# POPULATION STUDIES OF *ASCOCHYTA RABIEI* ON CHICKPEA IN SASKATCHEWAN

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**ABSTRACT**

An epidemic increase in severity and incidence of asochyta blight, caused by *Ascochyta rabiei* (Pass) Labrousse (teleomorph: *Didymella rabiei* (Kovachevski) v. Arx. Syn. *Mycosphaerella rabiei* Kovachevski), has occurred on chickpea (*Cicer arietinum* L.) crops in Saskatchewan over the past 5 growing seasons. In order to explore the nature of the outbreak, studies assessing population differences in pathogenicity and genetic variability were employed. Isolates of *A. rabiei* collected in 1998, 2001 and 2002 were inoculated onto 7 differential chickpea genotypes for pathogenicity testing. Significant isolate by differential interaction occurred, but accounted for a low proportion of the total variability suggesting no genotype specific relationship exists between *A. rabiei* and *C. arietinum*. Furthermore, it was found that when averaged over all differentials, the isolates from 2001 and 2002 caused significantly greater disease than isolates from 1998, suggesting that the disease epidemic is in part due to a shift in the population to overall greater aggressiveness. The largest increase in disease severity was observed on the cultivar 'Sanford', which was widely grown in commercial chickpea fields before 1999. To evaluate the genetic diversity of different *A. rabiei* populations, 30 isolates from 1998 and 30 isolates from 2002 were compared with random amplified polymorphic DNA fingerprinting. Several clusters of isolates collected from either 1998 or 2002 were approximately 60% genetic similar suggesting divergence of these populations of *A. rabiei*. However, analysis of molecular variance showed that over 90% of the variation occurred within populations. Pairwise differences and gene diversity over loci showed that genetic diversity of the 2 populations had the same amount of genetic variability. Analysis of mating type distributions revealed that the populations from 1998, 2001 and 2002 did not significantly depart from a 1:1 ratio suggesting random mating of each population. Further supporting the hypothesis of a randomly mating population, linkage disequilibrium for both 1998 and 2002 populations was very low.

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**DEDICATION**

To the one who has always known it all.

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| | |
|---|---|
| A | Adenine |
| AFLP | Amplified Fragment Length Polymorphism |
| AMOVA | Analysis of Molecular Variance |
| ANOVA | Analysis of Variance |
| AUDPC | Area Under Disease Progress Curve |
| bp | base pair(s) |
| C | Cytosine |
| CDC | Crop Development Centre |
| CTAB | Hexadecyl Trimethyl-Ammonium Bromide |
| DNA | Deoxyribose Nucleic Acid |
| EDTA | Ethylenediamine Tetracetic Acid |
| FLIP | Food Legume Improvement Program |
| G | Guanine |
| HPLC | High Pressure Liquid Chromatography |
| ICARDA | International Centre for Agricultural Research in the Dry Areas |
| ICC | ICARDA Chickpea |
| ICRISAT | International Crops Research Institute for the Semi-Arid Tropics |
| ILC | International Legume Chickpea |
| $I_A$ | Index of Association |
| ISSR | Inter Simple Sequence Repeat |
| MAS | Marker Assisted Selection |
| MAT | Mating type |
| MP-PCR | Microsatellite-Primed PCR |
| OMA | Oatmeal Agar |
| PAA | Plant Area Affected |
| PCR | Polymerase Chain Reaction |
| PDA | Potato Dextrose Agar |

| | |
|---|---|
| PDB | Potato Dextrose Broth |
| QTL | Quantitative Trait Loci |
| RAPD | Random Amplified Polymorphic DNA |
| RFLP | Restriction Fragment Length Polymorphism |
| ROS | Reactive Oxygen Species |
| RIL | Recombinant Inbred Lines |
| SDS | Sodium Dodecyl Sulphate |
| SSR | Simple Sequence Repeat |
| STMS | Sequence-Tagged Microsatellite Site |
| T | Thymine |
| UPGMA | Unweighted Pair-Group Method with Arithmetic Means |
| USDA | United States Department of Agriculture |
| VNTR | Variable Number Tandem Repeats |

# CHAPTER 1

## INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a self-pollinating diploid annual, with 2x=2n=16 chromosomes (Tekeoglu et al., 2000). It is the third most important grain legume in the world after common bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (Tekeoglu et al., 2000). Primarily, chickpeas are grown in the Indian subcontinent, West Asia, North Africa, Ethiopia, southern Europe, Mexico, Australia, the north-western United States and in the Brown and Dark Brown soil zones on the Canadian prairies (Reddy and Singh, 1984). Average yield of chickpeas worldwide is about 700 kg/ha which is much below its potential (Reddy and Singh, 1984; Singh et al., 1994). Yields are seen as low and unstable compared to other crops due to adverse effects of a number of biotic and abiotic stresses (Singh et al., 1994).

One of the greatest biotic stresses reducing potential yield in chickpea is ascochyta blight caused by *Ascochyta rabiei* (Pass.) Lab. (Singh and Reddy, 1996). *Ascochyta rabiei* has been reported in 29 countries world-wide (Nene and Reddy 1987). Symptoms of the disease include wilting leaf tips, leaf lesions, stem lesions causing stem breakage and lesions on pods resulting in seed infection. The two most damaging symptoms are stem breakage and pod infection (Reddy and Singh, 1990a).

*Ascochyta rabiei* is heterothallic, thus when two compatible mating types are present genetic recombination can occur resulting in ascospore production (Trapero-Casas and Kaiser, 1992a; Wilson and Kaiser, 1995). Isolates of both mating types and ascospores of *Didymella rabiei* have been found in western Canada indicating the occurrence of sexual recombination (Kaiser, 1995; Armstrong et al., 2001). Recombination could potentially lead to greater genetic and pathogenic variability in populations of *A. rabiei*.

Pathogenic variability in *A. rabiei* populations has been reported in almost all chickpea growing regions in the world, including India, Pakistan, Turkey, Syria, the Palouse region of the north-western United States and Canada (Vir and Grewal, 1974; Kaiser and Muehlbauer, 1984; Reddy and Kabbabeh, 1985; Singh, 1990; Chongo et al., 2004). Recently, several pathotypes of *A. rabiei* have been identified in western Canada with a broad range in pathogenic variation in the population (Chongo and Gossen, 2001; Chongo et al., 2004). Chongo *et al*. (2004) also confirmed the presence of genetic variability among *A. rabiei* isolates collected in the 1998 and 1999 growing seasons based on Random Amplified Polymorphic DNA (RAPD) molecular markers.

Despite recognition of the destructiveness of *A. rabiei* in chickpea production world-wide, very little headway on controlling the disease through resistance breeding has been made in the past century. Resistance in breeding lines of chickpeas to ascochyta blight is not durable due to the high variability of *A. rabiei* populations wherever chickpeas are grown (Vir and Grewal, 1974; Reddy and Kabbabeh, 1985; Singh, 1990; Singh and Reddy, 1993a). Resistance break down is possibly the greatest challenge in breeding for resistance to ascochyta blight in chickpea (Singh and Reddy, 1991).

Cultivars available in western Canada, lack complete resistance to *A. rabiei*. Partial resistance in cultivars adapted to western Canada tends to break down after the onset of flowering (Chongo and Gossen, 2001). Partially resistant cultivars contribute to the development of new pathotypes of the disease by imposing selection pressure, possibly resulting in increased virulence or aggressiveness within the pathogen population (Singh et al., 1992). Sanford is currently one of the few large kabuli chickpea cultivars adapted to the short season growing conditions of Western Canada, and pathogenic diversity studies of *A. rabiei* have indicated that resistance in Sanford may be short lived (Chongo et al., 2004).

With a genetically diverse population of *A. rabiei*, it is important not only to develop cultivars with durable forms of resistance, but also to monitor changes in the population structure to anticipate resistance breakdown in existing chickpea cultivars.

The objectives of this project were as follows: i) To assess whether isolates of *A. rabiei* have become more virulent or aggressive by comparing recently collected

populations of *A. rabiei* with populations collected in 1998 and characterized by Chongo et al. (2004); ii) To evaluate the level of genetic diversity of current populations of *A. rabiei* with RAPD molecular markers and compare with the 1998 population of Chongo et al. (2004); and iii) To determine mating types of individuals from 1998, 2001 and 2002 populations and assess if mating type distribution has changed over years. The present study evolved from a previous study on a population of *A. rabiei* in Saskatchewan collected primarily from 1998 conducted by Chongo et al. (2004). Wherever possible, similar methodology was used in order to allow comparison of the studies.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Chickpea production

Chickpeas (*Cicer arietinum* L.) are a high value crop that is potentially well suited to growing conditions in the Canadian prairies. Chickpeas have been produced in Saskatchewan since 1995, when only 1000 ha were seeded. An increase in acreage to 467 400 ha in 2001 can be attributed to low staple crop prices and increasing marketing opportunities. Furthermore, chickpeas can easily be incorporated into a traditional wheat-fallow cropping system which expands the rotation and increases crop diversity (Chongo et al., 2000).

At the peak of production in 2001, Canada was the 3rd largest producer of chickpeas in the world after India and Turkey (FAO, 2004). Acreage has been declining steadily since, and Canadian production of chickpeas was only 67 Kt in 2003 as compared to 455 Kt in 2001 (FAO, 2004). Export of chickpeas grown in Canada has been declining since 2000 from 179 Kt to an expected 35 Kt in 2004 (Agriculture and Agri-food Canada, 2004). In 2003, average yield of chickpeas in Canada was 1078 Kg/ha, well above the world average of 687 Kg/ha (FAO, 2004). World-wide, chickpea production over the past decade has varied between 7000 and 9000 Kt with 9 million to 12 million ha harvested (FAO, 2004).

There are approximately 33 fungal, 7 viral and 1 bacterial disease that have been reported on chickpea in different parts of the world (Nene, 1980).

## 2.2 Distribution and impact of ascochyta blight on chickpea

The expansion of chickpea production in Saskatchewan is partially limited by ascochyta blight along with lack of early maturing varieties suitable for the short growing season on the Canadian prairies. Ascochyta blight has been recorded in at

least 35 chickpea producing countries (Nene et al., 1996) and is considered the most devastating disease of chickpeas worldwide resulting in substantial yield loss (Kaiser, 1973). The disease was first introduced to western Canada in 1973 through movement of infected chickpea germplasm (McKenzie and Morrall, 1975).

Given favourable environmental conditions, yield loss may reach 100% (Navas-Cortés et al., 1998). In 1999 within Saskatchewan, yield loss was as much as 96% possibly due to cool, wet weather, which persisted throughout the growing season (Chongo et al., 2000).

## 2.3 Taxonomy, nomenclature and morphology

The casual agent of ascochyta blight of chickpea is the ascomycete *Ascochyta rabiei* (Pass) Labrousse (teleomorph: *Didymella rabiei* (Kovachevski) v. Arx. Syn. *Mycosphaerella rabiei* Kovachevski). The pathogen was first described as *Zythia rabiei* in 1867 by Passerini from France (Khune and Kapoor, 1980). The name *Phyllosticata rabiei* (Pass.) has been suggested by some researchers, but Singh et al. (1997) argued that the conidial structure was lacking an apical appendage and a surrounding gelatinous sheath, which are typical features of *Phyllosticata* spp. It has also been suggested that the anamorph be named *Phoma rabiei* as *Phoma* spp. contain mostly one-celled conidia (Kovačevski, 1936; Labrousse, 1931; Luthra and Bedi, 1932; Sattar, 1934 and Sprague, 1932; Bruns and Barz, 2001) and about 5% bicelled conidiospores (Khune and Kapoor, 1980). Singh et al. (1997) found that conidia from a culture of *A. rabiei* exhibited apical thickening of the cells, enteroblastic development of successive conidia in a phialidic mode, and condial septa that originated from in growth of the lateral wall and attained the thickness of a final septum from the beginning of growth. These are all typical feature of the genus *Phoma*. Additionally, the *A. rabiei* isolate lacked physical features of other *Ascochyta* spp. such as basal frills on seceded conidia and bounding of the outer conidial wall and the inner conidiogenous cell wall distal to the conidiogenous locus. Bruns and Barz (2001) found that isolates of *A. rabiei* showed a high variability in the number of cells per spore and the number of nuclei, but usually there were 1, 2 or 4 nuclei per spore indicating that mitosis occurs in the spores.

