CHAPTER 4

CYTOLOGY OF BASIDIOSPORE FORMATION AND EXAMINATION OF HAPLOID VIRULENCE IN *PUCCINIA HELIANTHI*

4.1 INTRODUCTION

It has been shown that virulence in rust fungi can be conditioned by single genes that are inherited in Mendelian fashion. Flor(1955,1956b) in a study of the genetics of virulence in flax rust *Melampsora lini* Sesm. and of resistance in flax concluded that for each gene determining resistance in the host there was a specific gene conditioning virulence in the pathogen. Virulence was most often found to be recessive to dominant avirulence. This was the basis for the gene-for-gene hypothesis which was examined further by Person(1959).

The genetic nature of virulence in the Uredinales suggests that the creation of new virulent pathotypes involves genome modification. The four mechanisms of modification suggested by Day(1974) were mutation, heterokaryosis, parasexual recombination and sexual recombination. Evidence that new pathotypes of rust may arise from parasexual recombination following heterokaryosis was considered by Burnett(1975) to be circumstantial. Burnett(1975) also discussed extra-chromosomal inheritance of virulence. Other recent work with the genetics of virulence of flax rust has shown the existance of genes that can inhibit expression of avirulence and can lead to unexpected segregation ratios in genetic analyses of some pathotypes (Lawrence, Mayo and Shepherd,1981; Jones,1988).

Mutations to virulence are thought to be important sources of new pathotypes when sexual recombinations are not possible because of failure to complete the life cycle (Statler,1987). Chemical mutagens and radiation have been used under laboratory conditions to demonstrate that new pathotypes of *Melampsora lini* (Flor,1956a; Flor,1958; Flor,1960; Statler,1985a), *M.medusae* (Prakash and Heather,1986), *Puccinia recondita* (Statler,1985b; Statler,1987), *P.coronata* (Zimmer, Schafer and Patterson,1963) and *P.graminis* (Watson,1957) can arise through mutation.

In nature the frequency of mutations to virulence may be low but the large size of pathogen populations results in the constant appearance of new virulent mutants (Day, 1978). Stakman, Levine and Cotter(1930) recovered a novel virulent mutant from a culture of *P.graminis* f.sp.*tritici* only after culturing it through successive generations for 13 years. These mutants may not be fit to survive. However, Watson(1957) showed that induced mutations to virulence in the laboratory paralleled the changes in virulence of the *P.graminis* population that occurred in the field.

Novel pathotypes of rusts may also arise through heterokaryosis. Somatic hybridization resulting from hyphal fusions and reassortment of the dikaryotic nuclei was used to explain the isolation of new pathotypes when urediniospores of two pathotypes of *Puccinia striiformis* were mixed and used to infect a susceptible host (Little and Manners,1969a). Nelson, Wilcoxson and Christensen(1955) also attributed a demonstrable variation in virulence in *P.graminis* in the absence of the sexual stage to heterokaryosis.

Meiotic recombination has been a source of new pathotypes in fungi where the sexual cycle can be completed. This has been demonstrated in nature where a greater diversity of alleles for virulence, expressed phenotypically as different pathotypes, can be found in sexually reproducing populations than in asexual reproducing populations. Stakman and Loegering (in Stackman and Harrar,1957) found 43 pathotypes of *Puccinia graminis* around bushes of the alternate host, *Berberis* spp, in Pennsylvania. Only five pathotypes were found in surveys in other areas of the state. Similar results were recorded in Israel where undescribed pathotypes of oat crown rust *P.coronata* were recovered from aecia on the alternate host *Rhamnus palaestina* (Wahl, Dinoor, Halperin and Schreiter, 1960). Groth and Roelfs(1982) found that a sexual population of *P.coronata* in the United States had 10-20% more virulence genes than asexual populations. In Canada, Martens, Clark and Seaman(1985) found greater virulence in sexual populations of *P.graminis* f.sp. *avenae* than in asexually reproducing populations. Pathotypes once developed, are exposed to selection pressures so that only reproductively fit forms will survive (Al-Kherb, Roelfs and Groth,1987).

The sexual cycles of some rust pathotypes have been completed in the laboratory. These often illustrate the heterozygous nature of the genotypes for virulence. Self-fertilization studies can result in the production of novel progeny. Wilcoxson and Paharia(1958) selfed Race 111 of *P.graminis* f.sp. *tritici* and identified 15 pathotypes among the progeny. Jabbar Miah and Sackston(1970b) selfed four pathotypes of *Puccinia helianthi* and demonstrated the heterozygous nature of the genotypes of the isolates used.

It is necessary to understand the nuclear cytology of the rust fungus studied before the inheritance of traits can be fully appreciated. The nuclear events in the life cycle of a rust fungus are nuclear fusion, meiosis and dikaryotization (Hiratsuka,1973). Teliospores in the 'classical system' as typified by *P.malvacearum* (Petersen,1974) are formed from the dikaryotic vegetative mycelium. The two haploid nuclei in the teliospore undergo karyogamy to form a true diploid nucleus. Meiosis occurs in the metabasidium formed when the teliospore germinates, resulting in four haploid nuclei that migrate into the four basidiospores formed.

Basidiospores are usually represented in texts as being uninucleate (Alexopoulus,1962; Talbot,1971; Burnett,1975). Anikster(1983) suggested that basidiospores of many rust fungi were more commonly binucleate. A mitotic

division of the single nucleus of the basidiospore resulted in the spore becoming binucleate. There seems to be inconsistencies in the use of terminology in the literature for describing cells containing two nuclei. This is evident when trying to differentiate between cells containing genetically similar or dissimilar nuclei. Hawksworth, Sutton and Ainsworth(1983) defined a dikaryon as having two genetically distinct haploid nuclei but defined binucleate cells as dikaryotic. No distinction was made therefore for cells containing two genetically similar nuclei. Rieger, Michaelis and Green(1976) considered that binucleate and dikaryotic cells each contained two nuclei which could be termed homokaryotic if genetically similar or heterokaryotic if genetically different. If the two nuclei of the basidiospores are the product of mitotic divisions then the spores are homodikaryotic.

Following germination of basidiospores and infection the binucleate condition of the cells of the pycnial thallus is reduced to uninucleate. Transfer of pycniospores results in fertilization of the pycnia and the formation of the aecial stage. Several variations on this scheme have been reported among the Uredinales (Olive,1953; Petersen,1974). Craigie(1959) described nuclear behaviour in the dikaryotization of haploid pycnia of *P.helianthi*.

If virulence is recessively inherited then the presence of an allele for virulence will not be expressed in a dikaryon heterozygous for that allele. The haploid nature of basidiospores should allow the expression of virulence to the host of the dikaryon. In autoecious rusts the host species for the monokaryon is the same as that for the dikaryon. This allows comparison of the uredinial spore and basidiospore virulence spectra (Flor,1959; Statler and Gold,1980; Kolmer, Christ and Groth,1984).

The studies reported in this chapter were to be a preliminary for a more extensive investigation of the genetics of virulence of *Puccinia helianthi* in Australia. Nuclear behaviour from dikaryotic mycelia to basidiospore infection was examined. The virulence spectra of basidiospores derived from a number of teliospore cultures were examined on a set of sunflower differential lines and some self-fertilizations (sib) were made.

4.2 EXPERIMENTAL

4.2.1 Cytological studies on the life cycle of *Puccinia helianthi* from teliospore formation to intra-epidermal vesicle formation.

The objective of this investigation was to study the cytology of the life cycle of *P.helianthi* from intercellular dikaryotic hyphae through teliospore formation and germination to basidiospore infection. Whole leaf staining was employed for some of the work because tissue sectioning

apparatus was not available. Several biological stains with specificity to chromatin or its components were tested in this study.

Materials and Methods.

The Feulgen technique of Williams and Mengden(1975) with the modifications for ambient temperature hydrolysis suggested by Prakash('980) was found to give reliable results. Regressive staining with Heidenheins haematoxylin following the schedule of Clark(1973) provided good staining of nuclei in metabasidia. Fast Green was sometimes used as a counter-stain for haematoxylin. Mounting feulgen-stained material in aceto-carmine was sometimes applied to darken nuclear structures. Carnoys fluid No.2 proved to be a satisfactory fixative. Other techniques that were trialed and failed or were considered inferior included the schedules for HCl-Giemsa (Goates and Hoffman,1979), acetocarmine (Smith,1947), propiono-carmine (Lu,1962) and the haematoxylin technique without fixation of Olive(1941).

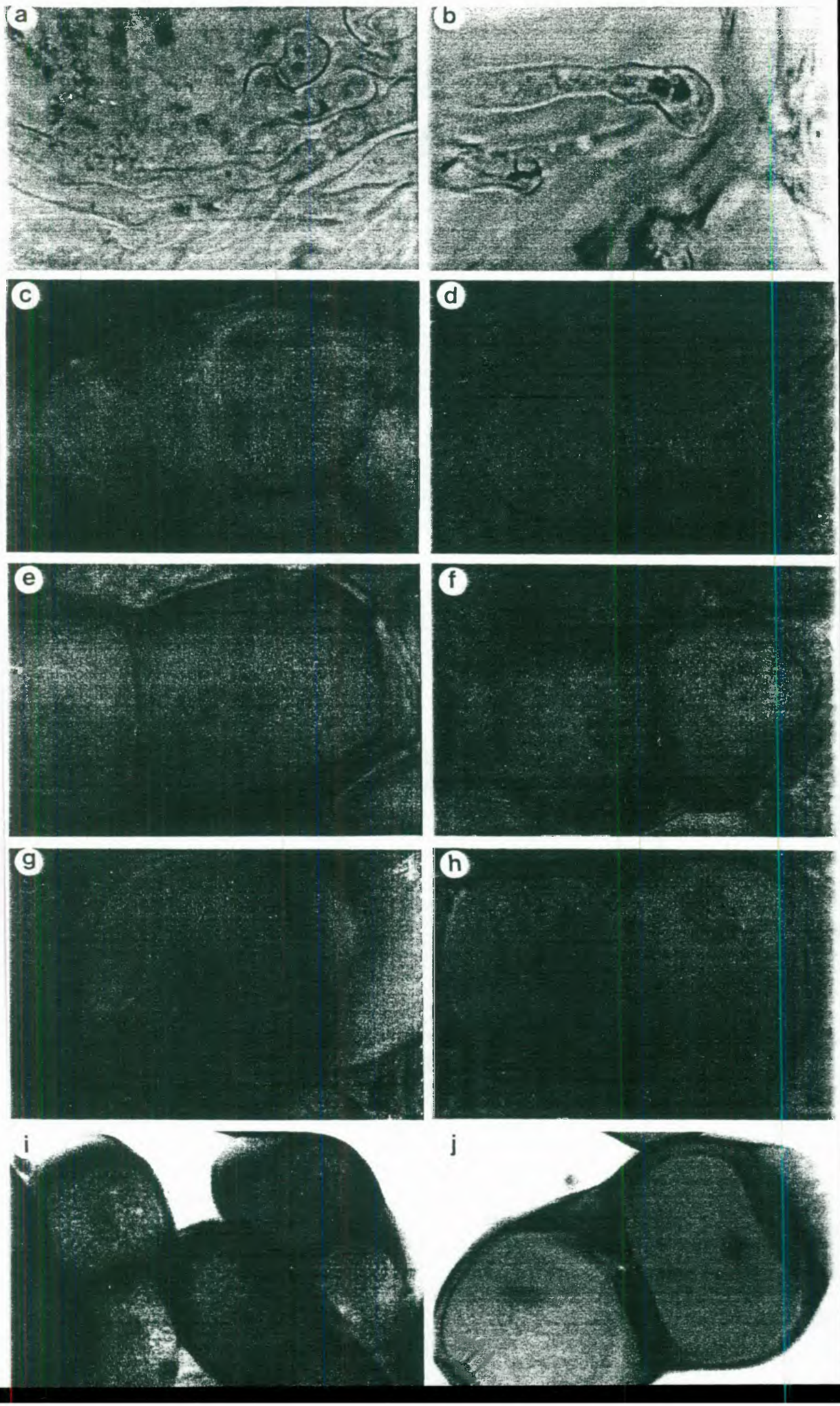
Fresh sunflower leaf tissue bearing telia was subjected to the Feulgen technique described by Williams and Mengden(1975). The hydrolysis in hydrochloric acid allowed the tissue to be teased and squashed. The intercellular vegetative hyphae were then exposed as were the hymenial cells of the telia. Whole leaf staining was also used for examination of intra-epidermal vesicles. Germinating teliospores were suspended over leaf discs of the susceptible sunflower line S37-388 in a moist chamber for 24h. The discs were then stained by the Feulgen technique. Host cell and fungal nuclei but not the cytoplasms were stained. Careful microscopy allowed the intra-epidermal vesicles to be observed.

