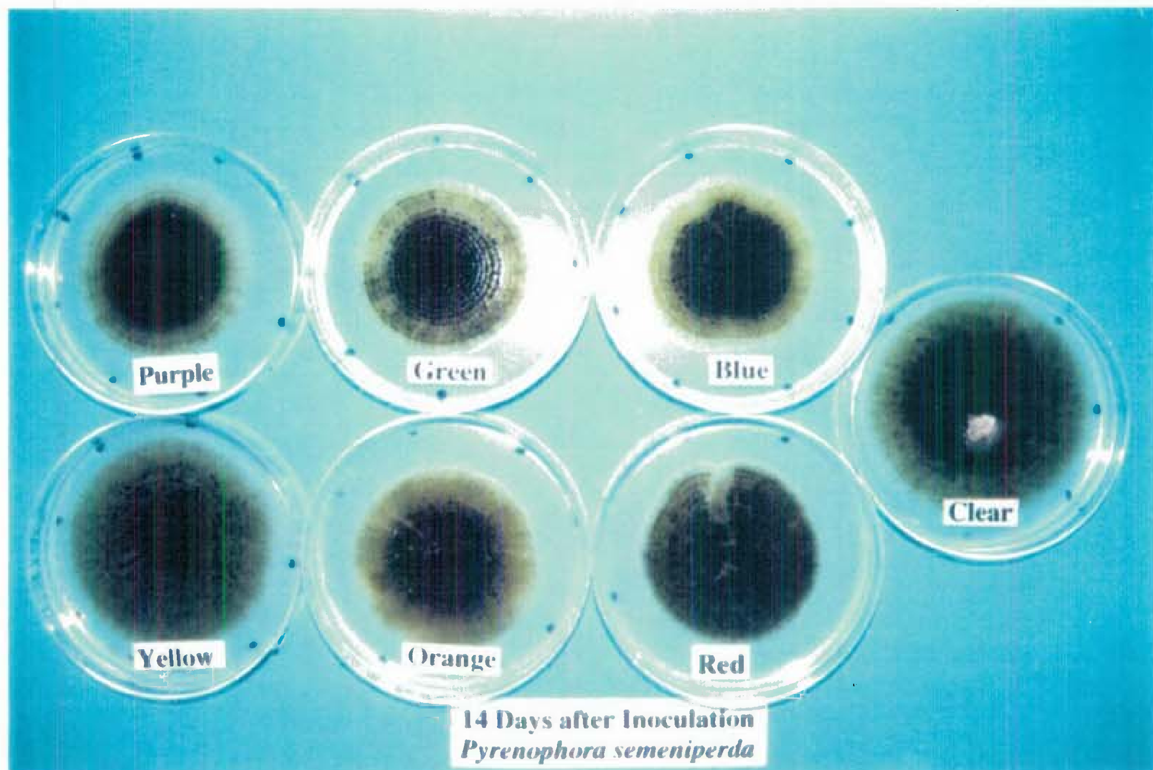

Fig. 3.7. Effect of light quality on colony growth of *P. semeniperda* (isolate 580681) grown on 1/4 PDA at 14 days after inoculation.

Table 3.8. Biomass of colonies of isolate 580681 grown in different liquid culture media. Data within the same column followed by a common letter were not significantly different according to Tukey HSD ($P>0.05$).

Medium	Biomass Production (g)
V8	0.351 ±0.05**a**
MEB	0.264 ±0.06**ab**
MAB	0.225 ±0.05**ab**
Fries	0.190 ±0.02**bc**
T&B	0.133 ±0.01**bcd**
SAB	0.124 ±0.01**cde**
LEB	0.104 ±0.001**cde**
CDB	0.053 ±0.002**de**
SWB	0.002 ±0.001**e**

3.3.7 The effect of wounding on sporulation.

Sporulation decreased with increasing wound size (Table 3.9). Wounding colonies with either a 3 or 5 mm borer resulted in the production of significantly more conidia ($P<0.01$). Colonies that were wounded with a 9 mm borer or not wounded produced significantly fewer conidia ($P<0.01$).

Table 3.9. The effect of different wound sizes on sporulation of *P. semeniperda* (isolate 580681). Data within the same column followed by a common letter were not significantly different according to Tukey HSD ($P>0.01$).

Wound Size (mm)	Sporulation (conidia ml^{-1} x 10^3)
3	17.2 ±2**a**
5	17.0 ±1.6**a**
7	8.7 ±0.3**b**
9	4.2 ±0.2**c**
Unwounded	4.6 ±0.5**c**

3.3.8 Integrated optima for mass production of conidia.

Exposure of colonies of *P. semeniperda* to NUV and cool white fluorescent light had a marked effect on sporulation. Sporulation for groups of treatments that were exposed to NUV was always significantly greater ($P<0.05$) than for the same group exposed to cool white fluorescent light (Table 3.10).

For those treatments exposed to NUV, wounding the mycelium resulted in consistently greater conidia formation. These differences were not always significant but colonies which were wounded, exposed to NUV and not wrapped with Parafilm® resulted in significantly ($P<0.05$) larger numbers of conidia being formed than any other treatment tested.

Sealing the culture plates with Parafilm® had a marked detrimental effect on sporulation. Plates unsealed for the duration of the experiments resulted in significantly greater ($P<0.05$) conidia production regardless of the other treatments. Similarly, plates that were sealed for only half of the experiment duration generally resulted in significantly greater ($P<0.05$) conidia production than for plates sealed for the entire duration of the experiments.

Table 3.10. The relative effects of NUV, white fluorescent light, wounding and sealing culture plates with Parafilm® on sporulation by *P. semeniperda*. Data followed by a common letter were not significantly different according to Tukey HSD (P>0.05).

Treatment Groups	Treatments*	Sporulation (conidia ml^{-1}) ($x10^3$)
I	WP+UV	4495 ±868**de**
	WP±UV	9624 ±1113**c**
	WP-UV	49033 ±2981**a**
II	UP+UV	1798 ±264**ef**
	UP±UV	4743 ±511**d**
	UP-UV	30809 ±1037**b**
III	WP+WL	124 ±52**h**
	WP±WL	1389 ±799**fg**
	WP-WL	3805 ±297**de**
IV	UP+WL	264 ±91**gh**
	UP±WL	242 ±133**gh**
	UP-WL	2464 ±242**de**

*Wounded (W) or unwounded (U).