The teleomorph, *Didymella rabiei*, was transferred from the *Mycosphaerella* species based on characteristics of *D. rabiei* that more closely resembled *Ascochyta* species such as a larger ascomata, the arrangement of nonfasciculate asci, the presence of pseudoparaphyses and the structure of the ascospores (Wilson and Kaiser, 1995).

## 2.4 Epidemiology of the ascochyta blight

### 2.4.1 Symptoms and disease cycle

Symptoms of infection on leaflets, petioles and young branches first appear as epinasty and loss of turgor, followed by water soaking and necrosis (Alam et al., 1989). Depending on the resistance level of the chickpea genotype, a necrotic disease reaction will result to varying degrees (Porta-Puglia et al., 1996). Mature lesions appear as tan-coloured necrotic areas that may develop dark margins when they mature. Advanced lesions girdle the stem and lead to defoliation, breakage and eventually death of the plant (Höhl et al., 1990; Chongo and Gossen, 2001). Asexual overwintering structures, called pycnidia, develop, often concentrically, within lesions (Nene and Reddy, 1987). The disease initially appears in small areas in the field but spreads quickly when optimal conditions for the disease prevail (Kaiser, 1973). Overwintering of the fungus in infested chickpea stubble is the main source of inoculum for subsequent growing seasons (Luthra et al., 1935; Kaiser, 1973; Navas-Cortes et al., 1995).

Under suitable conditions, the development of the teleomorph of the fungus, *Didymella rabiei*, can occur on infested chickpea stubble. Pseudothecial initials develop on residue in the fall and continue to develop into the following spring. In the USA, pseudothecia mature at the beginning of March and release ascospores over a two-month period with the majority of ascospores released in mid-April (Trapero-Casas and Kaiser, 1992a). In Canada, ascospores have been found in spring and summer (Armstrong et al., 2001), but the exact time of release and when release terminates has not been documented. Ascospores are suspected to initiate blight epidemics in fields up to 15 km from the source field through wind transfer (Kaiser, 1997) and disease has appeared in fields free of chickpea debris using pathogen-free

chickpea seed has been planted (Trapero-Casas and Kaiser, 1992a). In addition, ascospores aid in the survival of the pathogen between growing seasons on infested debris (Kaiser et al., 1987; Navas-Cortés et al., 1995).

### 2.4.2 Disease spread

The primary means of spreading *A. rabiei* over large geographical distances is through movement of infected seed (Kaiser and Hannan, 1988; Kaiser, 1997). The fungus is able to survive in infected chickpea seed in storage and maintain it's viability for over 5 years (Kaiser, 1987). Furthermore, infected seed can also be a source of inoculum from year to year (Kaiser and Hannan, 1988).

Ascospores, produced in asci within the pseudothecia, are forcibly discharged into the air (Kaiser, 1987). The ascospores are carried by wind and may be carried over great distances (Kaiser, 1992; Trapero-Casas and Kaiser, 1992a). Trapero-Casas et al. (1996) found that ascospores of *D. rabiei* are a major source of primary inoculum for epidemics of the disease in southern Spain. Conidia of *A. rabiei* can cause repeated secondary disease cycles within fields throughout the growing season (Wilson and Kaiser, 1995) through rain-splashing conidia, which ooze from pycnidia (Kaiser, 1973; Chongo and Gossen, 2001). The disease can also be spread by vehicles, people or animals moving through the crop (Kaiser, 1973).

The agronomic practice of minimizing tillage for moisture conservation leaves chickpea debris on the soil surface of an infected field. This may contribute to the development of *D. rabiei* pseudothecia during the winter months (Trapero-Casas and Kaiser, 1992a). Pseudothecia and pycnidia were found to remain viable for at least 2 years when left on the soil surface, but were only viable for 2 to 5 months after debris was buried (Navas-Cortés et al., 1995).

### 2.4.3 Environmental conditions

Temperature, rainfall and wind are the environmental factors that have the greatest influence on disease development of ascochyta blight (Weltzien and Kaach, 1984; Nene and Reddy, 1987; Trapero-Casas and Kaiser, 1992b). The amount of rain is closely correlated with disease severity as it is crucial for pseudothecia maturation,

discharge of ascospores and the infection process (Akem, 1999). The disease can reach epiphytotic proportions when the relative humidity is greater than 60%, leaf temperatures are between 10 and 20°C, combined with more than 150 mm of annual rainfall or a leaf wetness period of at least 7 hours (Reddy and Singh, 1990c; Trapero-Casas and Kaiser, 1992b). Disease severity increases as the duration of wetness periods exceed a minimum of 6 hours, and with increasing temperatures to a maximum of approximately 20°C (Trapero-Casas and Kaiser, 1992b). Lower and upper temperature limits for infection and disease development are about 5°C and 30°C, respectively (Trapero-Casas and Kaiser, 1992b). In order for an epidemic of ascochyta blight to occur a monthly average temperature of at least 8°C and a monthly rainfall of at least 40 mm is needed (Ketelaer et al., 1998).

### 2.4.4 Teleomorph

Pseudothecial development requires no nutrients other than those provided by the chickpea debris (Trapero-Casas and Kaiser, 1992b). Generally, pseudothecia are arranged in rows on chickpea straw and are erumpent, dark brown to black, subglobose and 120-270 μm in diameter with an inconspicuous ostiole when mature (Trapero-Casas and Kaiser, 1992a). Asci which protrude from pseudothecia of *D. rabiei* are cylindrical to subclavate, 8-spored and 50-80 x 10-12 μm with a bitunicate wall (Trapero-Casas and Kaiser, 1992a). Ascospores are hyaline and two-celled (Akem, 1999), ellipsoidal to biconic, strongly constricted at the septum and 9.5-10 x 4.5-7 μm in size (Trapero-Casas and Kaiser, 1992a). Estimated potential for ascospore production is 15 000 ascospores per square millimetre of infested tissue (Trapero-Casas and Kaiser, 1992a). Trapero-Casas and Kaiser (1992a) found that temperature had limited influence on the induction of pseudothecia, but a large effect on their maturation. Long dry periods can delay maturation of ascospores (Navas-Cortés et al., 1998). Navas-Cortés et al. (1996) found that the occurrence of rain had a greater effect on ascospore discharge than the amount of rainfall.

The teleomorph of *A. rabiei* was first observed in the Palouse region of the United States in 1988 by Kaiser (1990), and in western Canada in 1999 by Armstrong et al. (2001). Sexual recombination of *A. rabiei* results in greater genetic diversity and

more virulent genotypes of the pathogen (Kaiser, 1997). It is thought that the teleomorph is more widespread than what has been reported (Kaiser and Hannan, 1987).

## 2.5 Infection process and histology of pathogenesis

Ascochyta blight rapidly spreads affecting all plant parts above the ground at any crop growth stag, resulting in a rapid collapse of tissue and spread of necrotic lesions (Shtienberg et al., 2000). The fungus infects the plant by direct penetration of the epidermis and/or the hydathodes after the formation of appressorium-like infection structures (Höhl et al., 1990; Köhler et al., 1995). Appressoria are not melanized, thus penetration occurs not only through mechanical force but also through the action of hydrolytic enzymes (Tenhaken, 1992). The fungus spreads subepidermally in the apoplastic space, mainly intercellularly, invades cells and causes a rapid collapse of leaf tissues and plasmolysis. This eventually results in the formation of necrotic areas and the development of pycnidia (Höhl et al., 1990; Köhler et al., 1995). Pycnidia generally develop near the vascular regions of plant tissue, possibly for structural support as all other cell tissue is destroyed within a lesion (Köhler et al., 1995). The main direction of fungal growth is from the leaflet through the vascular region of the petioles to the stems. Eventually the leaflets, petioles and stems are filled with fungal hyphae and collapse (Köhler et al., 1995). In moderately or severely blighted leaves, Gaur (2000) found that both the production of chlorophyll 'a' and chlorophyll 'b' was reduced significantly. This was attributed to inhibition of production by *A. rabiei* or enhanced activity of chlorophyllase (Gaur, 2000).

Fungal exotoxins are responsible for changes in cell morphology on susceptible cultivars in the infection process in advance of invading fungal hyphae (Pandey et al., 1987; Höhl et al., 1991). Phytotoxins that have been isolated from infected chickpea tissue include solanapyrones A, B, C (Alam et al, 1989; Höhl et al., 1991; Latif et al., 1993) and cytochalasin D (Latif et al., 1993). Solanapyrones A, B and C have also been isolated from culture filtrates of *Alternaria solani*, the casual fungus of blight of potato and tomato (Ichihara et al., 1983). Different isolates of *A. rabiei* differed in the amount and types of toxin compounds produced (Latif et al.,

1993). Solanapyrone A is the most toxic followed by solanpyrones C and B (Kaur, 1995). Solanapyrones A, B and C are active individually as well as additively in combination (Alam et al., 1989; Kaur, 1995). Latif et al. (1993) and Kaur (1995) found yield of phytotoxins from different isolates of *A. rabiei* were well correlated with their degree of pathogenicity on chickpea. Additionally, there is considerable heterogeneity among fungal isolates at the biochemical level (Latif et al., 1993). Cultural conditions also affect toxin production (Chen and Strange, 1991).

*A. rabiei* isolates with greater aggressiveness are more toxigenic and the more susceptible genotypes are more toxin sensitive (Latif et al., 1993). It is possible that toxin production by pycnidia is inhibited on the leaves of resistant genotypes (Platerosanz and Fuchs, 1978; Höhl et al., 1991), that decreased toxin sensitivity is based on a higher toxin threshold or that resistant genotypes possess detoxification mechanisms (Johal and Briggs, 1992).

Copper amine oxidase (CuAO) is a soluble protein that is abundant in the extracellular fluids of many legumes, and is associated with defence responses and the balance of reactive oxygen species such as hydrogen peroxide which kills invading pathogens. It has been demonstrated that CuAO expression increases in response to both wounding and infection with *A. rabiei*. Furthermore, CuAO activity is greater in cultivars of chickpea with greater resistance to *A. rabiei*. Increase in levels of CuAO is signalled by jasmonic acid and reduction to basal levels of CuAO in plant is prompted by salicylic acid and abscisic acid. These signalling molecules appear to trigger CuAO activity not only at the site of wounding or infection, but also systemically throughout the plant. Increased lesion size caused by *A. rabiei* occurred when CuAO production was inhibited with 2-bromoethylamine as there was more damage to the cortical parenchyma and sclerenchyma cells of the plant. Additionally, lesion morphology was affected by inhibition of CuAO as lesions grew lengthwise instead of girdling the stem (Rea et al., 2002).

## 2.6 Control methods for ascochyta blight

### 2.6.1 Resistance in chickpea to ascochyta blight

#### 2.6.1.1 Breeding for resistance

Since the use of resistant cultivars is the most practical way to control ascochyta blight, breeding for genetic resistance is a major objective of chickpea improvement programs world-wide (Muehlbaur and Singh, 1987; Reddy and Singh, 1990b). Even though ascochyta blight has been known for almost 90 years, little progress has been made on its control with host-plant resistance (Singh, 1992; Reddy and Singh, 1990b; Singh and Reddy, 1991). The development of varieties resistant to ascochyta blight has been slow over the past 60 years due to lack of high-level, stable sources of resistance (Reddy et al., 1992). Screening for resistance to *A. rabiei* showed that the amount of resistant germplasm is very small and that chickpea has a narrow genetic base (Reddy and Singh, 1984; Singh and Reddy, 1993a; Udupa et al., 1993).

Breeding for horizontal resistance was attempted at the International Centre for Agricultural Research in the Dry Areas (ICARDA) but abandoned when positive results could not be achieved (vanRheenen and Harware, 1994). Recently attempts have been made to include several resistance genes into single lines (gene pyramiding) which includes crossing resistant cultivars of different origins (Singh et al., 1994; vanRheenen and Harware, 1994). Research at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) has concentrated on developing cultivars by pyramided resistance genes by using marker-assisted selection (MAS), as it was otherwise difficult to identify lines containing combinations of different genes (Singh and Reddy, 1996).