Examination of metabasidial formation was obtained by staining teliospores that were germinated *in vitro*. Glass slides were cleaned in absolute alcohol and coated on one side with two layers of 1% water agar. This was achieved by dipping each slide into molten agar and wiping one surface clean on paper towel. When the first coat had solidified the procedure was repeated. A large drop of an aqueous suspension of teliospores activated to germinate by cold-soak preconditioning (Chapter 2) was placed on the agar and excess moisture was withdrawn by placing a piece of filter paper against the drop. The prepared slides were placed inverted, each end resting on lengths of balsa wood, over wet filter paper in 20cm glass Petri dishes. These were then incubated at $17^0 \pm 2^0$C for various durations (8-16h) before sample slides were removed and dried at 30°C. After the agar had dried the slides were fixed and stained.

Results.

The vegetative mycelium from which telia of *P.helianthi* formed was intercellular and dikaryotic (Figure 4.1a,b). In the hymenial layer of the telium dikaryotic teliospores were formed (Figure 4.1c,d). Karyogamy occurred as the teliospores matured. The two nuclei came into contact and the

Figure 4.1 De-dikaryotization in *Puccinia helianthi*
a-b Dikaryotic vegetative intercellular hyphae
c-d Dikaryotic immature teliospores
e-h Chromosomes form and nuclei fuse
i Expanded diploid nuclei in teliospores
j Contracted diploid nucleus in each teliospore cell
(x1000, Feulgen technique)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chromosomes underwent synapsis and the subsequent rounding of the nucleus indicated the formation of the nuclear membrane (Figure 4.1 e-i). In the mature teliospore the nuclei were unexpanded (Figure 4.1j).

The nuclei were expanded as the teliospores germinated and the metabasidia emerged (Figure 4.2a,b). The nucleus entered the metabasidium and assumed a position in the middle of the cytoplasmic contents (Figure 4.2c,d). The chromosomes shortened and thickened during Prophase I (Figure 4.2e,f). The formation of a distinct metaphase arrangement was not observed. The daughter nuclei separated during Anaphase I (Figure 4.2g,h) and a brief Telophase I was entered as the nuclei were separated by a septum (Figure 4.2i,j).

The second meiotic divisions involved the smaller reduced nuclei (Figure 4.3a,b). Prophase II and Anaphase II did not occur synchronously in each cell of a metabasidium. The four Telophase II nuclei in each metabasidium expanded and were separated by septa (Figure 4.3c-f).

A basidiospore formed on the sterigma that developed from each cell of the metabasidium. The single nucleus of each metabasidial cell entered the developing basidiospore. The basidiospore nucleus often divided again to render the spore binucleate (Figure 4.3h-j).

The two nuclei migrated from the basidiospore into the developing intra-epidermal vesicle (Figure 4.4a). The nuclei moved apart and a septum was formed between them. From that stage on mycelial cells were uninucleate (Figure 4.4b-c).

4.2.2 Comparative virulence of basidiospores from field collections of teliospores of *Puccinia helianthi* on sunflower rust differential lines.

The study reported in this section was conducted to examine the diversity of virulence present in teliospores of *P.helianthi* collected in a number of regions of Australia over a number of years.

Material and Methods.

Collection of sunflower tissue bearing telia of *P.helianthi* was commenced in 1981. Usually, infected leaves were collected without regard to their maturity. Areas of leaf with a concentration of telia were excised and allowed to air-dry at room temperature for 48h. Tissue was then stored in brown paper bags in a domestic refrigerator at 4^{0}C until used.

Forty samples representing the period 1981-1989 were collected from various host lines growing in the regions; Central Queensland, Dawson-Callide, Darling Downs, northern New South Wales and Victoria (Table 4.1). Tissue of each collection bearing approximately 150 telia was cold-soak preconditioned for 3 weeks. An aqueous suspension of teliospores of each collection was then prepared by crushing and teasing at least 50 preconditioned telia in a droplet of distilled water. Each suspension was

Figure 4.2 First meiotic division in *Puccinia helianthi*
a-b Expanded generative nuclei in germinating teliospore cells
c-d Diploid nuclei in metabasidia
e-f Chromosomes in Prophase I
g-i Anaphase I
j Telophase I (Septum arrowed)
(x1000 a,b,e,g,i Feulgen technique; c Feulgen technique and acetocarmine; f,j haematoxylin; d,h haematoxylin and fast green)

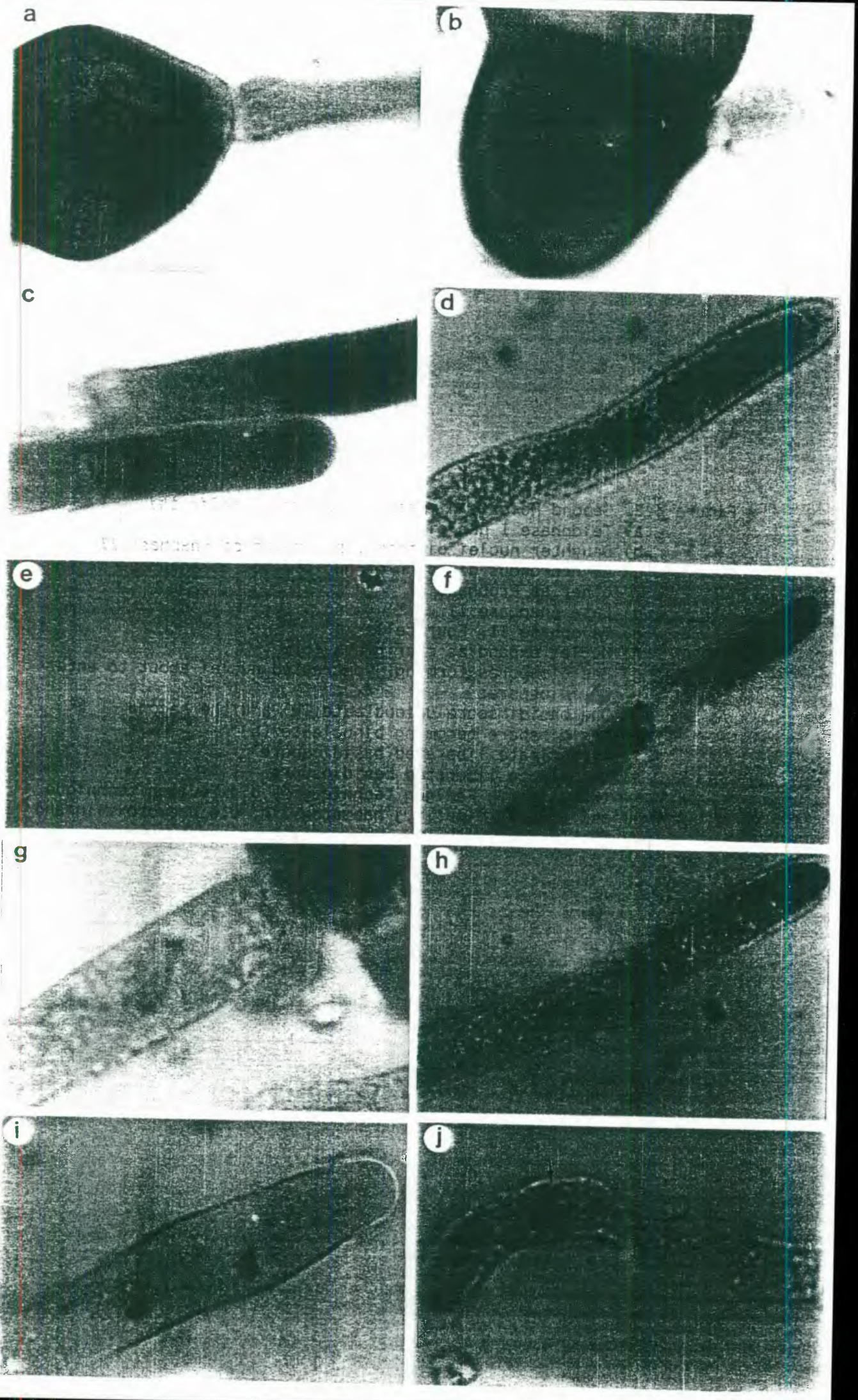
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Figure 4.3 Second Meiotic division in *Puccinia helianthi*

a) Telophase I nuclei
b) Daughter nuclei dividing, beginning of Anaphase II
c) Asynchronous division of nuclei. One at Anaphase II the other at Prophase II
d) Late Anaphase II
e) Telophase II. Four cells
f) Nuclei expand as sterigmata form
g) Basidiospores forming. Contracted nuclei about to enter basidiospores
h) One basidiospore uninucleate (u) and the second basidiospore becoming binucleate (b)
i) Uninucleate liberated basidiospore
j) Binucleate liberated basidiospore

(x1000 c,f,h Feulgen technique; a,b,g Feulgen technique and acetocarmine; i,j haematoxylin; d,e haemotoxylin and fast green)