*Parafilm® sealed culture plates for whole experiment (P+), half experiment (P±) or unsealed (P-).

*Illumination with near ultra-violet light (UV) or cool white fluorescent light (WL)

3.4 Discussion

Growth rates of *P. semeniperda* on solid media were greatest on those media containing host materials such as oatmeal and leaf extracts. Growth was also rapid when colonies were grown in liquid culture containing oatmeal (MAB) but was slight in liquid leaf extract media. This result contrasts with the findings of Brittlebank & Adam (1924) who reported uniform growth rates, though with variable vigour, on a wide range of media. Shoemaker (1966) reported rapid growth of *P. semeniperda* on 2 % malt agar, but, in the

present study isolates 580681 and 580534 grew slowly and mycelium was sparse when cultured on 2 % malt agar. In contrast, growth of isolate 580681 was rapid in malt extract broth. Few other reports of culture media effects on the growth of *P. semeniperda* exist in the literature. Sato & Takeda (1990) found that colony growth of *P. teres* was greatest on barley leaf agar and V8 juice agar while growth on oatmeal agar was similar to that on PDA. In contrast, Lohan & Cooke (1986) reported greatest mycelial growth on PDA and the least on clarified V8 juice agar. This indicates that there are culture media-isolate interactions in the *P. teres* complex and it would appear that the same occurs with *P. semeniperda*.

Although *P. semeniperda* grew faster on media containing oatmeal, all cultures for future experiments requiring measurements of radial growth were grown on 1/4PDA. This was because the opacity of the media containing oatmeal made accurate measurements difficult and time consuming.

When grown on MAM and CMA, both of which contained oatmeal, isolates 580681 and 580534 of *P. semeniperda* produced conspicuous black stromata whereas only stromatal initials were observed on the other media tested. Drechsler (1923) reported the formation of sclerotia below the surface of the medium when *P. semeniperda* was cultured on a medium containing little organic material. Wallace (1959) postulated that the submerged sclerotia *sensu* Drechsler (1923) were really undeveloped synnemata. Paul (1969) described the development of stromata with setose conidiophores on grass seed. The majority of conidia observed in the present study were formed on aerial mycelia. The stromatal initials produced on media not containing oatmeal superficially resembled sclerotia as described by Drechsler (1923). The stromata observed in the present study were larger than those reported by Shoemaker (1966) but similar to those found by Brittlebank & Adam (1924). Stromatal development of 3 to 4 mm a month described by Wallace (1959) was much slower than stromatal growth of up to 5 mm in 14 days observed in the present study. Stromatal development was also observed in colonies grown in liquid culture. The role of stromata produced under submerged conditions is uncertain, since no conidia were observed in association with them. However, the possibility exists that conidia were formed on stromata and then dislodged. Dislodged conidia may have germinated to produce secondary colonies.

The role of stromata in the life cycle is also uncertain. However, stromata are characteristically present on seeds infected with *P. semeniperda*, in association with conidia, which may act in the dispersal of the organism.

The results for sporulation in most experiments were presented in 2 ways: conidia cm^{-2} and conidia $colony^{-1}$. Conidia per unit area of colony is the conventional unit of sporulation in the literature (Onesirosan & Bantarri, 1968; Deadman & Cooke, 1985; Hunger & Brown, 1987; Strandberg, 1987). When considering the practicalities of preparing conidial suspensions for use in mycoherbicide efficacy tests and other epidemiological studies, it is important to know the conditions that maximise conidial production particularly when laboratory resources are limited and large numbers of plants are to be inoculated. For this reason it is also important to know optimal conditions for sporulation in terms of conidia $colony^{-1}$. For example in the present study, similar numbers of conidia cm^{-2} were produced on MAM and CV8 ($P>0.05$). However, 9 times as many culture plates of colonies grown on CV8 were required to produce the same conidial yield as 1 plate of MAM. It is useful therefore, for sporulation data to be expressed in terms of conidia cm^{-2} and conidia $colony^{-1}$. Some differences occur in the literature in the way in which sporulation data for *Pyrenophora* spp. are expressed. For example, Platt, *et al.* (1977) and Platt & Morrall (1980) expressed sporulation of *P. tritici-repentis* as conidia per plate even though they calculated the values using colony diameters. Evans, Hunger & Siegerist (1993) expressed sporulation of *P. tritici-repentis* as conidial density per Petri dish but did not calculate colony areas.

P. semeniperda did not require host material to be incorporated into the medium to promote good sporulation but when measured by conidia $colony^{-1}$, the best was observed on MAM which contained oatmeal. Paul (1969) found that conidia of *P. semeniperda* formed irregularly, but that best development occurred on media low in nutrients or on those media that included host material. Kreitlow & Bleak (1964) reported that *P. semeniperda* grew and sporulated sparsely on PDA whereas isolates cultured on water agar sporulated earlier and more prolifically. The isolates tested in this thesis did not sporulate on water agar. *P. semeniperda* sporulated heavily on media containing V8 juice, although colony sizes were very small. Similar findings have been reported by workers for other *Pyrenophora* spp. Sato & Takeda (1990; 1991) reported that optimal conidia formation of *P. teres* occurred on V8 medium, some sporulation on barley leaf agar and little or no sporulation on oatmeal agar. Other workers have also found that media containing V8 juice supported good sporulation by *P. teres* (Lohan & Cooke, 1986), *Pyrenophora graminea* Ito & Kurib. (Grbavac, 1981) and *P. tritici-repentis* (Kahn, 1971; Platt *et al.*, 1977; Evans *et al.*, 1993).

The temperature requirements for optimal growth of *P. semeniperda* were similar to those reported by Wallace (1959). He found that lateral growth was greatest at 25 °C and that good growth occurred at 30 °C on PDA. Wallace (1959) further reported that growth ceased at 35 °C, but resumed on transfer of cultures to 25 °C. That was not the case in the present study where incubation at 35 °C appeared to kill the mycelium. It is postulated that the cardinal temperatures for growth of *P. semeniperda* are: min., 5 °C; optimum, 23.2 ±0.5 °C; max., between 30 and 35 °C. Similar optima have been reported for other species; *P. graminea* (Singh & Saksena, 1973); *P. teres* (Sato & Takeda, 1990) and *Pyrenophora bromi* (Died.) Drechs. (Carter & Dickson, 1961). Platt *et al.* (1977) reported an optimum temperature of 25 °C for growth of *P. tritici-repentis* and also found that density of aerial mycelium was greatest at 5 and 35 °C.