Chickpea breeders are also looking to wild relatives of chickpea as novel sources of resistance genes. A major effort of many chickpea breeding programs around the world has been the screening of wild *Cicer* species as they may be a valuable source of resistance (Singh et al., 1994). Resistance to ascochyta blight has been found in accessions of *C. echinospermum* P.H. Davis (Collard et al., 2001, 2003), *C. reticulatum* Ladiz. (Collard et al., 2001, 2003), *C. bijugum* K.H. Rech. (Collard et

al., 2001, 2003; Harware et al., 1992), *C. judaicum* Boiss. (Collard et al., 2001; Singh and Reddy, 1993b) and *C. pinnatifidum* Jaub. and Sp. (Collard et al., 2001; Singh and Reddy, 1993b). Singh and Ocampo (1993) and Singh et al. (1999) have found that both *C. reticulatum* and *C. echinospermum* are cross-compatible with *C. arietinum*. Therefore, these species are valuable sources of immediate resistance that can be introgressed into cultivated chickpea cultivars. For non-cross-compatible species, the rescue of ovules and their in-vitro culture could be a solution for crossing reproductive barriers for successful crosses between mates with genetic incompatibilities (Singh and Ocampo, 1997).

Many lines of chickpea germplasm that show resistance to ascochyta blight in the vegetative stage do not maintain resistance through the reproductive growth stage (Reddy and Singh, 1984; Chongo and Gossen, 2001). New growth on plants tended to be less susceptible to *A. rabiei* than older growth on resistant cultivars, and all parts of the plant were highly susceptible on susceptible varieties (Chongo and Gossen, 2001). Additionally, leaves had lower ascochyta infection compared to the stems suggesting that gene expression differs in stems and leaves (Chongo et al., 2001). Reddy et al. (1992) found that desi germplasm had higher resistance than kabuli germplasm with desi-type cultivars having a greater level of resistance during the vegetative stage, but increasing susceptibility at the podding stage (Singh and Reddy, 1993a). Reddy and Singh (1984) and Singh and Reddy (1993a) suggested that there was a positive association of blight resistance with later maturity, increased plant height and erect growth habit for both kabuli and desi accessions, but seed size was negatively associated with resistance in kabuli-type accessions.

Most studies on biochemical, morphological and physiological characteristics of chickpea genotypes with differing levels of resistance have been inconclusive (Sattar, 1933; Ahmed et al., 1952; Hafiz, 1952; Vir et al., 1975; Koundal and Sinha, 1983; Chand et al., 1988). However, Sarwar et al. (1996) found that 2 resistant cultivars had a significantly thicker stem hypodermis than 2 susceptible cultivars. They hypothesized that the thickness of the stem hypodermis may contribute to host resistance by protecting the cortical and vascular tissues from fungal attack by providing a shielding effect.

#### 2.6.1.2 Resistance genes

There is a lot of controversy in the literature on the inheritance of genes conferring resistance to ascochyta blight (VanRheenen and Haware, 1994). Vir et al. (1975) and Eser (1976) first reported a single dominant resistance gene in different desi cultivars. Since then there have been reports of 1 recessive gene for blight resistance in a kabuli cultivar and 1 dominant gene in several kabuli cultivars (Singh and Reddy, 1983), 2 dominant genes in several lines and 1 recessive gene in another line (Tewari and Pandey, 1986), 2 complementary recessive genes (Kusmenoglu, 1990; Santra et al. 2000), 1 recessive and 5 dominant genes for resistance with 2 complementary dominant genes that control resistance and interallelic interactions influencing the simple Mendelian segregation of these genes (Dey and Singh, 1993) and 2 major complementary recessive genes with the possibility of several minor modifying genes taking part (Tekeoglu et al., 2000). The quantitative nature of the inheritance of resistance was explained by the presence of minor genes (Tekeoglu et al., 2000). Muehlbauer and Singh (1987) found evidence that other genes modified the expression of blight resistance. Malik (1990) concluded that the genetic control of quantitative variation of ascochyta blight resistance was complex after attempting to fit simple and more complicated Mendelian models to extensive data sets.

Santra et al. (2000) located 3 Quantitative Trait Loci (QTL) with interval mapping of RILs from a resistant line of *C. arietinum* crossed with a wild relative of chickpea, *C. reticulatum*. QTL-1 and QTL-2 together accounted for an estimated average of 48% of the variation in blight reaction over two seasons, and QTL-3 was a minor QTL which also contributed to resistance. It is thought that QTL-1 and QTL-2 coincide with the 2 recessive genes reported by Kusmenoglu (1990) and Tekeoglu et al. (2000).

Udupa and Baum (2002) were the first to map resistance in the chickpea genome to specific pathotypes. Pathotypes I (least aggressive), II (moderately aggressive) and III (highly aggressive) were identified by Udupa et al. (1998). Using the chickpea landrace ILC-3279 which is resistant to Pathotype I and II, and a susceptible cultivar, a RIL population was derived to study the genetics of resistance to different pathotypes of *A. rabiei*. They found that resistance to pathotype I was

conferred by a major locus and resistance to pathotype II was conferred by two major loci (Udupa and Baum, 2002). Mendelian genetics and QTL analysis both showed the same number of genes controlling resistance to both pathotypes and mapped the respective genes to the same regions in linkage groups. The resistance gene *ar1* (pathotype I-specific resistance gene) and *ar2a* (one of the pathotype II-specific resistance genes) were located on linkage group 2. The majority of resistance genes in cultivars screened seem to be linked, as shown with *ar1* and *ar2a* (Udupa and Baum, 2002). In contrast, Cho et al. (2004) inoculated F7-derived RILs from an intraspecific cross of the resistant line FLIP84-92C and a susceptible line with isolates representing pathotypes I and II. A QTL for resistance to pathotype II and 2 QTLs for pathotype I were found on LG4A and LG2+6, respectively. In addition, a single gene could explain the majority of the quantitative resistance to pathotype I, and was needed to confer resistance to pathotype II along with the QTL identified on LG4A.

The functions of several genes were identified by isolating, sequencing and comparing cDNAs of chickpeas infected with *A. rabiei* with other plant species (Ichinose et al., 2000). Plausible functions of isolated cDNAs included: i) defence related pathway genes such as reinforcement of cell walls, PR-proteins, phytoalexin biosynthetic enzymes and reactive oxygen species (ROS) scavenging enzymes; ii) genes involved in signal transduction; iii) genes for expression such as transcription factors and translation; iv) catabolic pathway genes and v) genes encoding proteins for primary metabolism (Ichinose et al., 2000). A gene product isolated by Ichinose et al. (2000) was homologous with a gene encoding a glycine rich protein (GRP) in alfalfa. GRPs were also characterized in chickpea in response to wounding by Cornels et al. (2000), who hypothesized that GRPs may be responsible for sealing of vascular elements in the apoplastic space during later phases of *A. rabiei* invasion (Köhler et al., 1995). Extracelluar β-1,3-glucanase, a PR-protein, has also been purified and characterized in *A. rabiei* infected chickpea (Hansell and Barz, 2001). β-1,3-glucanase is found in the intercellular fluid of the apoplastic space between cells where growth of the fungus is stopped in a resistant cultivar (Hanselle and Barz, 2001). Two gene products found by Ichinose et al. (2000) were homologous with β-1,3-glucanase genes from tobacco and pea. However, Hanselle and Barz (2001) found that the amount of

β-1,3-glucanase accumulation did not differ significantly between a susceptible and resistant cultivar, thus cannot solely be the reason for resistance.

### 2.6.2 Chemical control

Fungicidal seed treatments are important in preventing disease initiation and development from infected seed. Kaiser and Hannan (1988) found that the most effective fungicidal seed treatment for ascochyta blight was benomyl and thiabendazole both of which reduced, but did not eradicate the incidence of seedborne *A. rabiei* in the treated seeds. Systemic fungicides have generally not been effective against *D. rabiei* (Shtienberg et al., 2000). An effective systemic fungicide would be useful in battling post-infection detection allowing better management of the disease and potentially reducing the number of fungicide applications (Shtienberg et al., 2000).

According to Kaiser and Hannan (1988), control of only one aspect of the disease cycle will probably not prevent infection in regions where favourable conditions for ascochyta blight persist. Furthermore, the application of fungicide is often not economical because multiple applications throughout the growing season may be needed in situations where weather conditions are favourable for disease development over long periods (Reddy and Singh, 1984; Reddy and Singh, 1990a; Singh and Reddy, 1996; Collard et al., 2001).

### 2.6.3 Integrated disease management

The use of resistant cultivars is the most effective and economical management strategy for ascochyta blight although the level of resistance currently available is not sufficient to withstand high disease pressure under favourable disease conditions (Nene and Reddy, 1987; Akem, 1999; Chongo et al., 2000). A combination of disease management utilizing moderate host plant resistance, foliar and seed treatment fungicides, and cultural controls such as deep ploughing of infested crop debris and a crop rotation of at least 4 years have been shown to be economical production practices (Reddy and Singh, 1990b). Other cultural practices such as reducing plant canopy thickness and planting pathogen free seed help minimize disease epidemics (Akem, 1999).

Monitoring seed infection is crucial in an integrated disease management system. The traditional method for testing seed-borne infection is to plate infected seeds on media to grow the pathogen. This testing is very laborious and the targeted pathogen may sometimes be overgrown and masked by other fungi. Thus Phan et al. (2002) developed a diagnostic polymerase chain reaction (PCR) test based on SCAR primers in order to detect *A. rabiei* in infected leaves and seed of chickpeas. They found that the test could detect very low levels of the pathogen in samples and could distinguish *A. rabiei* from a range of other chickpea pathogens but was not specific enough to distinguish *A. rabiei* from other *Ascochyta* species. However, the test was quantitative as the intensity of the PCR product correlated with measured amounts of template DNA used in the amplification reaction. It is expected that pathogen diagnostics based on PCR technology will become an important tool to integrate disease management for seed health testing programs and quarantine procedures (Phan et al., 2002).

## 2.7 Definitions of terms describing host-pathogen interactions

Two types of pathogen specificity are described by plant pathologist, plant species specificity (otherwise known as basic compatibility) and cultivar specificity (Heath, 1981, 1987). Plant species specificity determines the host species and cultivar specificity determines cultivar range within a given host range, although it is possible that host-pathogen interactions controlling each type of specificity may coexist in the same tissue (Heath, 1981). Plant resistance to plant species specificity of a pathogen has been termed basic resistance, non-host resistance, basic incompatibility, general resistance, multigene resistance or broad resistance, whereas host-genotype or cultivar resistance is parasite-specific and superimposed on basic compatibility and may express a gene-for-gene interaction with the pathogen (Heath, 1991; Prell and Day, 2001). Moreover, it is possible that a gene-for-gene interaction may govern both types of resistance to both levels of pathogen specificity (Heath, 1991).

A physiological race is described by the Commonwealth Mycological Institute as “parasites (particularly fungi) characterized by specialization on certain cultivars of one host species”, although the term race has not formally been recognized as a taxon

in the International Code of Botanical Nomenclature (Caten, 1987; Bos and Parlevliet, 1995). Caten (1987) argues that different types of races need to be defined, since 'race' has been used to describe very different aspects of pathogenic variation which has resulted in several concepts of 'race' in plant pathology. 'Simple races' are defined by one or two genes determining toxin production of necrotrophic fungi, whereas 'physiologic races', or 'virulent races', are defined primarily by biotrophic fungi when reactions are analyzed on a set of differential hosts differing in sources of qualitative resistance. In contrast, isolates should be classified into 'aggressive races' if they do not interact differently between various host genotypes (Caten, 1987). 'Biologic races' as described by Caten (1987) are major intraspecific groups differentiated by morphological features and other characteristics.