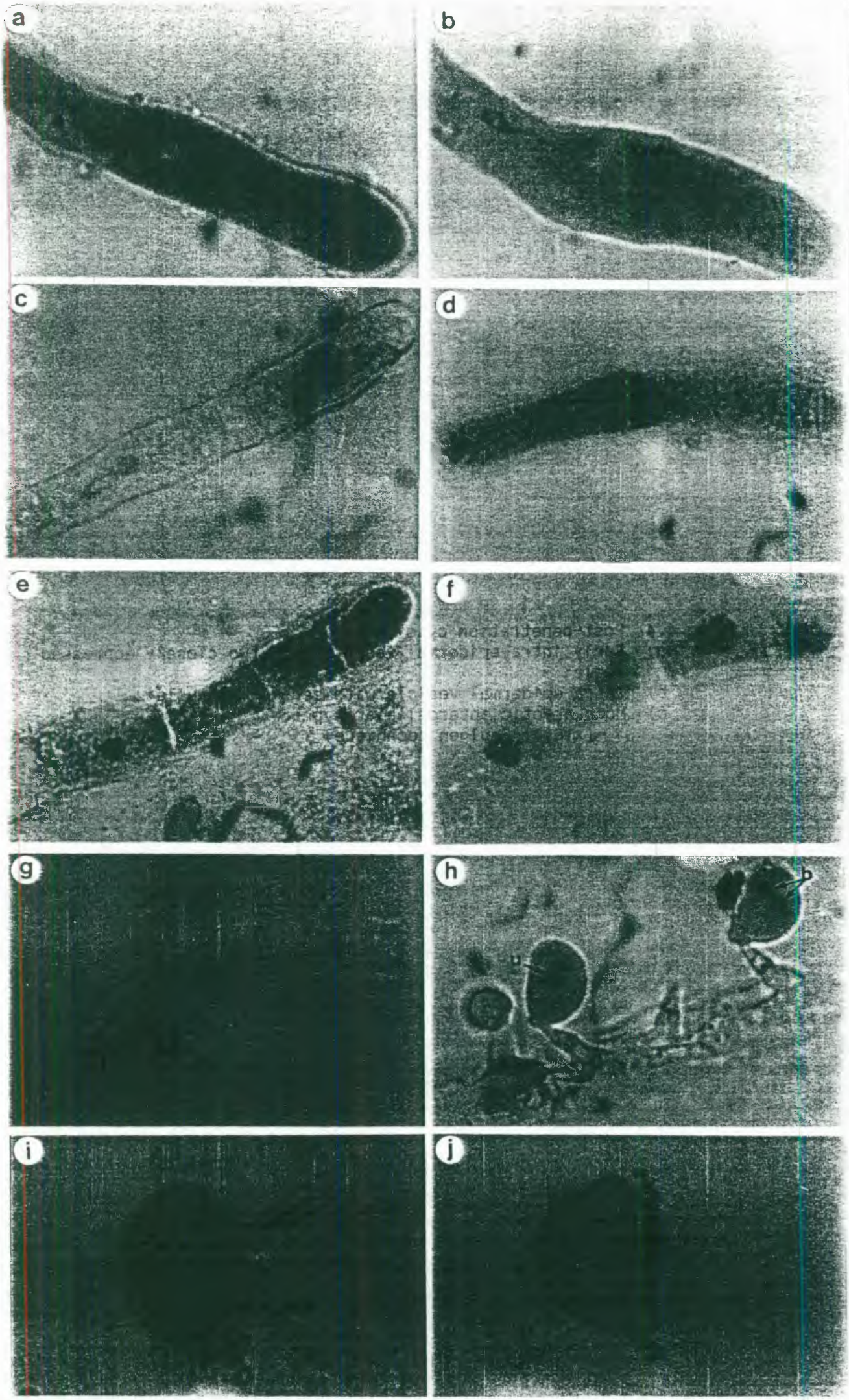
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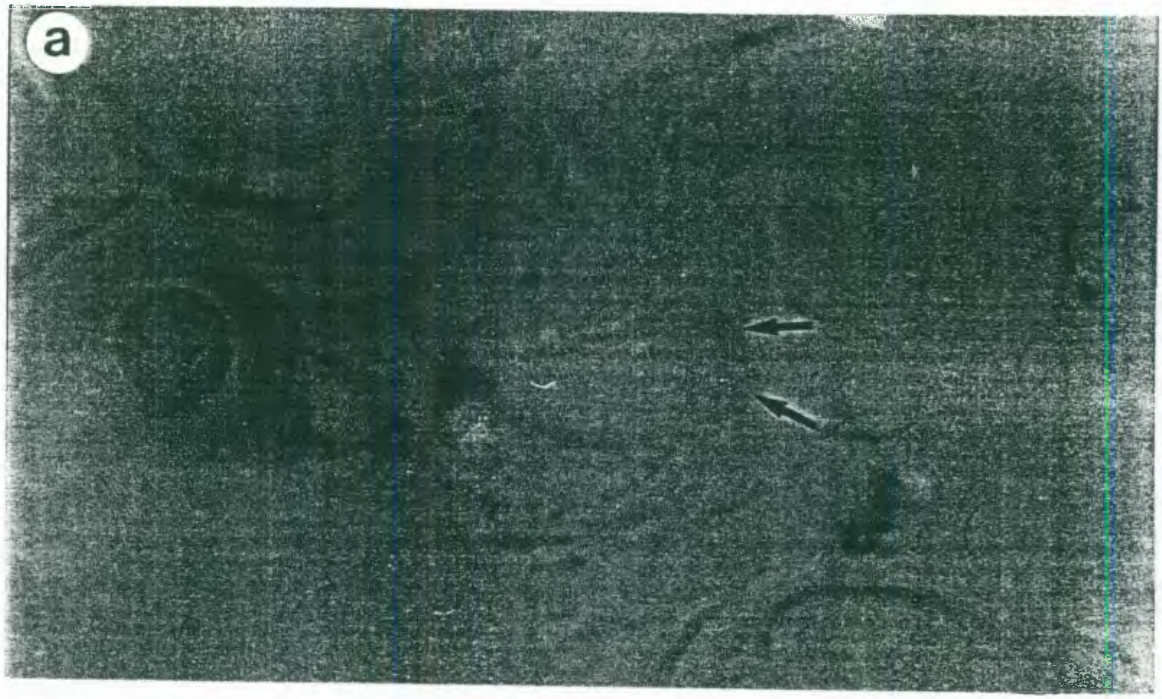
Figure 4.4 Post-penetration cytology
a) Early intra-epidermal vesicle with two closely appressed nuclei
b) Intra-epidermal vesicle with seperating nuclei
c) Monokaryotic intercellular hyphae
(x1000, Feulgen technique)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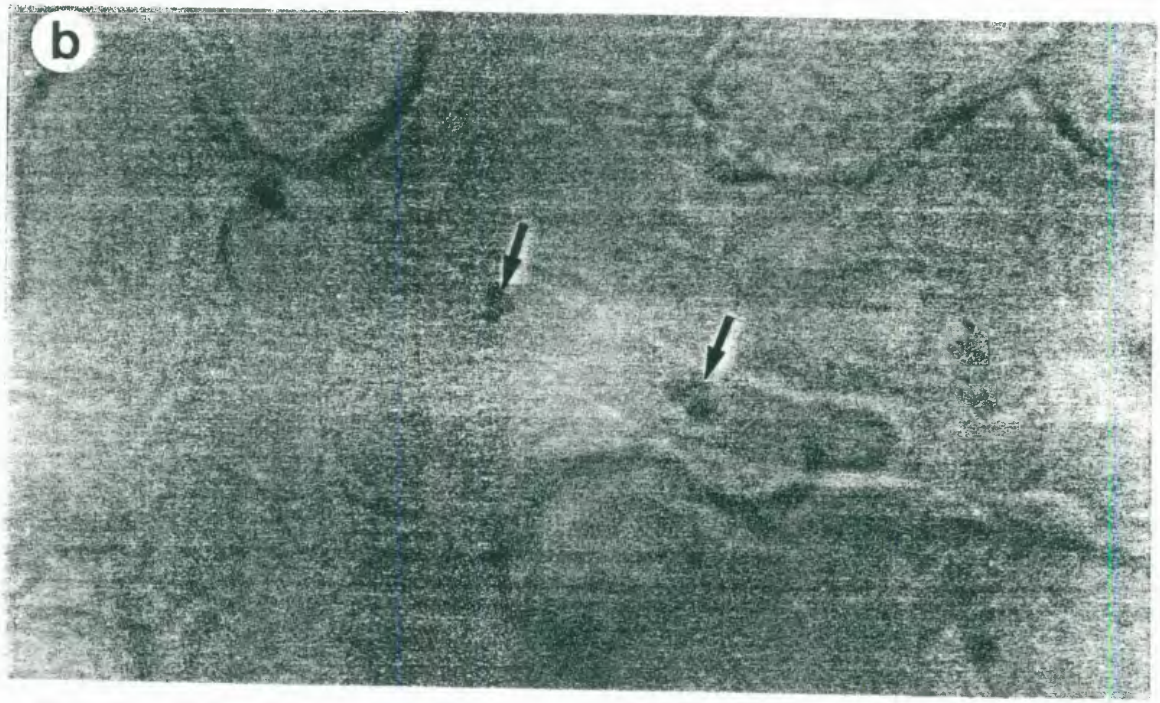
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made up to 2ml with more distilled water and then distributed onto solidified 0.5% water agar contained in 21.5 x 30.5 x 1.5cm transparent plastic trays.

The trays containing the inoculated agar were inverted over sunflower seedlings growing in 4 x 7 compartmentalized polystyrene seedling trays. Trays were 20 x 32cm with each compartment 5cm square. The 28 compartments allowed two replicate seedlings of 14 sunflower rust differential lines to be grown in each tray. The two replicate seedlings were separated by planting the different lines in a repeating sequence (Figure 4.5).

Sequence	1	5	9	13	3	7	11
	2	6	10	14	4	8	12
	3	7	11	1	5	9	13
	4	8	12	2	6	10	14

Sunflower Lines

1. S37-388	5. HA-R1-2-3	9. HA-R5-3-1-5	13. 70111
2. S37-388RR	6. HA-R2-1-1-1-1	10. 70019-0-7	14. 70127
3. CM29	7. HA-R3-1-1-1-1	11. 70096	
4. PhRR3	8. HA-R4-1-2-2*	12. 70099	

* - sometimes replaced by the Pacific Seeds selection(80223-3-4) from Saenz Pena 74-1-2.

Figure 4.5 Sequence of seedlings of sunflower rust differential lines planted in trays.

Each inoculated tray was suspended over a seedling tray by placing it on four lengths of dowel embedded in the media of the four corner compartments. The seedlings were sprayed with atomized water before the assembly was enclosed in clear plastic film. The inoculation chambers were incubated in a plant growth room at 17±2°C in a 12h photoperiod. The film, inoculated tray and dowels were removed after 48h and the seedling trays were returned to the glasshouse where they were kept at approximately 28/15°C day/night.

Teliospore collections were tested in groups of ten over the period March, 1988 to June, 1989. Each collection was tested twice on separate occasions. Assessment of seedling reaction was made 14-18 days after the teliospores were suspended over the seedlings. The reactions of the seedlings

Table 4.1 Source of teliospore collections used for comparison of virulence.