The temperature requirements for optimal sporulation of *P. semeniperda* were likewise similar to those demonstrated for other species of *Pyrenophora* (Onesirosan & Bantarri, 1969; Platt *et al.*, 1977; Sato & Takeda, 1990). Conidia were formed over the range 10 to 30 °C with optimal sporulation occurring between 18 and 20 °C. However, the results obtained may have been confounded at 25 and 30 °C by high humidity. At these temperatures, Petri plates placed on the thermo-gradient plate had free water on the underside of the lids which in many cases dripped onto the growing colonies. This was not the case with Petri plates incubated at the other temperatures. Although high humidity (100 % RH) has been shown to favour conidiation in *P. tritici-repentis* (Platt & Morrall, 1980) sporulation of *P. semeniperda* may have been retarded by the presence of free water. As is the case for many fungi, the optimal temperature for sporulation of *P. semeniperda* appears to be lower than the optimal temperature for mycelial growth (Lily & Barnett, 1951).

Germination of *P. semeniperda* conidia occurred over the range 5 to 35 °C, with the optimal temperatures being 20 to 30 °C. Although no mycelial growth was observed at 35 °C, some germination occurred at this temperature. This may have been due to a lag in the time for the interior of the Petri plate to reach 35 °C on placement in the incubator since Wallace (1959) reported that germination of conidia of *P. semeniperda* occurred within 1 h. The temperature optima for germination of conidia were similar to those for mycelial growth. The germinability of conidia produced at 30 °C was lower than that for the conidia produced at the other temperatures tested. The reason for this loss of germinability is uncertain. It has been shown for *P. graminea* that increasing temperatures resulted in a decrease in conidium size

(Teviotdale & Hall, 1976). This decrease in size may affect germinability of the conidia produced.

P. semeniperda produced more biomass under acidic conditions. No growth occurred in moderate to highly alkaline media. The use of various buffer solutions to control the pH of the culture media may have influenced the growth of colonies. However, the medium of Tomás & Bockus (1987) which was not amended with any buffer solution (initial pH 7.0) produced a biomass similar to those at pH 6.6 or 7.3. Other pH studies have utilised synthetic media amended with varying amounts of HCl and NaOH (Singh & Saksena, 1973; Holmquist, Walker & Stahr, 1983). However, a preliminary study showed that amending media pH using these solutions resulted in large fluctuations in pH over time. When buffer solutions were used to amend pH, the media remained relatively pH stable over several weeks. It was concluded that the use of pH stable media was preferable for testing pH limits as required in this study (Lily & Barnett, 1951). Another method of testing pH effects on fungi is the use of pH gradient plates (Sacks, 1956; Sacks, King & Schade, 1986). However these were difficult to prepare and could not be easily replicated.

The pH optima for growth of other species of *Pyrenophora* were generally more acidic than those reported here. Singh & Saksena (1973) reported maximum growth of *P. graminea* at pH 6, whilst very little growth was apparent at pH 4 and no growth was observed at pH 10 and 11. Kapoor (1970) also found that pH 6 was the optimum for growth of *Bipolaris australiensis* (M. B. Ellis) Tsuda & Ueyama. However, the fluctuations in pH over the range tested were large. It was also demonstrated that growth was poor at low pH (Kapoor, 1970).

Sporulation of *P. semeniperda* expressed using both methods of measurement was greatest at pH 5.34 and in the MAM without the addition of buffer solution. The optima (pH 5.44 and 5.71) are similar to that reported for *B. australiensis* by Kapoor (1970). The addition of buffer solution to the media did not appear to influence sporulation compared to MAM without amendment, but the radial growth (data not presented) was slightly less. Consequently, the unamended MAM produced more conidia colony^{-1}. The pH optima for sporulation of *P. semeniperda* are close to the optimum for mycelial growth. Although fewer pH concentrations were tested for sporulation than growth, it appears that the range for sporulation and growth were similar.

Radial growth of *P. semeniperda* was significantly greater in darkness or when cultures were exposed to a 12 h photoperiod than when exposed to constant illumination or a

6 h photoperiod. Carter & Dickson (1961) found that the greatest radial colony growth of *P. bromi* occurred when cultures were grown in the dark at 24 °C or at 16 °C under 12 h photoperiod. *P. semeniperda* appears to grow faster when exposed to at least 12 h darkness. Depression of growth rate resulting from sub-optimal conditions is generally greater in illuminated than un-illuminated cultures (Carlile, 1965). The possibility exists that the differences in growth rate of *P. semeniperda* attributed to different photoperiods, may be as a result of sub-optimal nutritional conditions. Cultures for these experiments were grown on 1/4PDA rather than the optimal medium (MAM) for accuracy of data acquisition. Carlile (1965) suggested that under optimal conditions, 'light' and 'dark' metabolism is of equal effectiveness in supporting growth, and that departures from optimal conditions affect the processes differently.

P. semeniperda produced more conidia when exposed to both light and darkness than when exposed to either constant illumination or darkness. No other reports of experiments of this nature exist in the literature for *P. semeniperda*. However, this finding is consistent with the findings of many other workers on a range of fungi including other members of the genus *Pyrenophora*. Khan (1971) and Raymond, Bockus & Norman (1985), reported that *P. tritici-repentis* required a light period for conidiophore formation and a dark period for conidia production. Similarly, Platt *et al.* (1977) found that optimal sporulation of *P. tritici-repentis* occurred when cultures were exposed to a 12 h light/dark cycle. They reported that sporulation occurred only when colonies had received a minimum of 3 h darkness and 1 h of light. Onesirosan & Bantarri (1969) demonstrated that abundant sporulation by *P. teres* occurred only if cultures were exposed to 12 h of light alternating with 12 h of darkness. Similarly, Sato & Takeda (1991) reported that *P. teres* required a diurnal 12 h photoperiod, and that sporulation gradually decreased with an extension or reduction in the photoperiod. In contrast, Deadman & Cooke (1985) described abundant sporulation of *P. teres* occurring on dark-incubated detached leaves, but not on dark-incubated PDA. They postulated that differences in sporogenic processes occurred between a pathogen on its natural substrate and the same pathogen cultured on an artificial medium. This evidence may have significant implications for the study of light induced sporulation. *P. graminea* has also been observed to require a light/dark alternation to produce abundant conidia (Houston & Oswald, 1946; Teviotdale & Hall, 1976). Other related species which have been reported as requiring both a light and a dark phase for sporulation include: *P. bromi* (Carter & Dickson, 1961), *P. avenae,*

Bipolaris oryzae (Breda de Haan) Shoem. (Leach, 1962), *Drechslera dictyoides* (Drechsler) Shoem. (Vargas & Wilcoxson, 196?), and *Drechslera catenaria* (Drechsler) Ito (Leach, 1967).