VanDerPlank (1982) described 'race-non-specific resistance' or 'horizontal resistance' as resistance against all pathogen races. This type of resistance is considered to be genetically stable, quantitative, incomplete and controlled by many minor genes in a polygenic fashion, so that disease reaction is normally distributed with continuous variation. Quantitative resistance in plants is usually found in response to infection with necrotrophic and saprophytic fungi and is thought to have increased durability and stability as it slows epidemic development. This type of resistance is thought to be highly influenced by the environment. The opposite of race non-specific resistance as described by VanDerPlank (1982) is 'race-specific resistance' or 'vertical resistance' which provides resistance only to certain virulent phenotypes of a pathogen. This type of resistance is considered qualitative as it is controlled by only a few major genes which provide complete resistance to a pathogen. Race-specific resistance occurs mainly in response to infection of plants with biotrophic fungi and bacteria to which the gene-for-gene concept applies. This type of resistance is considered non-durable as it only delays the onset of epidemics.

Arguments against VanDerPlank's classification system of types of resistance to plant pathogens are quite common. In fact, there are many inconsistencies and controversial uses of the terms pathogenicity, aggressiveness and virulence in plant pathology when describing pathogens attacking plants. Bos and Parlievliet (1995) argued that aggressiveness described whether, and to what extent, a pathogen can

attack an organism, while the term virulence denotes the ability of a parasite to cause disease in the host plant, thus making a parasite a pathogen. Pathogenicity was defined as the result of both aggressiveness and virulence, as the overall disease-inducing capacity and should be measured as the amount of disease development or the degree of infection (caused by aggressiveness) as well as the severity of the symptoms produced on a range of host genotypes (Bos and Parlievliet, 1995). Bos and Parlievliet (1995) argued that virulence/avirulence as described by VanDerPlank (1982) is inappropriate to describe what happens during a pathogen attack. A better description is that resistance is induced by an elicitor produced by a gene in the pathogen, whereas malfunction, mutation or absence of this gene results in normal aggressiveness. If there is a lack of specific interactions between known plant genotypes and differing pathogen genotypes, Bos and Parlievliet (1995) also suggested that race non-specific resistance may be involved and the pathogen should not be classified into a pathotypes or races as this only applies to gene-for-gene interactions (VanDerPlank, 1982).

For the purpose of this study, terms described by Caten (1987) and VanDerPlank (1982) will be used to describe resistance to the pathogen population studied. Aggressive races as defined by Caten (1987) will refer to isolates differing in aggressiveness and will be equated with pathogens evoking 'race-non-specific' resistance in plants as described by VanDerPlank (1982). Caten's (1987) description of a physiologic or virulent race will correspond to 'race-specific' resistance in the host (VanDerPlank, 1982). The term pathotype will be equated with race unless otherwise stated because they are essentially determined using the same approach in most studies on *A. rabiei*.

## 2.8 Pathogenic structure of *Ascochyta rabiei* populations

Population studies revealed that aggressiveness within a local population of *A. rabiei* can be variable within a few years of a population's documented existence (Jan and Wiese, 1991). Santra et al. (2001) found that five isolates from the Palouse region of the USA had a significant amount of pathogenic diversity which could be credited to the introduction of the pathogen to the region from different countries. Sexual

recombination during meiosis (Kaiser and Hannan, 1987; Trapero-Casas and Kaiser, 1987) and random genetic drift followed by selection can also contribute to diversity (Kohn et al., 1988; Milgroom et al., 1992; Morjane et al., 1994). High genetic diversity of *A. rabiei* and frequent recombination increases the likelihood of development of rare, more aggressive pathotypes, thus rendering current resistance sources ineffective (Chongo et al., 2004).

Different pathotypes of *A. rabiei* have been characterized based on differences in aggressiveness on differential host genotypes through artificial inoculation with the pathogen, similar to determining physiological races (Navas-Cortés et al., 1998). Unfortunately, physical assays of pathogenicity are inconsistent due to the limited control of the environment present in greenhouse experiments which can influence the aggressiveness within the same isolate/line combination (Udupa et al, 1998). Furthermore, biological pathotyping is laborious, time-consuming and requires stringent standard experimental conditions (Navas-Cortés et al., 1998).

In most pathogenicity studies, *A. rabiei* isolates have been classified into physiological races or pathotypes based on reaction pattern on a set of differential chickpea genotypes. Arbitrary points on the rating scales have been selected that are assumed to separate a susceptible from a resistant reaction. However, there is rarely a clear resistant or susceptible reaction on chickpea when infected by *A. rabiei*. The term "pathotype" is used synonymously with "race" in several studies on populations of *A. rabiei*, and in some studies different pathotypes essentially represent levels of aggressiveness of isolates (e.g. Jamil et al., 2000). Even though this system has allowed pathologists to quickly analyze and present disease reaction results, it appears to be unsuitable to describe the data. The approach has proven to be unreliable because of lack of reproducibility, suggesting this pathosystems may be more complex than what a traditional race or pathotype system can describe.

Pathogenic variability among isolates of *A. rabiei* has been reported from India (Vir and Grewal, 1974; Singh, 1990; Singh and Pal, 1993; Ambardar and Singh, 1996; Navas-Cortés et al., 1998), Syria and Lebanon (Reddy and Kabbebeh, 1985; Singh and Reddy, 1993; Udupa and Weigand, 1997), the Palouse region of the USA (Jan and Wiese, 1991; Navas-Cortés et al., 1998), Italy (Porta-Puglia et al., 1996), Pakistan

(Qureshi and Alam, 1984; Hussain and Malik, 1991; Jamil et al., 1993; Jamil et al., 1995; Jamil et al., 1997; Navas-Cortés et al., 1998; Jamil et al., 2000), Spain (Navas-Cortés et al., 1998), Australia (Khan et al., 1999), Tunisia (Hamza et al., 2000) and most recently in Canada (Chongo et al., 2004). These studies have used from 3 to 15 differential host genotypes, classified 11 to 130 isolates of *A. rabiei* into 3 to 14 pathotypes.

## 2.9 Genetic structure of *Ascochyta rabiei* populations

In order to understand the co-evolution of plant pathosystems, it is necessary to analyze the genetic variation in pathogen populations in time and space. Classical biological pathotyping using a set of differential genotypes is laborious, expensive, time-consuming, requires standardization of the test environment and cannot be replicated in other countries since pathogenic fungi are subject to quarantine regulations and cannot be exported or imported (Weising et al., 1991).

Fungal genotypes are most reliably characterized with DNA polymorphisms (Morjane et al., 1994; Jamil et al., 2000). DNA polymorphisms have been used increasingly in recent years to analyse genetic identity, variability and relatedness in *A. rabiei*. High levels of genetic variation in *A. rabiei* can be detected by using DNA markers, such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLP), Amplified Fragment Length Polymorphism (AFLP) markers and different forms of microsatellite markers (Udupa et al., 1998; Santra et al., 2001; Peever et al., 2004). Geistlinger et al. (2000), Phan et al. (2003a) and Peever et al. (2004) have found that microsatellite markers converted to PCR Sequence Tagged Microsatellite Site (STMS) markers are a relatively quick, reliable method that detects a suitable amount of variability for genome analysis.

### 2.9.1 RAPD fingerprinting

RAPD analysis is a PCR application, which uses arbitrary oligonucleotide primers that bind to homologous base sequences in the genome, thus detecting differences in the nuclear genome (Welsh and McClelland, 1990; Williams et al., 1990; Mitra et al., 2000). RAPD analysis does not require any prior knowledge of the

genome as in RFLP studies (Rowe et al., 1997). RAPDs are simple, quick and avoid the use of radioisotopes (Rowe et al., 1997). Many phylogenetic studies of *A. rabiei* have utilized RAPD markers, but problems with reproducibility and suitable levels of polymorphisms have caused researchers to explore other marker technologies. The use of synthetic oligonucleotides complementary to microsatellite motifs as hybridization probes to reveal RFLP is a more sensitive detection method than RAPDs (Morjane et al., 1994).

Many genetic studies on *A. rabiei* have utilized RAPD markers to detect levels of similarity between and within populations of the pathogen. A wide range in the number of polymorphisms detected by the RAPD technique in *A. rabiei* has been recorded. As few as 3 RAPD primers have been used to completely delineate a sample of 30 Italian isolates (Fischer et al., 1995). On the other hand, 48 RAPD primers were used on populations from different chickpea growing regions in the world, and cluster analysis grouped these isolates by geographic origin (Santra et al., 2001). Microsatellite markers have also been used in conjunction with RAPD primers in order to more effectively characterize the *A. rabiei* genome (Udupa et al., 1998; Lichtenzveig et al., 2002; Phan et al., 2003a).

### 2.9.2 Microsatellite fingerprinting

Simple Sequence Repeats (SSR) or Variable Number Tandom Repeats (VNTR) can be used as a co-dominant marker system and are based on variation in repetitions of a core microsatellite repeated sequence of approximately 2 to 10 bp (Tautz and Renz, 1984; Weising et al., 1991; Akkaya, 1992; Geistlinger et al., 1997a). Tandem repeats are often spread at multiple sites (up to $10^5$) throughout the genome (Beckmann and Weber, 1992; Wang et al., 1994). Microsatellite marker systems can produce both simple banding patterns of polymorphism at 1-2 loci or more complex banding patters of up to 26 scorable bands from DNA of *A. rabiei* depending on the combination of digestion enzyme used and the motif (Weising et al., 1991; Geistlinger et al., 1997b).

At least 25 different microsatellite motifs have been found in the *A. rabiei* genome (Weising et al., 1991; Morjane et al., 1994; Geistlinger et al., 1997b;

Geistlinger et al., 2000; Phan et al., 2003a; Peever et al., 2004). Lichtenzveig et al. (2002) screened 37 STMS primer pairs and found that 13 (35%) showed clear and reproducible polymorphic amplicons. A unique clonal lineage as defined by its fingerprint haplotype was found when 22 isolates of *A. rabiei* from the USA, Pakistan, Syria, Turkey and Tunisia were amplified with 20 primers sequences that were designed for *A. rabiei* microsatellites (Geistlinger et al., 1997a; Geistlinger et al., 2000).

Recently, microsatellite markers have been utilized to map the genome of *A. rabiei*. Lichtenzveig et al. (2002) mapped 14 STMS and 36 RAPD primers to the genome of *A. rabiei*. Ten linkage groups were found and 19 (38%) markers were unlinked, 8 of which were STMS markers, suggesting the markers were not evenly distributed throughout the genome. By backcrossing $F_1$ progeny of *D. rabiei* it is hoped that loci can be mapped to pathogenicity by analyzing aggressiveness of isolates. This will generate a better understanding of the *A. rabiei – C. arietinum* interaction (Lichtenzveig et al., 2002). Similarly, Phan et al. (2003b) used 9 STMS, 63 AFLP and 37 RAPD markers on an F1 population between an Australian and American isolate to construct a detailed genetic linkage map identifying 18 major and 10 smaller linkage groups less than 20 cM in length. Unfortunately, the maps generated by Lichtenzveig et al. (2002) and Phan et al. (2003b) cannot be compared as only STMS markers were common to both studies.

By using microsatellites as oligonucleotide probes in order to reveal RFLP markers, 50 isolates from a single field in Tunisia were studied (Morjane et al., 1994). They found that haplotypes of *A. rabiei* were heterogeneous and patterned throughout the field in a mosaic (Morjane et al., 1994). Furthermore, each location was dominated by a different haplotype and several haplotypes occurred in more than one (but not all) locations. These results suggested that clusters of identical haplotypes developed at infection loci within the field and then were spread by rain splash. A single host plant could be coinfected with up to four different haplotypes of the pathogen suggesting that *A. rabiei* populations within a single chickpea field contained isolates with large amounts of minor genetic variation (Morjane et al., 1994). On the other hand, Udupa et al. (1998) found that there was very little or no variation within

and among isolates collected from a single lesion, plant or field and that over 80% of lesions from a single field belonged to a single genotype.