Collection Accession #	Date of Collection	Locality	Host
Pht - 2	1981	Queensland	Mixture
3	Mar,1982	Darling Downs	Sunfola
4	May,1982	Biloela	Sunfola
5	Apr,1983	Darling Downs	Unknown Hybrid
8	May,1983	Gunnedah	Suncross 52
10	May,1983	Capella, Central Queensland	Hysun 31
12	May,1984	Retro, Central Queensland	Cargill 205
13	Dec,1984	Capella, Central Queensland	Dynamite
14	Feb,1985	Strathmerton, Victoria	Suncross 52
15	Mar,1985	Cambooya, Darling Downs	Hysun 33
16	May,1985	Orion, Central Queensland	Sunfola
17	May,1985	Orion, Central Queensland	Pac.Exp.Hybrid #1
18	May,1985	Nobby, Darling Downs	Pac.Exp.Hybrid #2
19	Jun.1985	Orion, Central Queensland	Dynamite
21	Dec,1986	Flagstone Creek, Lockyer Valley	Dynamite
22	Dec,1986	Flagstone Creek, Lockyer Valley	Pac.Exp.Hybrid #3
24	Mar,1987	Bongeen, Darling Downs	Dynamite
27	Mar,1987	Hermitage, Darling Downs	Beauty
36	Feb,1988	Felton, Darling Downs	Pac.Exp.Hybrid #4
37	Feb,1988	Felton, Darling Downs	Dynamite
41	Apr,1988	Moree, Nth. New South Wales	Dynamite
43	May,1988	Flagstone Creek, Lockyer Valley	Pac.Inbred Line #1
44	May,1988	Moree, Nth. New South Wales	Advance
47	May,1987	Clifton, Darling Downs	Dynamite
51	Jun,1988	Capella, Central Queensland	Beauty
53	Jun,1988	Capella, Central Queensland	Cannon
54	Jun,1988	Emerald, Central Queensland	Pac.Exp.Hybrid #5
55	Jun,1988	Emerald, Central Queensland	Pac.Exp.Hybrid #6
57	Jun,1988	Clermont, Central Queensland	Dynamite
58	Jun,1988	Clermont, Central Queensland	Dynamite
59	Jun,1988	Moree, Nth. New South Wales	Dynamite
60	Jun,1988	Gindie, Central Queensland	Dynamite
61	Jun,1988	Capella, Central Queensland	Dynamite
64	Apr,1989	Bodua, Darling Downs	70019
66	May,1989	Delungra, Nth. New South Wales	70111
67	May,1989	Delungra, Nth. New South Wales	Pioneer F66
68	May,1989	Delungra, Nth. New South Wales	Hysun 24
69	May,1989	Delungra, Nth. New South Wales	Beauty
70	May,1989	Delungra, Nth. New South Wales	Hysun 34
71	May,1989	Delungra, Nth. New South Wales	Hysun 44

to infection by basidiospores were divided into four classes;

Immune - No macroscopic sign of infection

Fleck - Necrotic flecking but no pycnia

Depauperate Pycnia - Pycnial clusters <1mm in diameter, often with necrotic borders

Normal Pycnia - Pycnial clusters >2mm in diameter

Only the larger normal pycnia were capable of producing aecia. That class therefore was functional and represented the susceptible reaction. The other classes represented degrees of incompatibility.

Results.

An example of the results obtained is illustrated in Figure 4.6.

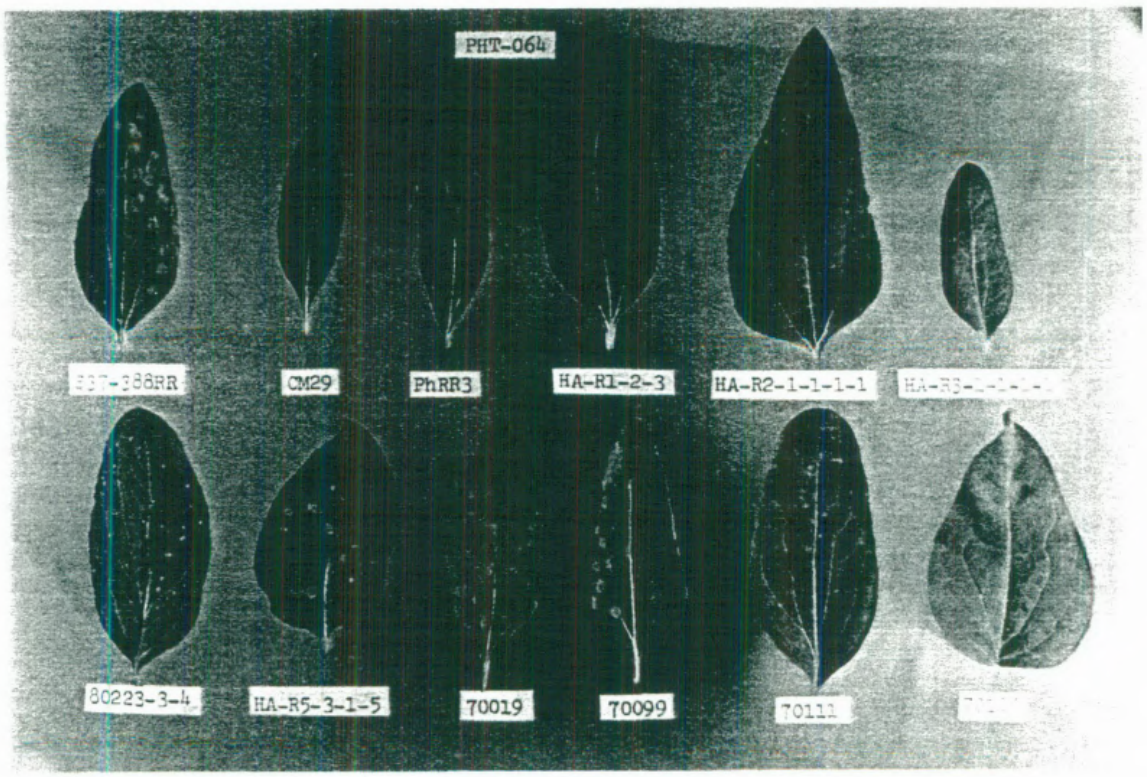

Figure 4.6 Reaction of 12 sunflower lines to infections from basidiospores derived from teliospore collection Pht-064. Note: Flecking on HA-R1 and 70111, depauperate pycnia on HA-R2 and 80223-3-4, normal pycnia on S37-388RR and 70019, a mixture on HA-R5 and no obvious reaction on CM29 and 70127 (S37-388 and 70096 have been omitted for photograph).

The data obtained (Table 4.2) show the range of virulence present in basidiospores from field collections of teliospores. All collections produced normal pycnial clusters on S37-388 and two collections (Pht-004 and

Table 4.2 Reaction types produced on 14 sunflower lines to infection from basidiospores from 40 field teliospore collections.

Puccinia helianthi Teliospore Collection (Pht-)

Sunflower Line	2	3	4	5	8	10	12	13	14	15	16
S37-388	4[1]	4	4	4	4	4	4	4	4	4	4
S37-388RR	2	2	2	2	2,3	2,3,4	2	2,3	2	2	2,3,4
CM29	2,3	1,2	1	2	2	1	2	1	2,3	2	1,2
PhRR3	4	4	2	4	2,3	2,3,4	4	2,3,4	4	2,3,4	4
HA-R1-2-3	2	2	1	2	2,3	2	2	2,3	2	2,3	2,3
HA-R2-1-1-1-1	2	2,3	2	2,3	2	2,3	2,3	2,3	2,3	2,3	2,3
HA-R3-1-1-1-1	1	2	1	2	2,3	2	1,2	1	1,2	1,2	2
HA-R4-1-2-2[2]	2,3	2,3	2	2	2,3	2,3	2	2	2	2	2,3
HA-R5-3-1-5	2	2,3	2	2	2,3	2,3	2,3	2,3	2,3	2,3	2,3
70019-0-7	2,3	2,3,4	2	2,3	2,3	2,3,4	2,3	2,3,4	2,3	2,3,4	2,3,4
70096	2	2,3,4	1	2	2	2	2,3,4	2	2	2,3,4	2
70099	2	2	1	2	2	2	2	2	2	2	2,3
70111	2,3	2,3,4	2	2,3	2,3,4	2	2,3	1	2,3	2	2
70127	1	1	1	2	2	1	1	1	1	1,2	2,3

	17	18	19	21	22	24	27	36	37	41	43
S37-388	4	4	4	4	4	4	4	4	4	4	4
S37-388RR	2	2,3	2	2,3,4	2,3	2,3	2,3,4	2	2	2,3	4
CM29	2	1	2,3	2	2	1	2	2	1	1	2
PhRR3	2	4	4	4	2,3,4	4	2,3,4	2,3,4	4	2,3,4	2
HA-R1-2-3	2	2,3	2,3	2,3	2	2,3	2	2,3	2,3,4	2	2
HA-R2-1-1-1-1	2	2,3	2,3	2,3	2,3	2,3	2,3	2,3	2,3	2,3	2,3
HA-R3-1-1-1-1	2	1,2	1,2	1,2	1,2	1,2	1,2	2,3	1	1	2,3
HA-R4-1-2-2	2,3	2,3	2,3	2,3	2,3	1	2	2,3	2,3	2	2
HA-R5-3-1-5	2,3	2,3	2	2,3	2	2,3	2,3,4	2,3,4	2,3,4	2	1,2
70019-0-7	2,3	2,3	2,3,4	2,3,4	2,3	2,4	2,3,4	2,3,4	2,3,4	2,3	2
70096	2,3	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4	2,3	2
70099	2	2	2,3	2	2	2	2,3,4	2,3,4	2,3,4	2,3	1
70111	2	2,3	2,3	2	2	2,3	2	2	2	2	1
70127	1,2	2,3	2,3	1	1	1	2	1	1	1	1

	44	47	51	53	54	55	57	58	59	60	61
S37-388	4	4	4	4	4	4	4	4	4	4	4
S37-388RR	2,3,4	2,3,4	2,3,4	2,3	2,3,4	2	2,3	2	2,3,4	2,3	2,3,4
CM29	1	2	2	2	2	2	2	1	2	1	2
PhRR3	3,4	4	2,3,4	4	4	4	3,4	2,3,4	2,3,4	4	2,3,4
HA-R1-2-3	2	2,3,4	2	2	2	2	2	2	2,3	2,3,4	2
HA-R2-1-1-1-1	2,3	2,3	2,3	2,3	2	2	2,3	2	2,3	2	2
HA-R3-1-1-1-1	1	1,2	1	1	1	1	1	1	1,2	1	1
HA-R4-1-2-2	2	2,3	2	2	2,3	2,3	2,3	2	2,3	2	2,3
HA-R5-3-1-5	2	2,3,4	2,3	2	2,3	2	2,3	2	2,3	2,3	2,3
70019-0-7	3,4	2,3,4	2,3,4	2,3	2,3,4	2,3	2,3,4	2,3,4	2,3,4	2,3,4	2,3,4
70096	2,3,4	2,3,4	2,3,4	4	4	3,4	4	2,3,4	3,4	2,3,4	2,3,4
70099	2,3	2,3,4	2,3,4	2	2,3,4	2	2	2	2,3,4	2,3,4	2,3,4
70111	2	2	2,3	2	2	2	2	2	2,3	1	2,3
70127	1	1	1,2	1	1	1,2	2	2	1	1	1

Sunflower Line	64	66	67	68	69	70	71	Percentage Collections Avirulent Only	Mixture	Virulent Only
S37-388	4	4	4	4	4	4	4	0	0	100.0
S37-388RR	3,4	4	3,4	2,3,4	3,4	2,3,4	4	55.0	37.5	7.5
CM29	2	2	1	1	2	2	1	100.0	0	0
PhRR3	4	3,4	4	4	4	4	4	10.0	35.0	55.0
HA-R1-2-3	2	2,3	2	1	2,3	2	2	92.5	7.5	0
HA-R2-1-1-1-1	2	2	1	2,3	2,3	2,3	2	100.0	0	0
HA-R3-1-1-1-1	1	4	2,3,4	4	2,3	3,4	4	87.5	5.0	7.5
HA-R4-1-2-2	2,3*	2,3*	2,3,4*	2,3,4*	3,4*	2,3*	3,4*	90.0	10.0	0
HA-R5-3-1-5	3,4	2,3,4	3,4	4	2,3,4	2,3	2,3	80.0	17.5	2.5
70019-0-7	4	3,4	3	2	2,3,4	3,4	2	37.5	60.0	2.5
70096	4	4	3,4	4	4	2,3,4	3,4	27.5	55.0	17.5
70099	4	2	2	2	2,3,4	2	2	72.5	25.0	2.5
70111	1	4	4	3,4	4	3,4	3,4	80.0	12.5	7.5
70127	1	3,4	4	2,3,4	3,4	2,3,4	3,4	85.0	12.5	2.5

1. Reaction types: 1- no visible reaction, 2- necrotic flecking, 3- depauperate pycnia, 4- healthy pycnia
2. HA-R4-1-2-2 replaced by Saenz Pena seln. 80223-3-4 where marked by *

Pht-17) were only virulent on that line. No collections were found with virulence to CM29 and HA-R2-1-1-1-1. Virulence to the other sunflower lines varied between and within teliospore collections (Table 4.2). For example, 55% of teliospore collections tested produced basidiospores which were avirulent on S37-388RR while only 7.5% of collections produced basidiospores which induced only compatible reactions. The remaining 37.5% produced a mixture of basidiospores which caused both avirulent and virulent reactions.