In the present study, *P. semeniperda* was observed to sporulate under conditions of continuous light and complete darkness. Other related species which have been observed to behave in this way include: *Drechslera poae* (Baudys) Shoem. (Lukens, 1960); *Bipolaris maydis* (Nisikado & Miyake) (Fukuk & Aragaki, 1972); and *Exserohilum turcicum* (Pass.) Leonard & Suggs (Cohen, Levi & Eyal, 1978). Photoinhibition of sporulation occurred on maize leaves infected with *E. turcicum* (Cohen, *et al.*, 1978).

Leach (1967) postulated that fungi could be categorised as 'diurnal sporulators' or 'constant temperature sporulators'. Diurnal sporulators had 2 distinct phases during photosporogenesis, an 'inductive phase' which leads to the formation of conidiophores and is stimulated by NUV and a 'terminal phase' which leads to the formation of conidia and is inhibited by NUV. Furthermore, the inductive phase occurred at relatively high temperatures, while the terminal phase occurred at relatively low temperatures. Constant temperature sporulators *sensu* Leach (1967) are stimulated by NUV, but sporogenesis cannot be divided into 2 distinct phases either in terms of light, or temperature. Examples of diurnal sporulators are *Alternaria dauci* (Kühn) Groves & Skolko and *P. tritici-repentis* (Leach, 1967; Khan, 1971) and examples of constant temperature sporulators include: *D. catenaria* and *Fusarium nivale* (Fr.) Ces. (Leach, 1967). The grouping of fungi into diurnal or constant temperature sporulators may be difficult, since isolates of the same fungus may behave differently. For example, Sato & Takeda (1991) reported that isolates of *P. teres* from Canada and Japan sporulated best after exposure to a range of different treatments. The results from the study reported in this thesis suggests that *P. semeniperda* is a constant temperature sporulator *sensu* Leach (1967). Sporulation occurred under constant temperature, and in continuous light, though optimal when alternated with darkness.

Although a large body of work has been reported for the effects of light quality on sporulation of fungi, little or none exists for the effects of light quality on radial growth. Radial growth of *P. semeniperda* was greatest when colonies were exposed to light in the band 500 to 700 nm. Photoinhibition of growth appeared to occur in colonies exposed to light from 400 to 540 nm and to a lesser extent, from 600 to 700 nm. In the treatment which was exposed to all the visible spectrum, growth was decreased relative to the 'yellow' treatment,

probably due to exposure to the other wavelengths. It is postulated that growth of *P. semeniperda* may be enhanced by exposure to light between 500 and 600 nm. Tan & Epton (1973) reported that black light (300 to 420 nm) retarded linear growth of *Botrytis cinerea* Pers. when compared to colonies grown in continual darkness.

The results for sporulation were the opposite of those obtained for radial growth. That is, *P. semeniperda* sporulated equally well under illumination from wavelengths in the band 400 to 540 nm, but poorly when exposed to longer wavelengths. Leach (1962) found that blue light (wavelength undetermined) stimulated sporulation of *P. avenae* and *B. oryzae*. In contrast, Onesirosan & Banttari (1969) described the inhibition of sporulation by wavelengths in the ranges 355 to 495 and 380 to 680 nm for *P. teres*. Other members of the family Dematiaceae have also been reported to have had sporulation inhibited by light of wavelengths between 400 and 515 nm (Aragaki, 1961; Zimmer & McKeen, 1969). Furthermore, Vakalounakis & Christias (1981;1985) described a reversible photoreaction of blue and NUV in sporulation of *Alternaria cichorii* Nattrass. They found that inhibition by blue light could be reversed by irradiation by NUV and *vice versa*. A similar reaction has been reported for *B. cinerea* (Tan, 1974; Tan & Epton, 1974).

It would appear from the results presented in this chapter that control of growth and sporulation of *P. semeniperda* and light quality may be linked. For example, under certain light conditions nutrients may be more readily available to the fungus and vegetative growth may result (Carlile, 1965). Conversely, under light conditions sub-optimal for growth, the fungus may expend more nutrients in an effort to reproduce and hence colonise where nutrients are more readily available.

P. semeniperda sporulated more prolifically after wounding. James, Summerell & Burgess (1991) reported that wounding mycelium of *P. tritici-repentis* did not significantly increase conidial production although a positive trend was observed. Other workers routinely removed aerial mycelium with an instrument to enhance sporulation by *P. tritici-repentis* (Raymond *et al.*, 1985; Hunger & Brown, 1987; Evans *et al.*, 1993). Wounding of mycelium as a method of enhancing sporulation has also been demonstrated for *P. teres* (Al-Tikrity, 1987), while depressing the formation of aerial mycelium by using centrifuged V8 juice has also been considered to enhance sporulation by *P. graminea* (Grbavac, 1981). Wounding mycelium is also a common practice for inducing members of the genus *Alternaria* to sporulate (Douglas & Pavek, 1971; McRae, Heritage & Brown, 1983). The size of mycelial

wounds also influenced sporulation of *P. semeniperda*. An increase in conidial production was associated with a decrease in wound size. That is, conidial production increased with more wounds per unit area. A similar result has been reported for sporulation of *Alternaria carthami* Chowdhuri (McRae, Heritage & Brown, 1983).

A convenient and effective method for producing large amounts of inoculum was devised after experimentation with light (NUV and cool white), sealing Petri plates with Parafilm® and wounding colonies. As mentioned above, *P. semeniperda* was found to sporulate best when exposed to light of wavelengths shorter than 500 nm. A commonly used, cheap and easy way of providing this environment is with the use of NUV fluorescent tubes (Commonwealth Mycological Institute, 1983). Leach (1962) suggested the use of NUV light as a regular means of inducing sporulation of many fungi rather than more elaborate nutritional studies. Although cool white tubes emit some wavelengths in the NUV range, *P. semeniperda* consistently exhibited more prolific sporulation when exposed to a mixture of NUV and cool white light, than cool white light alone.