### 2.10 Relationship between pathogenic and molecular variability

Many studies have not found any correlation between pathogenicity of isolates of *A. rabiei* and RAPD or microsatellite diversity (Navas-Cortés et al., 1998; Udupa et al., 1998; Hamza et al., 2000; Santra et al., 2001). In contrast, Weising et al. (1991) analyzed 6 isolates of *A. rabiei* with 4 microsatellite probes and 4 restriction enzymes and found that fingerprint data supported classification of isolates according to their aggressiveness. For example, 2 isolates showed low levels of aggressiveness on most host cultivars and shared identical genomic fingerprints, and the same held true for the highly aggressive isolates. When 53 Syrian isolates were screened with a combination of RAPD and a microsatellite markers, isolates with genotypic similarity correlated with pathogenicity data, with Pathotype III (the most aggressive pathotype) expressing the least amount of genetic diversity compared to isolates from Pathotype I and Pathotype II (Udupa et al., 1998). Similarly, when historical and contemporary populations of *A. rabiei* from the Pacific Northwestern US were analyzed, the two populations were divergent (Peever et al., 2004). They suggested that the difference in genotypes was associated with the change in the aggressiveness of the population that had been observed.

The number of primers used affected the detection of correlations between pathogenicity and molecular characteristics. For example, Fischer et al. (1995) used three primers on thirty isolates and found no pathotype-specific amplification pattern. Since single RAPD primers only cover approximately 0.2% of the total fungal genome, a large number of primers may be needed to detect correlations with pathogenicity. It was suggested that several hundred primers would be needed to observe such a correlation (Fischer et al., 1995).

## CHAPTER 3

# PATHOGENIC VARIABILITY IN *ASCOCHYTA RABIEI* POPULATIONS ON CHICKPEA IN SASKATCHEWAN

## 3.1 Introduction

Pathogenic variability among isolates of *A. rabiei* has been reported from almost all chickpea producing regions in the world. In India, Vir and Grewal (1974) first classified *A. rabiei* isolates into 6 races, followed by Singh (1990) who classified 13 isolates into 12 pathotypes with 12 host differentials. Singh and Pal (1993) identified 5 races among 11 isolates tested on 7 differentials. More recently, 18 isolates selected based on morphological data from a population of 76 isolates were classified into 10 races with 12 differentials by Ambardar and Singh (1996). In Syria and Lebanon, 50 isolates were grouped into 6 pathotypes with 6 differential genotypes (Reddy and Kabbebeh, 1985) and 53 isolates were grouped into 3 classes of differing virulence on 9 differentials (Udupa and Weigand, 1997). A study conducted in the Palouse region of the USA found 11 virulence forms from 39 isolates tested on 15 differential chickpea lines (Jan and Wiese, 1991). In Italy, 41 isolates of *A. rabiei* were divided into 3 groups based on their reaction on 13 genotypes and analyzed with cluster analysis (Porta-Puglia et al., 1996). Jamil et al. (2000) classified 130 isolates with 3 differential genotypes with varying levels of resistance into 3 pathotypes based on the isolates' aggressiveness. A study comparing pathotypes of isolates from different origins was carried out by Navas-Cortés et al. (1998) who found 11 pathotypes with 5 isolates from India 14 from Spain, 11 from Pakistan, 7 from the USA, 2 from both Morocco and Greece and 1 isolate from each Turkey, Italy and France. Seventeen representative genotypes of *A. rabiei* from the Beja region of Tunisia were tested on 8 differential genotypes and 5 highly virulent phenotypes were found using cluster analysis (Hamza et al., 2000). Despite this wealth of publications,

it is difficult to compare results among these studies on variability due to differences in the procedures, such as plant age of inoculation, differences among rating scales, and chickpea differential lines used (Navas-Cortés et al., 1998).

Most recently, a study by Chongo et al. (2004) used a set of 8 differentials, 5 kabuli (ILC 4421, FLIP 83-48, ILC 3856, Sanford and UC 27) and 3 desi (ICC 4200, ICC 4475 and ICC 6328) accessions to classify 40 isolates of *A. rabiei* collected mostly in 1998 from commercial chickpea fields in Saskatchewan. The isolates were grouped into 14 pathotypes based on resistant or susceptible reactions of the differentials, with 1 to 8 isolates in each pathotype group.

Udupa et al. (1998) suggested that as few as three differentials from each class (susceptible, moderately resistant and highly resistant) are sufficient for pathotyping *A. rabiei* isolates into 3 pathotypes based on increasing level of aggressiveness. The standard set of differentials used at ICARDA consist of 'ILC 3279' as a resistant genotype, 'ILC 482' as tolerant and 'ILC 1929' as a susceptible genotype (Jamil et al., 2000). Similarly, the United States Department of Agriculture (USDA) uses cultivars 'Dwelley' and 'French White' for pathotyping isolates into Pathotype I and Pathotype II respectively (Chen et al., 2004).

Since the last pathogenicity study on *A. rabiei* isolates in Saskatchewan in 1998 (Chongo et al., 2004), there have been epidemics of the disease on chickpea crops throughout the province, most notably in the growing season of 1999. Prior to this study there was a dramatic increase in ascochyta blight on the cultivar Sanford, which was a popular cultivar among chickpea producers in the mid to late 1990s.

The objectives of this study were to determine if isolates of *A. rabiei* collected in Saskatchewan in 2001 and 2002 were more aggressive than isolates collected prior to 2000, and to evaluate the suitability of a pathotype or race categorization of *A. rabiei* based on the collected data.

## 3.2 Materials and Methods

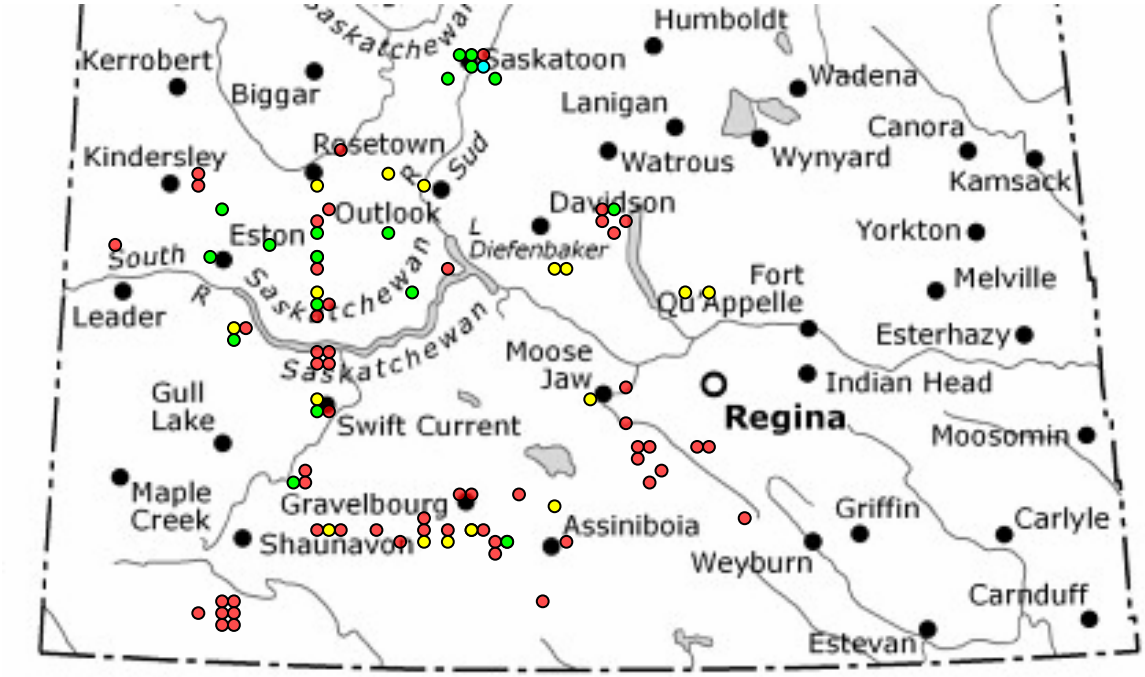
### 3.1.1 Isolate collection, pathogen isolation and isolate selection

An *A. rabiei* population collected in 1998 by Chongo et al. (2004) from 25 chickpea fields throughout Saskatchewan was compared with populations collected in

2001 and 2002. Diseased plant samples were selected from commercial chickpea fields throughout central and south western Saskatchewan in 2001 and 2002 in 21 and 56 fields respectively (Figure 3.1). A hierarchical sampling approach was used where several plants were sampled at 5 locations along a w-shaped transect within 1 acre of the field. At least one isolate from a diseased plant at each sampling location was isolated by placing sterilized infected plant tissue on potato dextrose agar (PDA) (DIFCO, Becton Dickinson & Co.) with 0.1g $L^{-1}$ of Streptomycin Sulfate (EM Science), transferring a single conidiospore from a sporulating culture and growing it on oatmeal agar (OMA). Spores were collected from the matured single-spore cultures and stored in a solution of 5 mg $L^{-1}$ skim milk powder dissolved in 20% glycerol and water in a cryofreezer at -70$^{o}$C. Isolates collected in 1998 and 2001 were cycled through UC 27, a susceptible host, to restore any loss in aggressiveness that may have occurred during storage. The final population of samples collected included 44 isolates from 1997-1999, 108 isolates from 2001 and 189 isolates from 2002 (Appendix 1). Isolates from 1997 to 1999, 2001 and 2002 analyzed in this study will be referred to as the populations of 1998, 2001 and 2002, respectively.

Forty isolates of the 1998 population were previously tested by Chongo et al. (2004) for pathogenic variability (Appendix 6). Thirteen isolates representing the previously tested 40 isolates from 1998, a random selection of 32 isolates from 2001 and a 54 from 2002 were tested for their pathogenic variability (Appendix 1). The 13 isolates representing the 1998 collection was a stratified sample with each isolate representing pathotypes designated by their ability to cause infection on a set of 8 chickpea differentials (Chongo et al., 2004). Comparison of this sub-sample with the larger population showed no significant differences from that of the larger population of 40 isolates tested previously by Chongo et al. (2004) (Appendix 2). Randomly selected isolates from 2001 and 2002 populations represented 18 and 53 fields sampled respectively.

**Figure 3.1**: Location of chickpea fields where *Ascochyta rabiei* isolates were collected in 1998, 2001 and 2002




*Green indicates fields were isolates were collected in 1998, Yellow indicates fields where isolates were collected in 2001 and red indicates fields were isolates were collected in 2002

### 3.1.2 Differential genotypes and inoculum preparation

Based on the differential set used by Chongo et al. (2004), seven differentials comprising three kabuli varieties (Sanford, CDC Frontier and Amit), one kabuli accession (ILC 3856), two desi accessions (ICC 4200 and ICC 4475) and a susceptible kabuli check (UC 27) were used to characterize isolates for aggressiveness and pathotype. Although ILC 4421, FLIP 83-48 and ICC 6328 were used by Chongo et al. (2004), they were not included as differentials in this study because of problems in seed supply and germination. Instead, Amit and Frontier were included, representing the highest level of resistance available in kabuli chickpea grown in Saskatchewan, and were considered resistant checks in this study.

Chickpea seedlings were grown in 10-cm-diameter plastic pots filled with Redi-earth (Terra-Lite Redi-Earth Peat-Lite Mix, Scotts-Sierra Horticultural products

Co., Marysville, OH, USA) and a slow-release granular fertilizer (Osmocote, Scotts-Sierra Horticultural Products Co., Marysville, Ohio, USA) at a rate of 4g per pot, with five seeds planted in each pot. Before inoculation, pots were thinned to 3 plants per pot. Seedlings were grown in a greenhouse at a temperature of approximately 20:16°C (day:night), with a 16-h photoperiod. One control pot per differential was sprayed with sterile distilled water only and included in each block.

Isolates of *A. rabiei* were grown in 5 or 10-cm-diameter Petri dishes on OMA for 7 to 14 days on a laboratory bench at room temperature. Cultures were flooded with sterile distilled water to dislodge conidia. Spore suspensions were diluted to 2 x $10^5$ conidia $mL^{-1}$ and plants were inoculated 21 days after seeding to runoff with an airbrush at a pressure of 40 psi. Plants were placed in a mist chamber for 48 hours post-inoculation.