Few collections produced basidiospores virulent on HA-R1-2-3, the first being Pht-037 which was collected in 1988. Virulence to PhRR3 was present in 1981 (Pht-002) while virulence to 70111 was found in a 1982 collection (Pht-003), again in a 1983 collection (Pht-008) but then not again until 1989 (Pht-066). Susceptibility of HA-R3-1-1-1-1 and 70127 was found in the same cultures from 1989 (Pht-066-071). HA-R5-3-1-5 was first susceptible to basidiospores from Pht-027 (1987) and was susceptible to some collections to which HA-R1-2-3 was resistant (Pht-027, Pht-036, Pht-064). Virulence to 70096 was first found in Pht-003 (1982) and to 70099 in 1987 (Pht-027).

4.2.3 Preliminary investigations on genetics of virulence in *Puccinia helianthi*.

4.2.3.1 Selfing of three groups of pycnia derived from three collections of teliospores.

At the commencement of these studies, it was thought that once teliospore germination could be manipulated, that a thorough examination of the genetics of virulence in a number of pathotypes of *Puccinia helianthi* could be conducted. This objective was hindered by two phenomena. Firstly, it was difficult to obtain teliospores of pure uredinial pathotypes and secondly it was difficult to procure consistent and reliable infection by aeciospores. The experimentation reported here is therefore preliminary in nature.

Materials and Methods.

In an experiment conducted during 1984 three field collections of teliospores were used to produce pycnia which were crossed within each collection and the pathotypes of the uredinial cultures ultimately obtained were determined. The collections used were Pht-002, Pht-003 and Pht-005 (Table 4.1) which produced healthy pycnial clusters on the sunflower line S37-388 but only flecking on S37-388RR and CM29. Each collection was tested separately at different dates. Aqueous teliospore suspensions of the culture were used to inoculate 0.5% water agar contained in 9cm diameter Petri dishes. The dishes were incubated under alternating 12h photoperiods at 18±2°C and were examined microscopically at 12h intervals to find when maximum teliospore germination and metabasidial formation occurred. After the majority of teliospores had germinated the dishes were placed, inverted, over

seedlings of S37-388 at growth stage V4 (Schneiter and Miller,1981). The inoculation chambers were incubated at 18±2°C under a 12h photoperiod for 48h before the chambers were removed and the seedlings were placed in a controlled environment cabinet.

Pycnia became visible on the adaxial surface of the leaves several days after exposure to basidiospores. Exposure to teliospore collections in which few spores were still germinating resulted in only a few pycnia (1-3) developing on any leaf. The distribution of pycnia reduced the chance that the mycelia of two colonies could fuse. The pycnial colonies were allowed to expand for a further week to ensure that a plentiful supply of pycnial nectar was present and that no aecia had prematurely formed. Such aecia could have been the product of accidental fertilization by nectar transfer or by hyphal fusion caused by infection by basidiospores of compatible mating types in close proximity.

Crosses between pycnia were made by using glass capillary tubes to obtain reciprocal transfer of nectar between pairs of selected pycnial clusters. Indelible ink was used to identify each colony with a code number. Aecia rapidly developed in successful crosses. Aeciospore discharge was prevented by maintaining the relative humidity of the controlled environment cabinet below 80% and ensuring that no water came into contact with the aecia.

Aeciospores were collected by gently placing pieces of filter paper against the aecial clusters. The filter paper was held in place with paper clips. The plants were then placed in a saturated humidity chamber. Care was taken not to damage the aecial cups. The aeciospores were discharged onto the filter paper as the aecial peridia absorbed moisture and dehisced. The pieces of filter paper were removed after 4-6h. They were then moistened with distilled water and pressed against the adaxial surface of leaves of seedlings of S37-388. Inoculated plants were kept in a saturated humidity chamber for 24h at about 20°C. The uredinial cultures, if produced, were used to inoculate seedlings of the sunflower rust differential lines S37-388, S37-388RR and CM29 and the sunflower hybrid Pacific Seeds Hysun 33. The procedure of Kochman and Goulter(1982) was used for multiple inoculations of single leaves. Pathotypes Race 3 and Race 1 were used for comparison.

Results.

For the three collections 133 reciprocal crosses (Pht-002,30; Pht-003,48; Pht-005,55) were made. Aecia were produced from 58 of the crosses and successful production of uredinia was achieved in only 24 of the aeciospore inoculations. All uredinial cultures obtained reacted identically on the sunflower lines on which they were inoculated and were different from Race 0 and Race 1 (Table 4.3).

Table 4.3 Reaction of 24 uredinial test cultures derived from completion of the sexual cycle of *Puccinia helianthi*

	Pathotype		
Sunflower Line	Race 0	Race 1	Test Cultures
S37-388	S[1]	S	S
S37-388RR	R	S	R
CM29	R	R	R
Hysun 33s[2]	R	R	S
Hysun 33r	R	R	R

1. S - Susceptible, infection types >3; R - Resistant, infection types 0,0;1,2 (Sackston,1962).
2. Plants of Hysun 33 segregated for reaction to the test cultures. Some plants (s) were susceptible while others were resistant (r). All were resistant to the control pathotypes.

The uredinial cultures obtained were distinct from Races 0 and 1 by the capacity to infect a group of plants of Hysun 33. Virulence to S37-388RR and CM29 was not obtained.

4.2.3.2 Rection of progeny generated from mass-selfing of pycnia produced on the sunflower line 70096.

The teliospore collection Pht-012 was used in this experiment. It was known to be virulent and produce healthy pycnia on the Pacific Seeds sunflower inbred line 70096. The experiment was designed to obtain a uredinial culture also virulent on 70096 by completing the sexual cycle.

Materials and Methods.

Cold-soak preconditioned teliospores of Pht-012 on 0.5% water agar contained in Petri dishes were suspended over seedlings of 70096 at G.S.V4 (Schneiter and Miller,1981) enclosed in plastic tubes. After 48h incubation at 17±2°C the tubes and Petri dishes were removed and the seedlings were taken to the glasshouse. No other rust infected sunflowers were growing in the glasshouse at the time.

Seven days after incubation necrotic flecks, depauperate pycnia and healthy pycnial clusters were present. Bulk transfer of pycnial nectar was performed 14 days after inoculation by using a Pasteur pippette to take up and bulk nectar from a number of healthy pycnia. The bulked nectar was then re-applied to the pycnia. Numerous aecia formed which were allowed to

develop. Leaves bearing aecia were harvested and suspended over healthy seedlings of 70096 in a saturated atmosphere for 24h. Aeciospores were discharged and fell onto the sunflower seedlings.

Results.

Uredinia formed on the seedlings of 70096 inoculated with aeciospores produced on other plants of the same line. However, many hypersensitive flecks representative of incompatible reactions also developed (Figure 4.8). All aeciospores derived from crossing healthy X healthy pycnia that developed on 70096 were therefore not equally virulent. A second attempt to repeat this experiment failed because aeciospore infections could not be obtained.

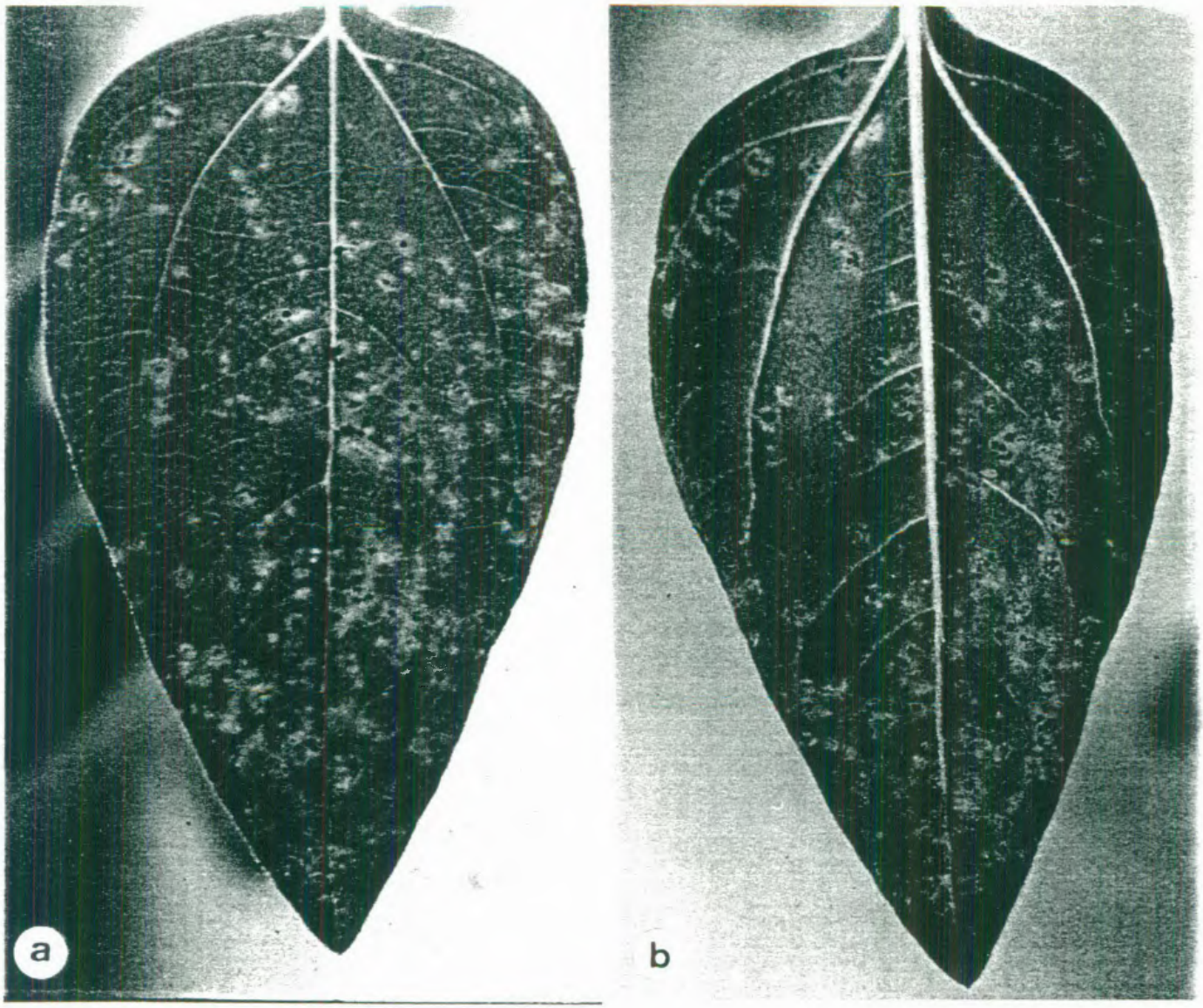

Figure 4.8 Reaction of 70096 to infection by aeciospores derived from the same line. Adaxial(a) and Abaxial(b) surfaces of same leaf.