A common laboratory practice when culturing fungi is to seal Petri plates with plastic wrap (such as Glad Wrap® or Parafilm®) to reduce contamination and/or water loss of the culture medium. The present study showed that the sealing of Petri plates with Parafilm® resulted in a reduced conidial yield. Colonies contained in Petri plates which were sealed for only half of the experimental period resulted in more conidia than those plates which were sealed for the whole experiment ($P<0.05$), and less than those that were unsealed ($P<0.05$). The reason for this could not be established although several possibilities exist. Sealing of Petri plates may lead to a build-up of metabolic by-products or "staling" compounds such as CO_2, which reduce the ability of the fungus to sporulate (Cotty, 1987). Another possibility is that the formation of ozone in irradiated media may cause a reduction in sporulation. Harding (1968) and Rich & Tomlinson (1968) both reported that ozone reduced the ability of *Penicillium* and *Alternaria solani* Ellis & Gibson, to sporulate. The sealing of Petri plates with Parafilm® may increase the concentration of ozone in culture. The characteristic odour of ozone is often encountered when using lamps which emit far-UV and NUV light.

3.5 Summary

Radial growth and sporulation (expressed as conidia cm^{-2} and conidia $colony^{-1}$) were optimal on Modified Alphacel Medium. Growth occurred over the temperature range 5 to less than 35 °C and was optimal at 23.2 °C. Sporulation occurred over the range 10 to 30 °C and was optimal at 19.2 °C when expressed as conidia $colony^{-1}$, and 18.3 °C when expressed as conidia cm^{-2}. The pH for maximal growth was 4.7, while that for maximal sporulation was 5.4 when expressed as conidia $colony^{-1}$, and 5.7 when expressed as conidia cm^{-2}. *P. semeniperda* required an alternating light/dark sequence for good sporulation. Growth was enhanced by light of wavelengths longer than 500 nm, while sporulation was enhanced by light of wavelengths shorter than 500 nm. Growth in liquid culture was greatest in V8 Juice broth, Malt Extract broth and Modified Alphacel broth, however, no sporulation was observed in these media. Sporulation was enhanced by mycelial wounding and illumination by NUV light. Sealing of Petri plates with Parafilm® had a inhibitory effect on sporulation.

CHAPTER FOUR

HOMOGENEITY AND PATHOGENICITY OF MONOCONIDIAL CULTURES OF *PYRENOPHORA SEMENIPERDA*

4.1 Introduction

Daniel *et al.* (1973) proposed that a criterion which bioherbicidal candidates must generally fulfil, is that of genetic stability and host specificity. This chapter will be concerned with phenotypic variation of isolates and their progeny and an appraisal of the homogeneity and pathogenicity of monoconidial isolates of *P. semeniperda* after long-term storage.

Sexual reproductive systems in fungi are common and can influence the genetic make-up of pathogen populations. Bioherbicides therefore, should be limited to those fungi that have no known sexual process, since variation may lead to biocontrol agents with decreased safety and effectiveness (Templeton, *et al.* 1979). However, genetic variability may occur in pathogens limited to asexual reproduction via other processes. For example genetic information may be exchanged between isolates via heterokaryosis and the parasexual cycle (Pontecorvo, 1956; Fincham, Day & Radford, 1979), or mutation of an isolate may occur either naturally or after exposure to mutagenic agents such as ultra-violet light and chemicals (Miller, Ford & Sands, 1989). Concerns about genetic variation are usually centered around introduced biocontrol organisms whose response in a new environment cannot be predicted and variation in endemic organisms which may lead to selection of an undesirable genotype capable of overcoming biotic or geographical constraints which have held the organism in natural equilibrium (Templeton, *et al.*, 1979). According to Leonard (1982), the chances of mutations occurring in the pathogen population of an endemic microbial herbicide is unlikely to differ from those already encountered in nature. He also postulated that the opportunities for adaptation by natural selection of the pathogen to non-target species would be limited because inoculum of a microbial herbicide originates from the same source rather than a naturally selected population from the previous year. Genetic variability in a pathogen used as a microbial herbicide would therefore, pose little or no hazard with respect to non-target species (Leonard, 1982; Weidemann & TeBeest, 1990).

as a microbial herbicide would therefore, pose little or no hazard with respect to non-target species (Leonard, 1982; Weidemann & TeBeest, 1990).

Alternatively, variation within bioherbicidal organisms can be used to advantage. For example, selection of an individual with the desired host specificity or biological characteristics may be possible (Miller *et al.*, 1989; Weidemann & TeBeest, 1990). Leonard (1982) suggested that bioherbicidal organisms can be selected periodically on the weed population to increase their virulence to match the increasing resistance in the plant host.

Unwanted mutations when culturing pathogens also pose problems for workers investigating bioherbicidal candidates. This can happen, for example, when isolates are repeatedly sub-cultured. Difficulties such as a loss of sporulation or a reduction in growth rate can be coupled with a loss of pathogenicity, and impede progress towards the commercialisation of potential bioherbicides. Efforts to reduce the chance of unwanted mutants in culture can be undertaken, such as re-isolation of isolates after passing through host plants and the use of monoconidial isolates when mass transferring for production of inoculum and other studies.

Carter & Dickson (1961) reported that 11 of 51 isolates of *P. bromi* had lost the ability to sporulate in culture after repeated transfer. Singh & Saksena (1973) found that great cultural variation occurred in isolates of *P. graminea* observed in India. Saltation, has been reported in Canadian isolates of *P. semeniperda* (Wallace, 1959). Furthermore, some of the isolates used in the studies reported in this thesis had also produced mutants in culture (Dr. E. Cother, personal communication, 1992).

The studies reported in this chapter were undertaken to elucidate the homogeneity of fresh and stored monoconidial isolates and their progeny. Furthermore, pathogenicity studies were done to investigate the infectivity of conidial propagules in relation to culture age, storage method and storage duration. The data from this research was used to ensure that relatively homogeneous isolates were used for other studies requiring both conidial and mycelial inoculum, as well as providing information on the best ways to store inoculum over protracted periods.

4.2 Materials and Methods

4.2.1 Colony variation when using different transferral techniques.

Growth characteristics were used to make an assessment of the phenotypic variability among cultures of *P. semeniperda* (isolate 580681) using four different transferral techniques.