### 3.1.3 Experimental design

Due to the large number of isolates studied which could not be handled simultaneously and the relative ease of inoculating all differentials at once, a split plot design was used. Isolates were main-plot treatments and the differentials the sub-plot treatments. In order to adjust for variability over time, main plots were arranged in a 10 x 10 simple lattice as described by Cochran and Cox (1992) (Appendix 3). The basic design was repeated twice to give four replications for every isolate-differential combination. Isolates selected for the study were randomly assigned numbers within the experiment (Appendix 1).

### 3.1.4 Disease rating scale

For calculating an isolates' aggressiveness and grouping into pathotypes, disease severity on each plant was assessed using a mixed quantitative and qualitative 0-9 rating scale as described by Chongo et al. (2004) with 0 = no symptoms; 1 = few very small (<2$mm^2$) lesions on leaves and stems, <2% plant area affected (PAA); 2 = very small lesions, 2-5% PAA; 3 = many small lesions (<2 to 5 $mm^2$), 5-10% PAA; 4 = presence of pycnidia, many small lesions, few large (>5$mm^2$) lesions, 10-25% PAA; 5 = many large lesions, 25-50% PAA; 6 = lesions coalescing, 50-75% PAA; 7 =

stem girdling, 75-90% PAA; 8 = stem breakage, >90% PAA; 9 = plant dead. Each plant was rated individually and then averaged over the three plants per pot for a disease value on each experimental unit. Rating was done every 3 days starting 4 days after inoculation for a total of 6 ratings which concluded 19 days after inoculation. Disease rating values for each isolate x differential combination were converted to area under the disease progress curve (AUDPC) for statistical analysis to avoid analyzing ordinal data. In the study by Chongo et al. (2004), disease assessment at 2 weeks after inoculation was used to determine pathotypes. Correlation analysis of rating values 2 weeks after inoculation and the AUDPC for this experiment revealed a correlation that was greater than 0.9, suggesting that the 2-week rating and AUDPC are comparable (data not shown).

### 3.1.5 Statistical analysis

#### 3.1.5.1 Analysis of variability among isolates and between populations

Adjusted least squares mean AUDPC values for each isolate, differential and isolate x differential combination were determined using the PROC MIXED function in SAS (SAS Institute, Cary, NC) (Appendix 4). The model for the main-plot treatment was tested by analyzing the example given in Cochran and Cox (1992) which was a simple 5 x 5 lattice repeated 5 times. Once tested, additional terms were added for the sub-plot treatments and interactions. Estimate statements were included in the SAS program to estimate standard errors of differences between isolate x differential means, between isolates and between differentials (Appendix 4).

Variation due to isolates and their interaction with differentials were further subdivided in terms of differences among isolates within populations (1998, 2001 and 2002) and between populations. The PROC MIXED function in SAS was used to obtain least square means comparing isolate and differential averages among the populations (Appendix 5). Years, differentials, isolates and differential-isolate interactions were considered fixed effects with all remaining effects considered random. Adjusted AUDPC was then analysed as a two-way analysis of variance (ANOVA) in MINITAB (MINITAP INC., State College, PA) to investigate the amount of interaction between differentials and isolates.

#### 3.1.5.2 Analysis of interaction between isolates and differentials

Azzalini and Cox (1984) describe quantitative and qualitative interactions which are of primary interest in studies of host-pathogen reactions. A quantitative interaction occurs when there is an increase in disease severity from one differential to another for one isolate, whereas another isolate causes no difference in disease severity between the same 2 differentials. A qualitative interaction occurs when there is a change in rank of disease reaction of two different isolates on two different cultivars. Quantitative interactions indicate a change in the classification of isolates due to overall aggressiveness, while qualitative interactions indicate a race-type pathogenic structure.

Interactions were considered to be significant if the calculated absolute value was greater than 2 times the standard error that was obtained in PROC MIXED by calculating the interaction effect for Isolates Ar-111-02 and Ar-85-01 on the differentials Amit and Frontier. This randomly selected combination of isolates and differentials were the first to occur in the data set. With a t-value of 2, the test is approximates the 5% significance level. If interactions were significant, the standard deviation between two differentials and the same isolates was multiplied by 3.29, based on Azzalini and Cox (1984), to determine the critical testing value. If the adjusted mean for comparison between two differentials with the same isolate is less than the critical value and comparison with the same two differentials with a different isolate is greater than the critical value, or *vice versa*, the interaction is considered qualitative as described by Azzalini and Cox (1984). All other significant interactions were considered quantitative.

## 3.3 Results

### 3.3.1 Variability between populations

Comparison of mean AUDPCs averaged over all differentials and all isolates for each year revealed that isolates from both 2001 and 2002 caused significantly higher disease levels than isolates from 1998 (Table 3.1). When disease severity on each differential, averaged over all isolates was measured, a significant increase in disease severity was found between 1998 and both 2001 and 2002 in Amit, CDC

Frontier, ILC 3856, Sanford and ICC 4200. There was no significant difference in disease caused by isolates from 2001 *versus* those from 2002 on any of the differentials or averaged over all differentials.

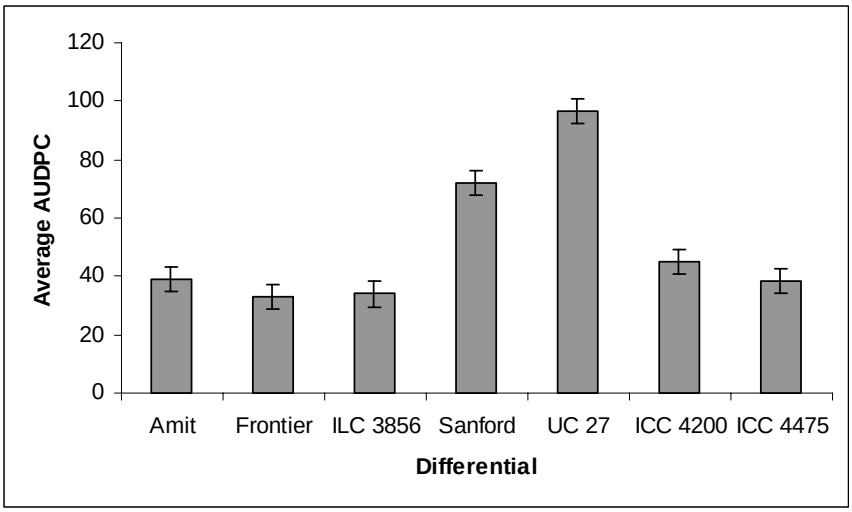
**Table 3.1:** AUDPC values averaged over 99 isolates of *Ascochyta rabiei* collected in 1998, 2001 and 2002, and evaluated on 7 differential chickpea genotypes

| Differential | 1998 | 2001 | 2002 | 2001-1998[1] | 2002-1998[1] | 2002-2001[1] |
|---|---|---|---|---|---|---|
| Amit | 32.2 | 42.2 | 43.3 | 10.0 ± 4.1** | 11.1 ± 3.9** | 1.2 ± 2.7 |
| Frontier | 21.8 | 38.1 | 40.0 | 16.3 ± 4.1** | 18.0 ± 3.9** | 1.8 ± 2.7 |
| ILC 3856 | 24.7 | 39.6 | 37.5 | 14.9 ± 4.2** | 12.8 ± 4.0** | 2.1 ± 2.8 |
| Sanford | 48.0 | 82.7 | 84.6 | 34.7 ± 4.1** | 36.7 ± 3.9** | 2.0 ± 2.7 |
| UC 27 | 99.6 | 96.3 | 94.4 | 3.3 ± 4.1 | 5.1 ± 3.9 | 1.9 ± 2.7 |
| ICC 4200 | 38.6 | 46.9 | 49.7 | 8.4 ± 4.1* | 11.1 ± 3.9* | 2.8 ± 2.7 |
| ICC 4475 | 37.5 | 39.0 | 38.7 | 1.5 ± 4.1 | 1.2 ± 3.9 | 0.3 ± 2.7 |
| **Mean** | 43.2 | 55.0 | 55.4 | 11.8 ± 3.3** | 12.3 ± 3.1** | 0.5 ± 2.2 |

[1] Difference in AUDPC-values in the respective years, standard error of the mean and significance level Note: Standard errors for comparing overall year means have 96 DF; those for comparing group means within differentials have more than 2000 DF; * Significantly different from zero at p = 0.05; ** Significantly different from zero at p = 0.01.

AUDPC-values were averaged over all isolates for each differential to evaluate the level of resistance in each genotype to the total population of *A. rabiei*. Amit, Frontier, ILC 3856 and ICC 4475 all had similar levels of resistance, whereas UC 27 was the most susceptible differential (Figure 3.2). Sanford was less susceptible than UC 27, but significantly more susceptible than the remaining differentials. Ranking of genotypes from resistant to susceptible resulted in the following structure: Frontier/ILC 3856, Amit/ICC4475, ICC 4200, Sanford and UC27 (Figure 3.2).

**Figure 3.2:** AUDPC-values averaged over 99 isolates of *Ascochyta rabiei* collected in 1998, 2001 and 2002 for each of 7 differential chickpea genotypes




### 3.3.2 Variability among isolates

Least-squares means from the mixed model analysis showed highly significant differences between *A. rabiei* isolates, chickpea differentials and the isolate x differential interaction. A two-way analysis of variance in MINITAB (MINITAP INC., State College, PA) was performed on the adjusted AUDPC means. Chickpea differentials accounted for most of the variability (Table 3.2). *Ascochyta rabiei* isolates contributed to variability at a higher level than the error term, suggesting that isolates also contributed to the total variance. The isolate x differential sum of squares had to be approximated because it was included in the error term and was estimated to contribute only 4.4% to the total variability.

To further investigate the highly significant isolate x differential term, a two-way ANOVA was performed on the differentials Frontier and Sanford. These two differentials were selected as Frontier was the most resistant differential and Sanford was the differential which indicated the greatest amount of change between populations. The interaction between isolates and these differentials accounted for only 0.4% of the total variability and isolates accounted for a much higher proportion of the total variability (Table 3.3).

**Table 3.2:** Two-way ANOVA of adjusted means of the area under the disease progress curve (AUDPC) based on disease severity of 7 differential chickpea genotypes inoculated with 99 isolates of *Ascochyta rabiei*

| Source of Variation | DF | Sum of Squares | Sum of Squares (as a % of Total Sum of Squares) |
|---|---|---|---|
| Isolates | 98 | 83701 | 17.5 |
| Differentials | 6 | 336299 | 70.4 |
| Error | 588 | 57338 | |
| *Isolate x Differential*** | | *21255* | *4.4* |
| *Error (adjusted)** | | *36646* | *7.7* |
| **Total** | 692 | 477392 | |

*Error term consists of the isolate x differential interaction plus the adjusted error. Error term is estimated by dividing the residual error by the number of replications (245.46/4 = 61.36), **Estimated adjusted sum of squares for interaction is Error – DF(error)*61.36 = (57338-588*61.36 = 21255)

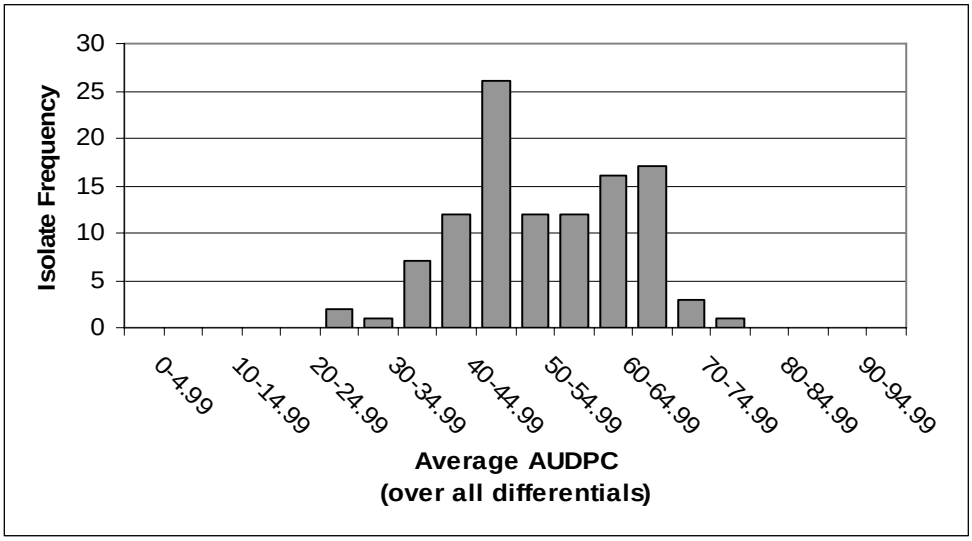
**Table 3.3:** Two-way ANOVA of adjusted means of the area under the disease progress curve (AUDPC) of disease severity on chickpea differentials Frontier and Sanford inoculated with 99 isolates of *Ascochyta rabiei*

| Source of Variation | DF | Sum of Squares | Sum of Squares (as a % of Total Sum of Squares) |
|---|---|---|---|
| Isolates | 98 | 50523 | 34.4 |
| Differentials | 1 | 89235 | 60.8 |
| Error | 98 | 7124 | |
| *Isolate x Differential*** | | *520* | *0.4* |
| *Error (adjusted)** | | *6604* | *4.5* |
| **Total** | | 146882 | |

*Error term consists of the isolate x differential interaction plus the adjusted error. Error term is estimated by dividing the residual error by the number of replications (269.57/4 = 67.39), **Estimated adjusted sum of squares for interaction is Error – DF(error)*67.39 = (7124.5-98X67.39 = 520)

The average adjusted AUDPC over all differentials for each isolate was calculated. The frequency distribution of these AUDPC values revealed a continuous distribution (Figure 3.3). Similarly, the frequency distribution of AUDPC for each individual differential revealed a continuous distribution (Figure 3.4).