The classical concept of dividing rust populations into pathotypes, depending on the infection types elicited when host lines known or thought to possess different genes for resistance to the fungus, dates back to Stakman and Piemeisel(1917). That and subsequent work on identifying virulent variants in rust populations has usually been concerned with the urediniophase since it accounts for the majority of economic damage done. Flor(1955) was able to show that virulence in several pathotypes of *Melampsora lini* was a recessively inherited character and required the presence of each allele in the dikaryon for expression. The heterozygote was avirulent.

The recessive nature of virulence allows a single allele for virulence to be present in the dikaryon without expression. A particular uredinial pathotype may therefore be homozygous for virulence to one resistance gene but be heterozygous at loci of other virulence genes. Without nuclear recombination by sexual or asexual means or deletion or mutation of the dominate allele then the presence of the recessive virulence genes in heterozygotes may not be identified. If it is assumed that virulence is chromosomally inherited and it can be shown that basidiospore nuclei are the meiotic products of teliospore germination then basidiospores have the potential of possessing segregants for virulence. Basidiospore infections by allowing expression of recessive alleles may therefore provide information on the potential of new virulent uredinial pathotypes to develop.

In this study nuclear behaviour was examined from teliospore formation to basidiospore infections. The vegetative hyphae of urediniophase of *P.helianthi* were found to be dikaryotic. Karyogamy occurred in the immature teliospores. The small size of nuclei of rust fungi made differentiation of chromosomes difficult. Nuclei of leaf epidermal cells of the sunflower line S37-388 were observed to be around 15 x 10um in dimension while the haploid nuclei in the intra-epidermal vesicles of *Puccinia helianthi* were around 5um in diameter. The inability to differentiate chromosomes made it impossible to determine the karyotype of *P.helianthi* and chromosomal behaviour during subsequent nuclear divisions involved in basidiospore formation. In other rust fungi the haploid chromosome numbers determined by light microscopy have been reported to be between 2 and 8 (Olive, 1953) while an electron microscopic study with computer enhancement suggested that *Uromyces phaseoli* var. *vignae* has 14 haploid chromosomes (Heath and Heath,1978).

To describe the divisions as meiotic was based on morphological similarity of the stages to those described for higher basidiomycetes and other species of rust fungi with exceptionally large nuclei (Savile,1939; Olive,1953; Olive,1965; Petersen,1974). The reduction in nuclear volume that occurred in *P. helianthi* following the division of the diploid nucleus in the

metabasidium suggests that the first meiotic division had occurred. The subsequent divisions of the two daughter nuclei were the second meiotic divisions and the division that occurred within the basidiospore were mitotic. The basidiospores were therefore homo-dikaryotic. The cytology of basidiospore formation in *Puccinia helianthi* can therefore be regarded as consistent with that of other rust fungi (Savile, 1939; Olive, 1953; Olive, 1965).

The binucleate basidiospores gave rise to uninucleate haploid cells following infection. The two homologous nuclei migrated into the intra-epidermal vesicle and then moved apart and were separated by a septum. The uninucleate condition was maintained until the pycnia which formed were fertilized (Craigie,1959).

Infections from the haploid basidiospores might therefore allow recessive virulence genes to be expressed by the haploid pycnial colonies. This is especially true for autoecious rust fungi in which the basidiospores infect the uredinial hosts. Flor(1959) found that basidiospores of Race 210 of *Melampsora lini* were virulent on all the differential flax lines which were susceptible to uredinial infections of that pathotype. Statler and Gold(1980) repeated the experiment with three pathotypes of *Melampsora lini* and 29 rust differential flax lines. Uredinial pathotypes Race 1 and Race 370 which were virulent on a line possessing the M^2 gene for resistance produced basidiospores which were virulent on that line. However, basidiospores of Race 370 were avirulent on the line possessing the N^1 gene although the urediniophase was virulent. In the other 84 host:pathogen interactions the basidiospores matched the virulence of the urediniospores or were virulent when the urediniospores were avirulent. The latter phenomenon may have indicated heterozygous alleles at the respective loci for virulence. Kolmer *et al*(1984) compared the virulence of basidiospores and the dikaryotic aeciospores and urediniospores of *Uromyces appendiculatus* and concluded that identical genes condition virulence in all spore forms.

In the present study it was possible to show that genes for virulence to certain sunflower lines were present in the *Puccinia helianthi* population for several years before the lines were identified as being susceptible to urediniospore infections. The sunflower lines PhRR3 and 70019-0-7 possess the genes for resistance used in two variations of the commercial hybrid Pacific Seeds Hysun 33. That hybrid was released commercially in 1984 after having been found to be free of sunflower rust in extensive field trialing in previous years (Pacific Seeds, *unpublished data*).

Basidiospores from teliospores collected in 1981 and 1982 (Pht-002, Pht-003) were virulent on PhRR3 and 70019-0-7, indicating that virulence to Hysun 33 was already present in the pathogen population prior to the breakdown of resistance. Similarly, basidiospores of Pht-003 collected in 1982 were virulent to 70096 and 70111 which are the sources of resistance used for the commercial sunflower hybrids Pac 378 and Hysun 34. The genes for

virulence were present in the rust population several years before these hybrids were released. The uredinial collections which were later found to be virulent on hybrids Hysun 33, Pac 378 and Hysun 34 (Chapter 5) may simply have developed from the recombination of virulence genes already present in the pathogen population.

Basidiopsores from field collections of teliospores can be used to screen potential breeding lines for resistance to sunflower rust. The two disadvantages of this technique that must be considered are i) not all pathotypes may produce teliospores and ii) virulent pathotypes can still arise through mutation. Attempts to obtain teliospores of various pathotypes of *P.helianthi* in the glasshouse failed. In the field most teliospore collections came from hybrids not known to possess pathotype-specific genes for resistance such as Dynamite, Hysun 31, Beauty and the open-pollinated line Sunfola. Although the pathotype Race 1 was identified in 1983 (Kochman and Goulter,1984) and became dominant on hybrids specifically susceptible to it,teliospores were not found on these hosts. Variability in capacity to form teliospores has been recorded in some rust fungi. Hoerner(1919,1922) isolated strains of *Puccinia coronata* which were believed to have lost the capacity to develop teliospores. The development of teliospores of *P.recondita* f.sp. *tritici* was found to be correlated with susceptibility of wheat lines. Teliospores formed more readily on moderately resistant lines (Jackson and Young,1967).

The development of pycnia on the sunflower line 70127 first occurred from teliospores collected in 1989 from the hybrid Hysun 44 (Pht-071) and other collections from the same locality. 70127 is the source of rust resistance for Hysun 44. The occurrence of uredinial and telial infections on Hysun 44 before haploid virulence was detected on 70127 suggests that the virulent uredinial pathotype may not have developed from recombination of existing virulence genes. Mutation was a likely source.

Teliospores were used in early studies on the pathogenic specialization of *Puccinia helianthi* between species of *Helianthus* (Arthur,1903; Kellerman,1903; Arthur,1904; Brown,1936). The first studies on genetics of virulence in sunflower rust made by Jabbar Miah and Sackston(1970b) produced many inconclusive results and were unable to verify that a gene-for-gene relationship existed in the *P.helianthi* : *Helianthus annuus* pathosystem. In the simple experiments conducted in this study it was found that selfing Pht-002, Pht-003 and Pht-005 did not result in segregation on S37-388RR and CM29. The potential exists therefore for making further studies of inheritance of virulence using these teliospore collections which are considered to be homozygous avirulent on certain host lines.

The mass-selfing of Pht-012 on 70096 and the segregation of virulence exhibited by aeciospores when re-inoculated onto 70096 suggests that virulence may not always be simply inherited. Flor(1941) considered the

incapacity of aeciospores of *Melampsora lini* to reinfect the source host as being 'homologous with heteroecism, although occuring in an autoecious rust'. Jabbar Miah and Sackston(1970b) suggested that non-nuclear factors were operating to modify the expression of virulence in sunflower rust. Virus-like particles (VLPs) consisting of double-stranded RNA have been identified in rust fungi including *P.helianthi* (Littlefield and Heath,1979) and have been shown to be transmissible (Lawrence, Boelen and Pryor,1988). Virus-like particles were found to modify the pathogenicity of *Leucostoma persoonii* (Nits.) Hoen. (Hammar, Fulbright and Adams, 1989). It is not known whether the presence of virus-like particles modifies the pathogenicity of rust fungi.

CHAPTER 5

DETERMINATION OF PATHOTYPES OF SUNFLOWER RUST (*PUCCINIA HELIANTHI Schw.*) IN AUSTRALIA.

5.1 INTRODUCTION

Many rust fungi are noted for exhibiting host specificity within a morphological species. In 1894, Jakob Ericksson demonstrated the existence of parasitically different forms in *Puccinia graminis* Pers. and some other rusts (Johnson,1953). To describe this host specificity Eriksson referred to the different forms as *formae specialis* or 'specilized forms'. Stakman and Piemeisal(1917) discovered that the *formae specialis* of *P.graminis* could be further sub-divided into more specialized units on the basis of their ability to infect different cultivars or lines within a host species. Because these groups were distinguished on the basis of physiological characters, in this case pathogenicity or virulence on specific host cultivars, they were termed physiologic races. The term physiologic race does not satisfactorily specify that virulence was the physiologic parameter of definition. A population of a parasite species in which all individuals have virulence in common irrespective of other physiologic characters was described therefore as a pathotype (Robinson,1976). The lines of a host species that reacted differently to the pathotypes were termed differential lines.

Specialization in sunflower rust collected from various species of *Helianthus* was investigated by Arthur(1903), Arthur(1904), Bailey(1923) and Brown(1936a). Bailey(1923) used cross-inoculation techniques to reveal the existence of at least three pathotypes of *P.helianthi*. Brown(1936a) cross-fertilized pycnia of rust isolates taken from 6 species of *Helianthus* and identified four distinct 'strains' based on the inter-sterility of the crosses between isolates taken from *H.annuus*, *H.petiolaris*, *H.tuberosus* and *H.subtuberosus*. The first identification of pathotypes of *P.helianthi* based on the infection of a set of differential lines of *Helianthus annuus* was made by Sackston(1962). He recognized four pathotype groups and suggested that with differentials of 'higher resolution' these groups could be further sub-divided. Hoes and Putt(1962) inoculated 14 inbred sunflower lines with several single pustule isolates of sunflower rust and identified 9 pathotypes of rust. Jabbar Miah, Hennessey and Sackston(1967) recognized 9 pathotypes derived through the selfing and hybridization of the four pathotype groups identified by Sackston(1962). This indicated the heterogenous nature of the pathotype groups but further utilization of the inbred sunflower lines used by Hoes and Putt(1962) and Jabbar Miah *et al*(1967) as differentials faltered because the inheritance of resistance in the lines was unknown. Antonelli (1985) recognized ten pathotypes in Argentina on a set of Argentinian

differential lines.