A 'micro-scalpel' was made to cut germ-tubes and to transfer germ-tubes and conidia to agar media. The 'micro-scalpel' was made of tungsten (W) wire approx. 4 cm long with a diam. of 0.076 mm which was threaded through the eye of a 15 mm hypodermic needle, until 1 cm of wire protruded at the end of the needle. Approx. 1 g of sodium nitrite ($NaNO_2$) was then placed in a small ceramic crucible (4 cm diam.) and heated with a bunsen burner until melting point. The tip of the tungsten wire was carefully dipped into the molten $NaNO_2$ until 0.5 to 1 mm of wire was left exposed at the needle tip. The $NaNO_2$ melted the tungsten wire and left a very fine, hardened point at the tip. This enabled the micro-manipulation of fungal material. A suitable handle for the hypodermic/wire manipulator was fashioned from a bamboo 'barbeque skewer'.

The four different transferral techniques assessed were:

i) Transfer of mycelial cores (3 mm diam.). A cores was excised with a sterile cork borer from the growing edge of 7 day old stock cultures and placed immediately onto a Petri plate containing 1/4PDA. This was replicated 10 times.

ii) Transfer of a single conidium. A conidium was removed using a dissecting needle from the sporulating periphery of a 14 day old stock culture and placed onto a Petri plate containing 1/4PDA. This was repeated 20 times so that 10 replicates resulted after conidial germination.

iii) Transfer of hyphal (germ-tube) tips. About 100 conidia were removed from the sporulating periphery of a 4 day old stock culture and placed on TWA containing 5% agar. This high concentration of agar was required because the surface of the medium offered resistance when cutting the tips of germ-tubes. The conidia were assessed after 12 h to determine those that had germinated to produce two germ-tubes with at least two septa. One germ-tube was excised using the tungsten 'micro-scalpel' by cutting between the two septa. Hyphal tips were placed on 1/4PDA and this procedure was repeated 50 times so that 10 replicates resulted after hyphal elongation.

iv) Transfer of conidia minus one germ-tube. After the germ-tube was excised from a conidium as described above, the remaining conidium with one germ-tube was placed on 1/4PDA. This procedure was repeated 20 times so that 10 replicates resulted after hyphal elongation.

Cultures were placed in an incubator in darkness and 20 °C for 14 days. Colony diameters were measured after 7 days. The experiment was repeated twice.

4.2.2 Growth and sporulation of monoconidial isolates.

Single conidia were removed from the sporulating edge of monoconidial stock cultures (isolates 580681 and 580534) and placed onto either PDA or MAM. Thirty Petri plates of each medium were inoculated and stored at 20 °C for 16 h. Each plate was then studied under a dissecting microscope (x 80) to ensure that the single conidium had germinated. If germination had occurred, the plate was placed under controlled conditions in an illuminated growth cabinet (20 ±1 °C, NUV at 13 µmol m^{-2} s^{-1}, 12 h photoperiod). Growth was measured at 3, 5, 7, 9, 11, and 14 days and sporulation was assessed at 14 days after inoculation. Twenty monoconidial isolates were used in each experiment. Due to the nature of the experiments, statistical analysis of all the data was not appropriate. However, an analysis of variance to determine differences between isolates was carried out using each monoconidial isolate as a replicate. Trials were not repeated.

4.2.3 Growth and sporulation of monoconidial isolate progeny.

The progeny of the monoconidial isolates initiated in the previous experiment were assessed for growth and sporulation characteristics. Progeny isolates were obtained by the methods outlined, except that conidia were obtained from only six of the 20 monoconidial isolates grown on PDA. The six isolates were chosen to represent the two fastest, two slowest and two median growing isolates after 14 (580681) and 11 (580534) days. Ten replicates were used to assess growth and sporulation characteristics as described in the previous experiment. Experiments were not repeated.

4.2.4 Cultural variation and pathogenicity after storage.

Monoconidial stock cultures of isolate 580681 were grown for 14 days and a sterile conidial suspension was made containing 2 x 10^4 conidia ml^{-1}. Five ml portions were

dispensed into sterile polycarbonate tissue culture vials and stored in either a domestic refrigerator (approx. 4 °C) or a domestic freezer (approx. -15 °C). After storage for one week, one, three, four, five, and six months the cultural variation and pathogenicity of the stored isolates were tested. Germination was assessed using the method described in section 3.2.2 of this thesis. The growth and sporulation of ten monoconidial colonies after storage at the two temperatures for the different periods of time were assessed by establishing 10 isolates on 1/4PDA for growth and MAM for sporulation. Growth was measured as colony diam. at 7 days, and sporulation was measured at 14 days after inoculation. Pathogenicity of stored conidia was assessed by inoculating 7 day old seedling wheat leaves with 50 µl of conidial suspension using the cover-slip method (section 2.4) and incubating for 24 h in darkness at 20 °C. The number of lesions produced on leaves was counted 10 days after inoculation.

4.2.5 Effect of culture age on conidial germination and pathogenicity.

Monoconidial cultures of isolate 580681 were initiated on MAM and grown at 20 °C in darkness. After 3, 6, 9, and 12 days growth, lines denoting colony growth were drawn on the bottom of the Petri plates by tracing around the colony circumference. At 14 days after inoculation, rings of mycelium and medium were cut from the cultures. Conidial suspensions were made from each ring (as described in section 2.3) and adjusted to a final concentration of 1000 conidia ml^{-1}. These suspensions represented conidia with ages of approx. 2-5, 5-8, 8-11, and 11-14 days. Conidial germination was measured. Seedling wheat leaves (10 day old) were inoculated using the cover slip method with 50 µl of suspension and incubated in the dark at 20 °C for 24 h. The number of lesions produced on leaves was counted 10 days after inoculation.

4.3 Results

4.3.1 Colony variation when using different transferral techniques.

Colony diam. after 7 days growth was significantly greater ($P<0.05$) when *P. semeniperda* was transferred as mycelial cores (mean = 25 mm). Growth initiated from single conidia (mean = 22.5 mm) or conidia without one germ-tube (mean = 23 mm) was significantly greater than that of isolates initiated from hyphal tips (mean = 19 mm) ($P<0.05$).

4.3.2 Growth and sporulation of monoconidial isolates.

Growth of monoconidial isolates was always poor on PDA when compared to that on MAM (P<0.01). After 14 days incubation all monoconidial isolates of 580534 grown on MAM had reached the edge of the Petri plates (colony diam. 88 mm). Colony diams. of isolate 580681 grown on MAM ranged from 71 mm to 88 mm, with an average of 78.5 mm (SD = 2.8). This constituted a significant difference between, isolates 580681 and 580534 (P<0.01) when each monoconidial isolate was used as a replicate in an analysis of variance.