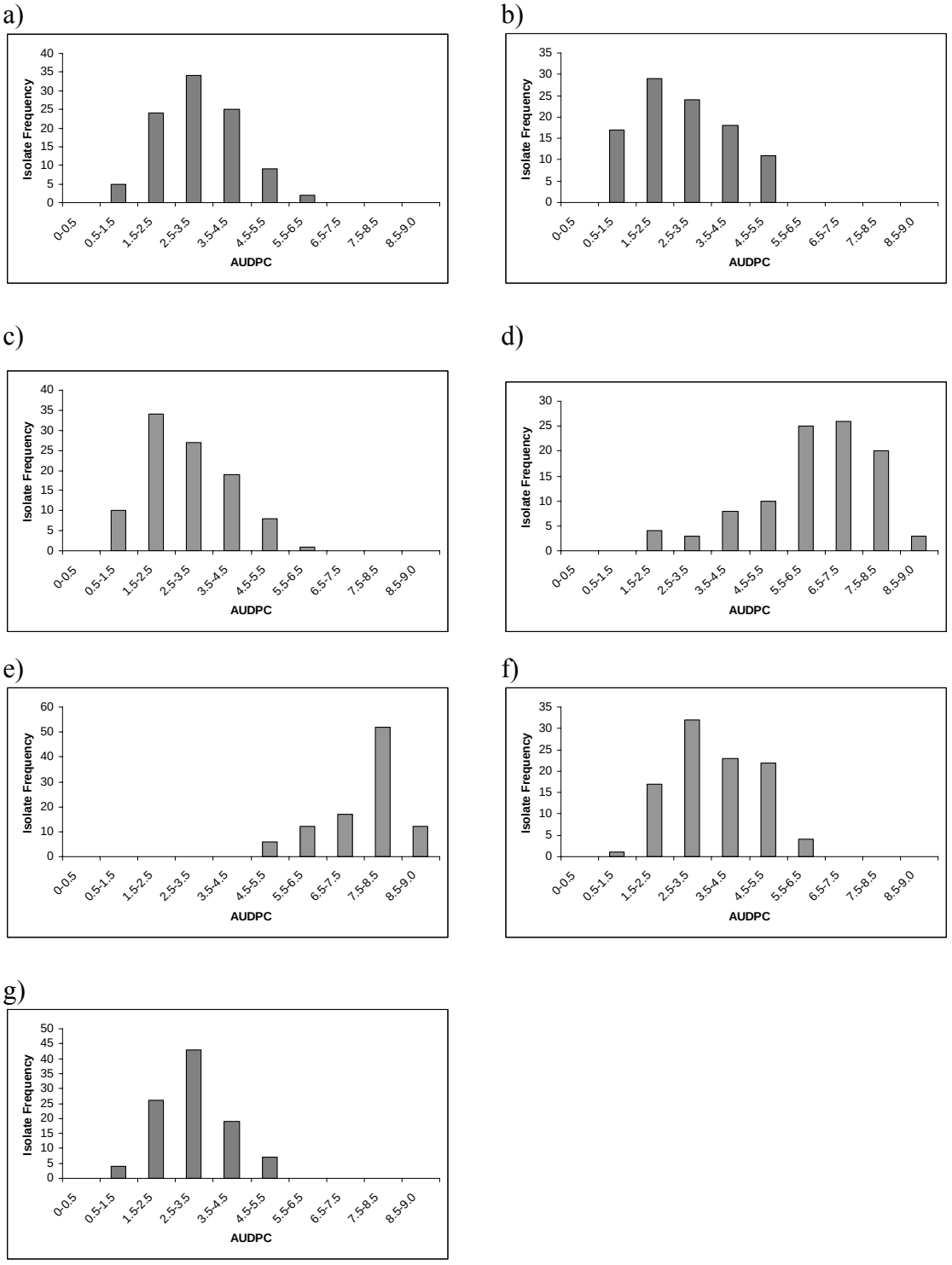
**Figure 3.3:** Frequency distribution of AUDPC of 99 isolates of *Ascochyta rabiei* averaged over all 7 chickpea differentials




### 3.3.3 Interaction Between Isolates and Differentials

The standard error of the difference between isolates Ar-111-02 and Ar-85-01 tested on Amit and Frontier was 15.8, thus interaction effects with an absolute value greater than 2 x 15.8 = 31.60 were considered significant in the 2 x 2 interaction tables. Of the possible 101 871 2 x 2 interactions, 90 554 or 88.89% showed no significant interaction, 11 316 or 11.11% showed significant quantitative interaction as described by Azzalini and Cox (1984) and 1 or <0.01% showed significant qualitative interaction. Since the frequency of qualitative interactions was far below the significance level used in the test (5%), it can be concluded that there was very little evidence for significant changes in rank in this data set.

**Figure 3.4:** Frequency distribution of AUDPC of 99 isolates of *Ascochyta rabiei* on 7 chickpea differentials




a) Amit; b) CDC Frontier; c) ILC3856; d) Sanford; e) UC 27; f) ICC 4200; g) ICC 4475.

## 3.4 Discussion

Greater overall disease severity caused by more recently collected isolates suggest that the pathogenicity of the population of *A. rabiei* has shifted and may continue to do so to more aggressive types in Saskatchewan. This is most likely due to selection for isolates with higher aggressiveness due to the use of genotypes with increased resistance. The commercially grown cultivar Sanford showed the greatest reduction of resistance due to changes in the fungal population. The increase in the severity of disease caused by isolates from 2001 and 2002 compared to 1998 on Sanford suggest the population of *A. rabiei* in Saskatchewan renders Sanford susceptible regardless of environmental conditions or disease management strategies. Widespread cultivation of the cultivar in Saskatchewan selected for isolates of *A. rabiei* able to overcome the resistance genes and mechanisms in Sanford.

Results from this study suggest that it is not possible to separate isolates of *A. rabiei* into physiological races or pathotypes based on susceptible or resistant reaction of each isolate x differential combination. If such a relationship occurred, it would be expected that the interaction between isolates and differentials would account for a much higher proportion of the total variation, especially when only the most resistant and a susceptible cultivar were considered. Furthermore, a continuous response in disease reaction over all isolates and differentials suggested disease resistance was quantitative in nature, and should be discussed in terms of levels of resistance in host genotypes and levels of aggressiveness in pathogen isolates. Further investigation showed very few significant interactions and only one qualitative interaction, which does not support a race structure in the *A. rabiei* population.

There are contrasting views on the diversity and stability of pathotypes or races of *A. rabiei* (Malik and Rahman, 1992; Udupa et al., 1998). Numerous pathogenicity tests of isolates world-wide have not provided evidence of race specificity and the lack of a genotype with complete resistance supports this hypothesis (Latif et al., 1993). Several pathogenicity studies showed that isolates identified as aggressive caused more disease on some chickpea genotypes than others and that some genotypes were more susceptible to all isolates of *A. rabiei* (Latif et al., 1993). In contrast, Reddy and

Kabbabeh (1985) classified 50 isolates into 6 races with race 6 the most aggressive and race 1 the least aggressive. A later study by Weising et al. (1991) found a different order of aggressiveness for the same isolates used by Reddy and Kabbabeh (1985) under different growth conditions (polyethylene house *versus* growth-chamber, respectively) and with a different set of differentials (Weising et al., 1991). Though it is possible that changes in ranking of the isolates in these studies support the hypothesis of race structure or genotype specificity in individuals in that particular population of *A. rabiei*, the change in ranking may more likely be due to differences in environmental conditions or methodology as much as to the different response to chickpea genotypes. Therefore, differences between the differentials and isolates that changed ranking may not have been significant.

Lack of distinct race structure has also been found in other *Ascochyta* species. Ahmed et al. (1996) used a differential set of 10 lentil genotypes to classify 84 *A. lentis* isolates. Although differential x isolate interactions were significant, the sum of the mean square values was only about 2.6% of the sum of mean square values due to differentials and isolates. Similarly, Wroth (1998) tested 99 isolates of *Mycosphaerella pinodes* on 10 differential field pea genotypes and found that it was not possible to separate isolates into distinct pathotypes due to a small variance component contributed by the isolate x differential interaction. Furthermore, a continuous distribution ranging from less aggressive to more aggressive isolates was found (Wroth, 1998), similar to the present study. Therefore, a strong argument can be made that it is not possible to separate isolates of *Ascochyta* species into distinct races or pathotypes.

These results suggest that pyramiding of multiple major and minor genes in commercial cultivars should improve resistance to ascochyta blight in chickpea crops, although it is possible that a genetically diverse outbreeding *A. rabiei* population may be able to overcome pyramided resistance within cultivars. The results also imply that future changes in the population of *A. rabiei* to greater aggressiveness can be expected as new cultivars, such as Frontier, are more widely grown. Thus, ongoing monitoring of the population with regular re-testing of released cultivars to confirm resistance status is imperative to manage resistance sources. The significance of seeking new

and different sources of resistance in chickpea to *A. rabiei* is also demonstrated in this study as it was shown how quickly an aggressive population of the pathogen could overcome resistance, exemplified by Sanford. Proactive resistance breeding for quick delivery of improved resistance is important in order to maintain production to supply export markets.

From this study, we can conclude that screening for ascochyta resistance in chickpea lines close to cultivar release is best done with a mixture of isolates or the most aggressive isolate found in a recent population of the pathogen. This would ensure that the resistance will remain effective against populations of the pathogen found in the field. However, the continuous variation in aggressiveness among isolates and the quantitative nature of resistance in chickpea to *A. rabiei* implies that only a moderately aggressive isolate should be used in selections of resistant parents for crosses to increase the likelihood of new effective combinations of resistance genes within a cultivar. New combinations of minor, additive resistance genes would hopefully provide increased resistance to that found in parental lines plus optimize the genetic background for other agronomic and quality-related traits.

# CHAPTER 4

## MOLECULAR VARIABILITY AND MATING TYPE DISTRIBUTION OF *ASCOCHTYA RABIEI* ON CHICKPEA IN SASKATCHEWAN

### 4.1 Introduction

Molecular analysis of *A. rabiei* DNA has been used most extensively to classify isolates genetically as well as quantify genetic variation within populations of the pathogen. Cluster analyses of RAPD data have shown that isolates of *A. rabiei* are generally grouped according to geographic origin among countries and within Canada (Santra et al., 2001; Chongo et al., 2004). There have also been studies that attempted to correlate molecular data to aggressiveness or pathotypes in order to hasten pathogenicity studies. For example, isolates from Pathotype III (most aggressive) were found to have less genetic diversity than Pathotype I (least aggressive) isolates suggesting that because of adoption of resistant cultivars there was selection pressure on populations of *A. rabiei* (Udupa et al., 1998). Molecular techniques are being used to map the genome of *A. rabiei*, so that genes responsible for aggressiveness can be more accurately marked (Lichtenzveig et al., 2002). Most recently, molecular markers for *A. rabiei* have been developed as a quarantine tool to avoid importation of infected seed in Australia (Phan et al., 2002).