Studies on virulence in sunflower rust have been hindered by the lack of a standardized means for identifing and describing pathotypes. Systems for designating pathotypes have been proposed by Sackston(1962), Antonelli(1985), Kochman and Goulter(1985) and Sackston, Bertero de Romano and Vasquez(1985). The international Sunflower Rust committee established in 1985 decided that pathotypes 'should be identified by a descriptive system listing all the resistance genes and/or differentials attacked rather than a code or sequential numbering system'(Kochman,1988).

The initial set of differential sunflower lines that the Sunflower Rust committee suggested be used are listed in Table 5.1. A number was assigned to describe each distnct locus for rust resistance. If the genotype was unknown then a letter was used to arbitaril y describe the locus or loci until the genotype was established. Table 5.1 also shows the reaction of the differential lines to the four North American pathotypes of *P.helianthi*. Under the system proposed by the Sunflower Rust committee of describing pathotypes on the basis of the resistance genes and/or differentials attacked the North American pathotypes would be described as: Race 1 = Race 0, Race 2 = Race 2, Race 3 = Race 1, Race 4 = Race 1,2.

The pathotype composition of sunflower rust in Australia was stable until 1983 with only Race 0 being identified (Kochman and Goulter,1982; Goulter,1983). Race 1 was detected in 1983 (Kochman and Goulter,1984). Since that time several private seed companies have released new sunflower hybrids that initially showed low levels of infection by sunflower rust. Crops of many of these hybrids have subsequently been found with moderate to high levels of rust severity. This would indicate that new pathotypes of *Puccinia helianthi* are developing or being selected for.

Most of the commercial hybrids in Australia are developed by private seed companies from propriety breeding inbred lines. The source of rust resistance in these inbred lines may be unknown. However the lines would be useful as rust differentials if available for public use.

The objectives of the experiments reported in this chapter were to show how a sunflower rust differential line for public release could be developed from a commercial hybrid and to determine the number of pathotypes in the sunflower rust population in Australia.

5.2 EXPERIMENTAL

5.2.1 Breeding of a rust differential sunflower line.

The experiment reported in this section was conducted to develop a sunflower inbred line that could be used as a rust differential line from a commercial sunflower hybrid.

Table 5.1 Sunflower genotypes suggested by the Sunflower Rust committee as rust differential varieties and their reaction to four North American pathotypes of *Puccinia helianthi*.

Number or Letter Assigned	Resistance Genotype[1]	North American Pathotype				Suggested sunflower lines
		Race 1 (Race 0)	Race 2 (Race 2)	Race 3 (Race 1)	Race 4 (Race 1,2)[2]	
0	rr	S[3]	S	S	S	S37-388
1	R1	R	R	S	S	CM69, CM90RR, S37-388RR, F164
2	R2	R	S	R	S	CM29, CM307-1
3	R3	?	?	?	?	CM403-4[5]
a=4	R4	R	R	R	R	HA-R1
b=5	R5	R	R	R	R	HA-R2
c[4]	?	R	R	R	S	P94
d	?	R	R	S	R	Impira INTA seln 11
e	?	?	?	?	?	LC20620
f	?	R	R	R	R	HA 340

1. Genotypes published by Sackston(1962), Jabbar Miah and Sackston(1970a) and Miller, Rodriquez and Gulya(1985).
2. Proposed nomenclature.
3. S- susceptible R- resistant. Reactions published by Sackston(1962), Gulya(1985), Yang(1986) and Yang, Antonelli, Luciano and Luciani(1986).
4. Letters assigned until genotypes have been determined.
5. Data on this line is obscure and the line is considered lost (Yang, Dowler and Luciano, 1989).

Materials and Methods.

The source of rust resistance was one plant of the sunflower hybrid Pacific Seeds Hysun 33 (designated Hysun 33s). This plant was one of a group found to be susceptible to infections from urediniospores derived from aeciospores resulting from crossing pycnia of Pht-002. The plant was resistant to pathotypes Race 0 and Race 1. The plant was grown to maturity and used as a pollen source. Plants of S37-388, the 'universal suscept', that had been emasculated manually with fine forceps were pollinated with the pollen from Hysun 33s. Crossing procedures followed the technique of Putt(1941).

Seedlings of the F_1 progeny were screened for resistance to Races 0 and 1. Resistant plants were selfed to obtain F_2 progeny. As well pollen from the resistant plants was used to pollinate emasculated plants of S37-388 to produce the first backcross. Resistant backcross (BC_1) progeny were used as pollen sources to pollinate the recurrent parent S37-388. Two further backcrosses were made using resistant backcross progeny and S37-388. Three BC_3F_1 plants resistant to Races 0 and 1 were selfed and BC_3F_2 segregation ratios were determined to the two pathotypes.

All rust inoculations were made when seedlings had reached G.S. V2 (Schneiter and Miller,1981). One first true leaf of each seedling marked with indelible ink was inoculated with Race 0 and the other primary leaf was inoculated with Race 1. A cotton bud (Q-tip) was used to apply a small deposit of a 20:1 (w/w) mixture of talc powder and urediniospores. Inoculated seedlings were placed in a mist chamber for 16h at 20^{0}C before being returned to the glasshouse bench. Fourteen days after inoculation the seedlings were examined. Resistant plants were identified as having hypersensitive flecking and no sori whereas large sori were present on susceptible plants. The flecking was so pronounced that it was possible to ascertain whether escapes had occurred.

Segregation ratios were determined for the F_2 progeny of four resistant F_1 plants of the cross S37-388/Hysun 33s as well as three plants of the Bc_3F_2 progeny.

Results.

No seedlings were found that differed in reaction to Race 0 and Race 1. Chi-square analyses were therefore performed without regard to pathotype used. The resistant phenotype of the F_1 progeny chosen for crossing suggested that resistance was inherited as a dominant character. The segregation ratios were therefore tested for goodness-of-fit to the ratios conferred by a single dominant gene (3:1), and two gene, one dominant and the other recessive (13:3) (Table 5.2).

Further selfing and screening of resistant selections yielded homogenous resistant plants. Five Bc_3F_5 selections were bulked and named PhRR3 (*Puccinia helianthi* Rust Resistant Gene 3). The analysis of the

Table 5.2 Reaction of F_2 and Bc_3F_2 progenies of the cross between S37-388 and Hysun 33s to *P.helianthi* Race 1.

Cross	No. of Plants		X^2 Value for		Probability	
S37-388/Hysun 33s	Resistant	Susceptible	3:1	13:3	3:1	13:3
F_2 #1	21	8	0.104	1.483	0.20-0.30	0.20-0.30
#2	67	19	0.485	0.631	0.30-0.50	0.30-0.50
#3	28	10	0.035	1.428	0.70-0.90	0.20-0.30
#4	42	13	0.055	0.862	0.70-0.90	0.30-0.50
Total	158	50	0.102	3.8184	0.70-0.90	0.05-0.10
Bc_3F_2 #1	28	10	0.035	1.428	0.70-0.90	0.20-0.30
#2	75	31	1.019	7.66	0.20-0.30	0.001-0.01
#3	110	32	0.460	1.335	0.50	0.20-0.30
Total	213	73	0.0419	8.6158	0.70-0.90	0.001-0.1

phenotypic reactions of the F_2 and Bc_3F_2 progenies indicate that a single dominant gene conditioned resistance to Races 0 and 1. The reactions of the differentials to three rust pathotypes was used to distinguish the gene in PhRR3, from the R1 and R2 genes in S37-388RR and CM29 respectively (Table 5.3).

Table 5.3 Reaction of four sunflower rust differential lines to three pathotypes of *P.helianthi*.

		Pathotype		
Differential	Gene	Race 0	Race 1	Race 3[1]
S37-388	rr	S[2]	S	S
S37-388RR	R1	R	S	R
CM29	R2	R	R	R
PhRR3	R3	R	R	S

1. Race 3 was a isolate derived from fertilization of pycnia on Hysun 33s
2. S - Susceptible R - Resistant

Table 5.3 demonstrates the ability of PhRR3 to differentiate sunflower rust pathotypes. Naming the locus R3 was made arbitarily since the sunflower line CM403-4 which is presumably the source of a previously described R3 gene for resistance has never been widely utilized as a differential and is now lost. Further genetic studies are required to demonstrate whether the R3 gene in PhRR3 is non-allelic to the R1 and R2 genes.

5.2.2 Analysis of the pathotype composition of the *P.helianthi* population in Australia.

The objective of this study was to investigate the possibility of detecting new pathotypes while their frequency was still low and so offer sunflower breeders timely warning of potential problems.

Materials and Methods.

Methods used to search for unrecognized pathotypes included:

i) Planting trap plots of the rust differential sunflower lines in conjunction with Pacific Seeds field evaluation trials in several areas of Queensland and New South Wales during 1985. This approach ceased because of the large seed quantities required. The ability of the inbred differentials to survive in the field was also unsatisfactory.

ii) Planting trap pots of differentials in the glasshouse and then exposing these in field sites for various lengths of time before returning them to the glasshouse. This approach was trialled in 1987 and was considered to have good potential. Problems involved with this method included the logistics of moving and retrieving the pots from areas distant from home base, maintenance of the pots in the field environment for a few days and protection of the seedlings from predators.

iii) Examination of commercial crops, field trials and breeding nurseries for presence of sori on lines or hybrids which had not previously been known to be susceptible. This was the surest means of detecting new virulences.

iv) Bulk samples of urediniospores collected from susceptible hybrids were used to inoculate the differential set.

Mono-uredinial isolates were made of any sori to be increased for analysis. Urediniospores from isolated single sori were used to inoculate the same host variety on which the sori were found. Inoculum from the sori that developed from these infections were either increased further by re-inoculating the host or used to inoculate the set of rust differential sunflower lines.

The differential set chosen consisted of the following thirteen sunflower lines.

S37-388 : the 'universal suscept' a Canadian selection from the heterogeneous cultivar Mennonite (Putt and Rojas,1955)

S37-388RR : R1 gene, from backcross between S37-388 and Morden Cross 69 (Putt,1978). Resistance gene in the latter line derived from wild *Helianthus annuus* (Putt and Sackston,1957). The selection used in these experiments was a Bc_2F_5 progeny of S37-388/ S37-388RR. This was made by the author because slight morphological differences had become apparent between S37-388 and S37-388RR after years of selfing.

CM29 : R2 gene, Morden Cross 29 (Putt and Sackston,1963), resistance gene derived from wild *Helianthus annuus* in Texas (Putt and Sackston,1957).

PHRR3 : R3 gene, Bc_3F_5 progeny of S37-388/Hysun33s described in 5.2.1

HA-R1-2-3 : R4 gene (Miller *et al*,1988), released as a S_3 composite from a single S_2 selection from the Argentinian open-pollinated cultivar Pergamino 71/538 (Gulya,1985). The line used here was an S_5 selection made in Australia by Pacific Seeds

HA-R2-1-1-1-1 : R5 gene (Miller *et al*,1988), released as a S_3 composite from a single S_2 selection from the Argentinian open-pollinated cultivar Impira INTA (Gulya,1985). The line used here was an S_7 selection made in Australia by Pacific Seeds.

HA-R3-1-1-1-1 : R4 gene (Miller *et al*,1988), released as an S_3 composite from a single S_2 selection from the Argentinian open-pollinated cultivar Charata INTA (Gulya,1985). The line used here was an S_7 selection made in Australia by Pacific Seeds.

HA-R5-3-1-5 : R4 gene (Miller *et al*,1988), released as an S_3 composite from a single S_2 selection from the Argentinian open-pollinated cultivar Guayacan INTA (Gulya,1985). The line used here was an S_6 selection made in Australia by Pacific Seeds.