No sporulation was observed by monoconidial isolates grown on PDA. Sporulation of isolate 580534 ranged from 3860 to 5660 conidia ml^{-1} with an average of 4640 conidia ml^{-1}. Sporulation of isolate 580681 ranged from 4120 to 5740 conidia ml^{-1} with an average of 4760 conidia ml^{-1}. Isolates 580681 and 580534 did not differ significantly (P>0.1), when each monoconidial isolate was used as a replicate in an analysis of variance.

4.3.3 Growth and sporulation of monoconidial isolate progeny.

In general the progeny of the monoconidial isolates grew and sporulated in a similar manner to the isolates from which they were derived. (Table 4.1). The groupings based on growth, (i.e. slowest two to fastest two growing isolates) stayed the same for parent isolate 580534, whilst the grouping from isolates derived from parent isolate 580681 was similar although the median isolates (580681#3 and #4) grew faster as progeny. The results for sporulation of monoconidial progeny were also close to that for the parent isolate.

4.3.4 Cultural variation and pathogenicity after storage.

No significant differences were observed in either colony growth or sporulation regardless of the duration of storage or the storage method (P>0.05, Table 4.2). The proportion of conidia that had germinated after storage was significantly greater in suspensions that had been refrigerated than those that had been frozen (P<0.01). A significantly smaller proportion of conidia germinated after storage in a freezer for > 1 week (P<0.05). In contrast, a significantly smaller proportion of conidia germinated after storage for 6 months in a refrigerator (P<0.05) than in a freezer. Significantly fewer lesions were produced when seedling wheat leaves were inoculated with suspensions that had been stored for > 3 months, regardless of storage method. However, conidia retained their infectivity

longer when stored in a refrigerator. No lesions were produced if conidia had been stored for > 4 months.

Table 4.1. Growth and sporulation of first generation progeny of *P. semeniperda* in relation to that of the parent monoconidial colonies. Growth (colony diam.) measurements are after 11 days (580534) and 14 days (580681). Progeny data are means of 10 replicates.

Isolate	Grouping	Parent isolate		Progeny isolate	
		Growth (mm)	Conidia ml^{-1}	Growth (mm)	Conidia ml^{-1}
580534#1	slowest	58	4450	62	4120
580534#2	slowest	60	3960	62	4060
580534#3	median	64	4840	68	4460
580534#4	median	64	5220	66	4580
580534#5	fastest	68	4020	68	4220
580534#6	fastest	68	4140	70	4320
Mean		**64**	**4438**	**66**	**4293**
580681#1	slowest	70	4120	72	4440
580681#2	slowest	72	4680	76	4380
580681#3	median	78	4200	88	4460
580681#4	median	78	4220	88	4340
580681#5	fastest	88	4760	88	4560
580681#6	fastest	88	4160	88	4240
Mean		**79**	**4357**	**84**	**4403**

4.3.5 Effect of culture age on conidial germination and pathogenicity.

The proportion of conidia that germinated was statistically similar regardless of conidial age (P>0.1). Significantly fewer lesions were produced when seedling wheat leaves were inoculated with conidia that were 2-5 days old (P<0.01). No significant differences in lesion numbers were detected when wheat seedling leaves were inoculated with conidia between 5 and 14 days old (Fig. 4.1)

Table 4.2. Variation in colony growth and sporulation, conidial germination, and pathogenicity of *P. semeniperda* (isolate 580681) after storage for up to 6 months in either a refrigerator or a freezer. Data within the same column and storage method followed by a common letter are not significantly different according to Tukey's HSD test (P>0.05). Data are means of 10 replicates over duplicate experiments.

Storage method	Storage duration	Colony diam. (mm)	Sporulation (con ml^{-1})	Germination (%)	Lesion no.
Refrig.	0 days	39 ±2	4145 ±611	99 ±1**a**	22 ±0.5**a**
Refrig.	1 week	40 ±0.5	4190 ±595	98 ±1.2**a**	23 ±0.7**a**
Refrig.	1 month	40 ±[illegible].5	4245 ±684	99 ±1**a**	24 ±0.4**a**
Refrig.	3 month	39 ±2	4330 ±624	99 ±1.5**a**	19 ±1.2**a**
Refrig.	4 month	38 ±2	4035 ±658	96 ±1.2**a**	13 ±1**b**
Refrig.	5 month	37 ±2.5	4385 ±662	97 ±1.4**a**	0
Refrig.	6 month	40 ±2	4305 ±598	89 ±4.2**b**	0
Freezer	0 days	39 ±2	4245 ±614	99 ±2.1**a**	23 ±1.2**a**
Freezer	1 week	38 ±1.8	3975 ±612	87 ±1.5**a**	22 ±1.5**a**
Freezer	1 month	39 ±1.5	4255 ±634	45 ±18**b**	16 ±2**b**
Freezer	3 month	38 ±1.5	4190 ±585	35 ±24**b**	14 ±1.6**b**
Freezer	4 month	39 ±2	4085 ±610	37 ±26**b**	12 ±1.1**c**
Freezer	5 month	39 ±3	4520 ±665	19 ±6**b**	0
Freezer	6 month	40 ±1.5	4235 ±605	29 ±11**b**	0

4.4 Discussion

Growth of freshly sub-cultured isolates was greatest when fungal material was transferred as cores of mycelium. Colonies initiated from cores of mycelium had many growing points and were reliant only on exogenous nutrients available from the culture medium. Colonies initiated from single conidia had fewer growth points for establishment and growth may have relied on nutrients supplied from the conidium until the new colony was able to assimilate nutrients from the culture medium (Prosser, 1983). Transfer of fungal material as hyphal tips resulted in significantly slower growth. This may have been because the nutrients available within a conidium were not available to hyphal fragments after excision from the parent

conidium. Furthermore, growth was initiated from a single growing point (van Etten, Dahlberg & Russo, 1983).