70019 : genotype undescribed but segregation studies suggest the R1 and R3 genes are present. An S_3 progeny from the Argentinian open-pollinated cultivar Pehuen INTA. Restorer line in some Pacific Seeds commercial hybrids.

70096 : genotype undescribed but segregation studies suggest the R3 gene plus a recessive gene condition resistance. An S_5 progeny from the Pacific Seeds 1981 Restorer population and used in a commercial hybrid.

70099 : genotype undescribed but segregation studies suggest the R_3 gene plus a novel dominant gene condition resistance. A sister S_2 selection of 70096 and used in a commercial hybrid.

70111 : genotype undescribed and segregation studies not finalized. From a pedigree cross involving Charata INTA and other rust resistant germplasm made by Pacific Seeds and used in commercial hybrids.

70127 : genotype undescribed and segregation ratios not finalized. S_4 selection from Charata INTA made by Pacific Seeds and used in a commercial hybrid.

Seedlings were grown in compartmentalized polystyrene seedling trays. Each tray consisted of sixty 5cm by 5cm compartments. This allowed four replicate seedlings of each differential line to be grown per tray. Inoculations were performed in the evening either by dusting the seedlings

with a mixture of talc powder and dry urediniospores of the test mono-uredinial isolate or by spraying with a suspension of the urediniospores in Pegasol 3440 special light mineral oil (Mobil Pty. Ltd.). Inoculated seedlings were incubated overnight in a mist-chamber at 20 ± 2°C. They were then maintained on glasshouse benches for 12-14 days before being scored for reaction to the test mono-uredinial isolate.

Reaction type was based on the following numerical rating system of Kochman(1988). The classes were:

0 - no visible reaction
$0_;$ - fleck reaction
1 - small uredinia (0.1 - 0.2mm diameter), associated flecking and necrosis
2 - uredinia 0.2 - 0.3mm diameter, associated chlorosis
3 - uredinia 0.3 - 0.4mm diameter, freely sporulating, little chlorosis
4 - large uredinia >0.4mm diameter with no chlorosis

Increasing values represent greater compatibility of parasite:host interaction. 0, $0_;$, 1 and 2 were classed as resistant reactions while 3 and 4 were susceptible.

Results.

Eleven distinct pathotypes were identified (Table 5.4) on the thirteen sunflower lines used. All sunflower lines, except for the HA-R3 and 70127, could be distinguished by the pathotypes found. This suggests that HA-R3 and 70127 have similar genotypes for resistance to sunflower rust. This is not suprising since both are derivatives of Charata INTA. The differential reactions of the other lines suggest that the genotypes for rust resistance in each of those lines was different.

5.3 DISCUSSION

The stable pathotype composition of the sunflower rust population in Queensland (Goulter,1983) has changed. It would seem that the introduction of hybrid sunflower cultivars in the early 1970's did not alter the pathotype composition. It was also thought that more extensive growing of hybrids possessing pathotype-specific resistance would increase selection pressure for the development of new virulent pathotypes of sunflower rust. As well it was recognized that variations in the composition might be detected with the introduction of other resistance genes. Those prophesies have eventuated.

The first new pathotype identified in Australia was Race 1 (Kochman and Goulter,1984). This appeared after hybrids which possessed the R1 gene for resistance (eg. Pacific Seeds Hysun 30) had been grown for nearly ten years. New hybrids with different genes for resistance were released in response to the occurrence of this new pathotype. Different sources of rust-resistance were introduced into the Australian sunflower population.

Table 5.4 Reaction of thirteen sunflower lines to eleven pathotypes of sunflower rust[1].

Differential Line	Genotype	PATHOTYPE										
		Race0	Race1	Race3	Pac 027	Pac 029	Pac 030	Pac 040	Pac 086	Pac 089	Pac 102	Pac 134
S37-388	rr	S	S	S	S	S	S	S	S	S	S	S
S37-388RR	R1	R	S	R	S	R	R	S	S	S	S	S
CM29	R2	R	R	R	R	R	R	R	R	R	R	R
PhRR3	R3	R	R	S	S	S	R	R	S	S	S	S
HA-R1	R4	R	R	R	R	R	R	S	S	S	S	S
HA-R2	R5	R	R	R	R	R	R	R	R	R	R	R
HA-R3	R4	R	R	R	R	R	R	R	R	R	R	S
HA-R5	R4	R	R	R	R	R	S	S	S	S	S	S
70019	R1+R3	R	R	R	R	R	R	R	S	S	S	S
70096	o[2]	R	R	R	R	S	R	R	R	R	S	R
70099	p	R	R	R	R	R	R	R	R	R	S	R
70111	q	R	R	R	R	R	R	R	R	S	S	S
70127	r	R	R	R	R	R	R	R	R	R	R	S
Suggested Name:	Race	0	1	3	1,3	3,o	4	1,4	1,3,4	1,3,4,q	1,3,4, o,p,q	1,3,4, q,r
Year Identified		<1983	1983	1984	1985	1985	1985	1986	1987	1987	1988	1989

1. Reaction types 0, 0;, 1 classed as resistant (R) and types 3 and 4 as susceptible (S).
2. Genotypes arbitarily chosen so as not to conflict with the letters assigned to other genotypes by Gulya and Masirevic(1988).

Many of the hybrids using these resistance genes have subsequently become susceptible to other previously undetected pathotypes. The origin of the pathotypes is unknown. They could be the products of genetic changes in the pathogen that occurred after the relevant hybrids were released. They may also have been present in the rust population at low frequency and were given a reproductive advantage with the wide spread utilization of new resistance genes which imparted a favourable directional selection pressure on the population (Leonard and Czochor,1980).

An interesting case study is that of the sunflower hybrid Pacific Seeds Hysun 33. This hybrid was released to farmers in 1984 after Hysun 32 was found to be susceptible to the pathotype Race 1. Hysun 33 was screened and found to be homogeneous for resistance to Race 0 and Race 1 before release. The rust resistance and yield performance of the hybrid resulted in it being widely grown. During 1984 a pathotype of sunflower rust virulent to a proportion of the hybrid plants was detected. The rapid appearance of this new pathotype, subsequently named Race 3, was suprising. Data from basidiospore infections (Chapter 4) suggested that the virulence gene/s for Hysun 33 were present in the rust population in 1982 and that Race 3 could have originated from completion of the sexual cycle. It is also interesting to speculate that the isolates of Race 0 compared by Kochman and Goulter(1982) and found to differ in aggressiveness were actually Races 0 and 3. The two pathotypes could not be differentiated by the sunflower rust differential lines then in use. Further studies could confirm the relative parasitic fitness levels of the pathotypes.

The origins of the other pathotypes are not known but relationships can be surmised. Isolate Pac027 (Race 1,3) was virulent on all plants of Hysun 33 and the virulence phenotype on S37-388RR (R1 gene) and PhRR3 (R3 gene) suggest a genotype combining the virulence genes of the two existing pathotypes, Races 1 and 3. A single mutation of the locus/loci for virulence in Race 3 to the o genotype would have created Pac 029. The narrow virulence of Pac 030 (Race 4) suggests it was derived from Race 0. Recombining the virulences of Races 1 and 4 produces Race 1,4 (Pac 040). Recombining the virulences of Race 4 with Race 1,3 or Race 1,4 with Race 3 would produce Race 1,3,4 (Pac 089). Whether the pathotypes Pac 089, Pac 102 and Pac 134 originated from the recombination of virulence genes from earlier pathotypes or were the products of single mutations in those earlier pathotypes will be more clearly understood when the genotypes for resistance in the sunflower lines 70096, 70099, 70111 and 70127 are analysed.

The phenotypes for virulence expressed by several pathotypes suggest that the resistance genotypes reported in several of the host lines may not be accurate. The lines HA-R1, HA-R3 and HA-R5 presumably possess the R4 gene (Miller *et al*,1988). The multiple resistance of HA-R3 indicates that it possesses more resistance genes than the R4 gene. It can also be inferred that since HA-R1 was resistant to Pac 030 (Race 4) but susceptible to Pac

040 (Race 1,4) that it may also possess the R1 gene. Senetiner *et al*(1985) thought that Pergamino 71/538 (from which HA-R1 was selected) possessed a single dominant locus as well as a second undetermined locus for resistance to three Argentinian pathotypes of sunflower rust. The three pathotypes were differentiated on Argentinian differential sunflower lines and cannot be directly compared to the pathotypes studied here. Yang *et al*(1986) concluded that since Pergamino 71/538 was susceptible to North America Race 2 but resistant to Races 0,1 and 1,2 it possessed genes different to the R1 and R2 genes. In contrast Gulya(1985) reported that the HA-R1 selection from Pergamino 71/538 was homogeneous for resistance to North American races 0, 1, 2 and 1,2. It is obvious that the International Standardization for describing pathotypes of sunflower rust and differential lines must be implemented so that meaningful communications can be obtained.

More pathotypes of sunflower rust will be identified in Australia with the development and utilization of new differential lines. It should be noted that the 11 pathotypes described were associated with commercial sunflower hybrids. Other pathotypes may be present at low frequency on the diversity of sunflower germplasm introduced into breeding nurseries and present on the wild *Helianthus* colonies that are prevalent in some areas of Australia (Dry,1985).

It would be advantageous if new 'single-gene' differential lines are developed. Monogenic differentials allow clear differentiation of pathotypes due to a narrowed range of reaction types produced (Roelfs,1986). They also allow the origin of new pathotypes to be more easily studied and would be useful in investigating the physiology of resistance (Flor,1954). PhRR3 is the first sunflower line produced purely to be used as a sunflower rust differential line. It fulfills the criterion of producing only a narrow range of reaction types. The S37-388 genetic background of PhRR3 ensures that any pathotype virulent to PhRR3 will form reaction type 3 - 4 sori. This will change if pathotypes are found to which S37-388 is resistant.

The description of the resistance gene in PhRR3 as R3 may cause confusion since that title had been used for the gene in CM403-4 (Miller *et al*,1988). Seed stocks of CM403-4 are not known and it is considered to be lost (Yang *et al*,1989). Morden Accession 403-4 was presumedly derived from outcrosses of S37-388 and wild sunflowers in Texas and possessed vigorous branching and purple flowers (Putt and Sackston,1957). The latter characteristics were dominantly inherited if outcrossing did occur. These characteristics were unsuitable for use in the generation of commercial hybrids. It is possible that CM403-4 was discarded in favour of more agronomically adapted lines. On the basis that the CM403-4 germplasm is extinct the gene in PhRR3 may retain the R3 epithet.

Jabbar Miah and Sackston(1970a) described the sunflower line M62-2672-2-r1 which was susceptible to North American Race 1 (Race 0) but resistant to the other three North American pathotypes. The reaction of PhRR3

may be similar to that line. PhRR3 is resistant to Race 1 but susceptible to Race 3. On the differential lines used by Jabbar Miah and Sackston(1970a) Race 3 would be indistinguishable from Race 0.

It can be recommended that future segregation studies on the genotypes of rust resistance should include inoculation with Race 0. Segregation ratios will then provide a better estimate of the total number of loci involved. For example, if Miller *et al*(1988) had used Race 0 in conjunction with Race 1,2 when examining the segregation ratios of S37-388/HA-R line F_2 progenies then the presence of other genes may have been indicated. Use of only Race 1,2 against which R1 and R2 are ineffective would not allow their presence to be detected.