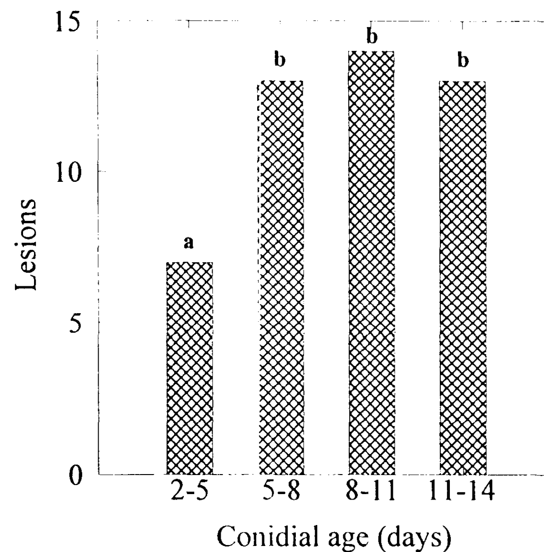

Fig. 4.1. The effect of culture age on pathogenicity of conidia of *P. semeniperda* expressed as the number of lesions produced on inoculated 10 day old wheat seedling leaves. Columns with a common letter do not differ significantly according to Tukey's HSD test (P>0.01).

Some phenotypic variation in the growth and sporulation of monoconidial isolates of *P. semeniperda* and their progeny was observed. The significance of this variation is uncertain because a statistical analysis of the data was not valid. The colony diameters of progeny were generally larger than the parent isolates but sporulation was similar. However, because fluctuations in the magnitude of both measures occurred, it would appear that this phenotypic variation is probably attributable to chance and not genetic variation.

The conidia of *P. semeniperda* are multicellular. However, cellular observations after staining with toluidine blue illustrated that the cells of *P. semeniperda*, in particular those of the conidium, are uninuclear (personal observation). Therefore, although germ tubes arise from different cells, the genomes should be the same since the vast majority of Ascomycete fungi are haploid (Fincham *et al.*, 1979; Kendrick, 1992). Mutation of isolates can also occur after heterokaryosis (Alexopoulos & Mims, 1979; Fincham *et al.*, 1979), which may be more likely to occur when cells are dikaryotic. Although a dikaryotic phase was not established for the isolates of *P. semeniperda* studied here, the possibility exists that one could occur later in the life-cycle, particularly when sexual fruiting bodies are formed (Talbot, 1971). The nuclear

condition of the hyphae of *P. semeniperda* during production of sexual bodies has not been reported. Morphological changes in isolates can also occur through altering growth conditions, such as the build up of "staling" compounds or changes in culture temperature (Prosser, 1983). It would appear that changes exhibited by the isolates of the present study would be more likely to occur for environmental rather than genetic reasons, because of their uninuclear hyphal cells.

The storage of conidial suspensions in a refrigerator or a freezer for up to 6 months did not significantly alter either growth or sporulation characteristics of cultures arising from the stored conidia. However, freezing did result in significantly reduced germinability of conidia. Loss of germinability of conidia may have been the result of lysis (Vegh *et al.*, 1988). Furthermore, pathogenicity of conidia decreased such that no lesions were produced on leaves inoculated with conidia that had been stored for more than 5 months. In a study on storage of a *Phytophthora* sp., Vegh *et al.* (1988) reported that radiating growth of frozen isolates decreased in comparison to controls, but that pathogenicity did not. Papavizas *et al.* (1984) found that the best temperatures to prolong shelf life of *Trichoderma* spp. were -5 to 5 °C. They also reported an increased shelf life when mycelial fragments were stored, and postulated that mycelium was more resistant to desiccation than conidia. The longevity of stored mycelium of *P. semeniperda* requires verification.

Conidia to be used in mycoherbicide studies are commonly desiccated prior to storage. Liquid inoculum is prepared by re-hydrating the conidia in the appropriate carrier formulation (Walker & Riley, 1982; Klerk, *et al.* 1985). In a preliminary study on the drying of *P. semeniperda* conidia using chemical desiccants such as $CaCl_2$ and $CaSO_4$ (data not presented), germinability and pathogenicity of conidial inoculum was reduced. Furthermore, air-drying of conidia resulted in substantial contamination of inoculum. This became more apparent when conidia were re-hydrated. The encapsulation of potential biocontrol agents in an alginate matrix has been suggested as a method to simplify production of pathogens used as mycoherbicides (Walker & Connick, Jr., 1983). In a pilot study, *P. semeniperda* was able to survive the encapsulation process and produce conidia from alginate pellets placed in a humid environment. Efficacy trials in the field were established with this form of inoculum (Chapter 8). A stable formulation, that is, one which has a shelf life of up to 2 years, is generally deemed to be a pre-requisite for a marketable bioherbicidal product (Churchill, 1982). However, this may not be essential. For example DeVine®, the first commercially

available bioherbicide, was distributed as a liquid and had a shelf life of only 6 weeks (Kenney, 1986). The results from the present study indicate that conidial suspensions of *P. semeniperda* may be stored under refrigerated conditions (4 °C) for up to 3 months without loss of germinability or pathogenicity

The age of conidia produced by *P. semeniperda* did not affect the proportion that germinated. However, inoculation of wheat leaves with the youngest conidia resulted in fewer lesions ($P<0.01$). This result suggests that an unknown factor required for pathogenicity is developed after conidium formation and may be related to conidium age. This contrasts with results obtained for *Botrytis fabae* Sardina by Last (1960). Last (1960) reported that the proportion of *B. fabae* conidia that germinated did not differ with respect to culture age (up to 40 days) but, infectivity of those conidia decreased dramatically from 100 % infectivity at 10 days to 1 % at 40 days. Kong, Kochman & Brown (1991) reported that infectivity of conidia of *Alternaria helianthi* (Hansf.) Tub & Nish decreased with conidium age and could be attributed to the loss of viability of ageing conidia. Although conidia older than 14 days were not tested in the experiments reported here, similar infectivity rates have been observed in other experiments (data not presented) with conidia of *P. semeniperda* up to 21 days old.

4.5 Summary

Growth of *P. semeniperda* was always greatest when fungal material was transferred as mycelial cores. Monoconidial isolates grew faster than isolates initiated from hyphal tips. Some variation occurred in the growth and sporulation of monoconidial isolates and their progeny. The variation observed in monoconidial isolates was attributed to chance and not genetic variation. As a result of these studies, stock cultures of *P. semeniperda* isolates were maintained by single-spore isolations. Conidia retained their germinability when stored under refrigerated conditions (approx. 4 °C) for up to 6 months. However, pathogenicity of the conidia decreased markedly after 3 months in storage. Conidia stored under frozen conditions (-15 °C) retained germinability and pathogenicity for only 1 week. The age of conidia produced in culture did not affect the proportion that germinated, however, conidia that were 2-5 days old produced significantly fewer lesions on inoculated wheat seedling leaves than those aged between 5 and 14 days.