Varying degrees of polymorphism have been detected using RAPD fingerprinting in a range of studies. Sarwar et al. (2000) used 27 RAPD primers to screen 4 isolates of *A. rabiei* from Pakistan and of those, 14 primers showed strong amplification and 3 to 16 polymorphic bands per primer. Santra et al. (2001) classified 37 Indian, 5 American, 3 Syrian and 2 Pakistani isolates with 48 polymorphic RAPD markers. Cluster analysis grouped these isolates by geographic origin. With only 3 primers selected from 14 previously screened, 30 Italian isolates

of *A. rabiei* revealed a high degree of genetic variation with each isolate showing a unique RAPD pattern (Fischer et al., 1995). Fifty-three Syrian isolates were screened with 14 RAPD primers producing 78 polymorphic loci and a single microsatellite probe/enzyme combination which defined 18 haplotypes (Udupa et al., 1998). Genetic similarity of isolates was correlated with pathogenic data for the same 53 isolates and genetic variation within each pathotype was determined. Some authors have found RAPD markers to be a reproducible, stable form of fingerprinting DNA of *A. rabiei*. For example, Fischer et al. (1995) found that RAPD patterns were used to re-identify isolates of *A. rabiei* successfully even when there was a long storage period. On the other hand, many studies have found low levels of polymorphism in RAPD markers. For example, Lichtenzveig et al. (2002) screened 232 RAPD primers and found that only 22 (9.5%) resulted in reproducible polymorphic bands.

*Didymella rabiei* is heterothallic with a bipolar, biallelic mating system (Wilson and Kaiser, 1995). Sexual reproduction is controlled by a single regulatory locus referred to as the mating type (MAT) locus as in other ascomycete fungi (Coppin et al., 1997; Nelson, 1996; Turgeon, 1998). Alternate sequences at the MAT locus are completely dissimilar and code for a regulatory gene (Metzenberg and Glass, 1990). A DNA binding protein containing an alpha domain is coded by MAT 1, or *MAT1-1*, and a DNA binding protein that contains a high mobility group domain is coded by *MAT1-2* (Coppin et al., 1997; Turgeon, 1998; Turgeon et al., 1993).

Traditionally, MATs in *A. rabiei* have been determined by crossing isolates with MAT-tester isolates (Armstrong et al., 2001; Kaiser and Kusmenoglu, 1997; Wilson and Kaiser, 1995). Conventional laboratory testing for mating type is tedious and time consuming, thus a MAT-specific, multiplex PCR assay was developed by Barve et al. (2003). Primers Com1 (common flanking primer), SP21 and Tail 5 were designed to amplify different size PCR fragments from *A. rabiei MAT1-1* and *MAT1-2* isolates (Appendix 7). The combination of Com1/SP21 amplifies about 400 bp of the *MAT1-1* idiomorph while the primers Com1/Tail 5 amplify about 700 bp of the *MAT1-2* idiomorph.

Kaiser (1997) reported world-wide distribution of the two MATs (*MAT 1-1* and *MAT 1-2*) in nature. When testing for mating type, Navas-Cortés et al. (1998)

found a 1:1 ratio of mating types in a sample of 17 isolates of *A. rabiei* collected in Spain and 7 isolates from the USA. Likewise, Barve et al. (2003) found an equal distribution of mating types in 2 different fields from the Pacific Northwest of the United States, and Kaiser and Okhvat (1996) found a close to 1:1 ratio in mating type in 9 isolates from Iran. In contrast, 5 isolates from India and 8 isolates from Pakistan, tested by Navas-Cortés et al. (1998) were exclusively *MAT 1-1* and a small population of isolates from each Greece, Italy and Morocco were all *MAT 1-2*. Populations of isolates from different fields and years in the US Pacific Northwest were analyzed for MAT by Peever et al. (2004). They found that 16 isolates from 1983 were exclusively *MAT1-1*, while other field populations from 1984, 1987, and 1998 through 2000 all had approximately equal proportions of each mating type. Kaiser and Küsmenoglu (1997) found that both MATs were present in a population of 145 *A. rabiei* isolates collected from 23 provinces in Turkey, but the population had a significantly higher frequency of *MAT 1-1* isolates. Navas-Cortés et al. (1998) suggested that there may be slightly greater selection for survival of one mating type over the other in some locations as was seen in the Turkish population studied by Kaiser and Küsmenoglu (1997). Even though only *MAT 1-1* has been identified in Australia, a natural occurrence of *D. rabiei* on chickpea stubble suggests both mating types are present (Galloway and MacLeod, 2003).

Initial studies on the frequency of *MAT1-1* and *MAT1-2* of *A. rabiei* in the chickpea growing regions of Saskatchewan in 1997 to 1999 found that both MATs were present and occurred at similar frequencies (Armstrong et al., 2001). They also found that isolates of both MATs were present on chickpea residue from the same area and occasionally from the same field.

The objective of this molecular diversity study was i) to determine if the 2002 population of *A. rabiei* in Saskatchewan has diverged genetically from the 1998 population; ii) to test the hypothesis of a 1:1 MAT ratio in 1998, 2001 and 2002 populations of the pathogen; and iii) to determine if the population is in equilibrium indicating that the population is outbreeding.

## 4.2 Materials and methods

### 4.2.1 DNA extraction

Two different extraction methods were tested on isolates of *A. rabiei*. DNA from 179 isolates (Appendix 6) was extracted with a mini preparation of the cetyltrimethyl-ammonium bromide (CTAB) method described below (4.2.1.1) and DNA from 25 isolates (Appendix 6) was extracted with the rapid fungal extraction method (4.2.1.2). A preliminary test comparing approximately 10 of the same isolates extracted with the different methods using number RAPD primers and the *MAT*-specific primers was performed, and did not show any difference in banding patterns, the quality or intensity of the bands. However, after screening a larger numbers of isolates by RAPD, we determined that the DNA extracted with the rapid extraction method may have resulted in less reliable banding patterns with more failed reactions. The ratio of readings at $OD_{260}/OD_{280}$ was used to determine the purity of the nucleic acid (4.2.1.3). When DNA of each extraction method was compared, the rapid fungal extraction method resulted in lower quality DNA compared to the CTAB method (Table 4.1). Furthermore, the $OD_{260}/OD_{280}$ of the isolates from the 1998 population used in the RAPD analysis was lower than those from 2002 used and the 2002 isolates were closer to the desired 1.8 value for ratios of readings at $OD_{260}/OD_{280}$.

**Table 4.1:** Comparison of ratios of readings at $OD_{260}/OD_{280}$ used to determine the purity of the nucleic acid for CTAB and rapid fungal extraction methods in 1998 and 2002 populations of *Ascochyta rabiei*

| Extraction Method | 1998 | | 2002 | |
|---|---|---|---|---|
| | # of isolates | Ratio | # of isolates | Ratio |
| CTAB | 18 | 1.91 | 126 | 1.86 |
| Rapid | 25 | 1.50 | 0 | n/a |
| Total | 43 | 1.67 | 126 | 1.86 |

#### 4.2.1.1 Genomic mini-prep CTAB extraction method

Single-spore isolates of *A. rabiei* were grown at room temperature in 100 mL flasks containing 50 mL of potato dextrose broth (DIFCO, Becton Dickinson & Co.)

on an orbital incubating shaker (LSI-2005RL, Lab Tech) at 120 rpm and 25°C. Mycelia from 5 to 7-day-old cultures were harvested by filtration and stored in 2 mL vials at -80°C. When needed, mycelia were freeze-dried for three days and pulverized with the help of two, 2 mm diameter plastic beads (VWR International). DNA was extracted following a modified mini-preparation protocol described by Chongo et al. (2004) based on the CTAB method. Extraction buffer [5M NaCl (Omni Pur, EMD), 10% CTAB (Sigma Chemical Company), 1.0M Tris (pH 8.0) (Omni Pur, EMD), 0.5M EDTA (pH 8.0) (EM Science), 1% β-mercaptoethanol (Omni Pur, EMD)] was added to pulverized mycelia, incubated, and DNA was extracted twice with chloroform (Omni Pur, EMD) and isoamyl alcohol (Omni Pur, EM Science) prior to precipitation with isopropanol (VWR International). Extracted DNA was washed with sodium acetate [1/10 vol. 3M NaOAc (BDH Biochemicals)] and 2.5 vol. 95% EtOH, and suspended in 50 µL deionized distilled $H_2O$.

#### 4.2.1.2 Rapid fungal DNA extraction method

A rapid fungal DNA extraction method with some modifications described by Cenis (1992) was used on 25 isolates (Appendix 6). Single-spored isolates of *A. rabiei* were grown in a 2 mL tube with 500 µL of potato dextrose broth (DIFCO, Becton Dickinson & Co.) for 72 hours at 25°C. An amount of 300 µL of SDS extraction buffer [200mM Tris HCl pH 8.5 (Omni Pur, EMD), 250 mM NaCl (Omni Pur, EM Science), 25 mM EDTA (EM Science), 0.5% SDS (BDH Biochemicals)] was added to each tube and ground for 4 minutes. DNA was precipitated by adding Sodium Acetate [3M (BDH Biochemicals)], pelleted by centrifugation, and washed with 500 µL of TE buffer [10mM Tris pH 8.0 (Omni Pur, EMD), 1mM EDTA pH 8.0(EM Science)]. An equal volume of isopropanol (VWR International) was added to re-precipitate DNA which was re-washed with 70% ethanol. After drying, DNA was dissolved in 10 µL TE Buffer [10mM Tris pH 8.0 (Omni Pur, EMD), 1mM EDTA pH 8.0(EM Science)].

#### 4.2.1.3 DNA quantification

In order to determine the quantity and quality of DNA, wavelengths were read at 260 nm and 280 nm with a spectrophotometer (Spectra Max 190, Molecular Devices). The ratio between the readings ($OD_{260}/OD_{280}$) was used to determine the purity of the nucleic acid and DNA concentrations were adjusted to 10ng $\mu L^{-1}$ and stored at -20$^{o}$C.

### 4.2.2 RAPD fingerprinting

#### 4.2.2.1 Isolate selection

Thirty randomly selected isolates from the 1998 population and thirty from the 2002 population were selected for molecular analysis (Appendices 1 and 6).

#### 4.2.2.2 Amplification and electrophoresis

DNA of each isolate was amplified in 20 µL of a solution made up of 1X Tris-Acetate (TAE) buffer (Invitrogen Life Technologies), 2.5 mM $MgCl_2$ (Invitrogen Life Technologies), 0.2 mM dNTPs each (Invitrogen Life Technologies), 1 Unit Taq DNA polymerase (Invitrogen Life Technologies), 0.2 µm primer and 10 ng genomic DNA. Amplifications was performed with a PTC-200 Peltier Thermal Cycler (MJ Research) with an initial denaturation step for 5 min at 94$^{o}$C, followed by 35 cycles of 30s at 94$^{o}$C, 30s at 37$^{o}$C and 30s at 72$^{o}$C, with a final extension for 7 min at 72$^{o}$C.

Amplified products were separated by electrophoresis in a 1% or 1.5% agarose gel (Invitrogen Life Technologies) in 1X TAE buffer [4.8g $mL^{-1}$ Tris (Omni Pur, EMD Science), Glacial acetic acid (Omni Pur, EMD), 0.5M EDTA pH 8.0)]. In each gel, a 1-Kb Plus DNA ladder (Invitrogen Life Technologies) was used as a molecular reference marker. Ethidium bromide (Omni Pur, EM Science) was mixed with the dissolved agarose at a concentration of 0.2µg $mL^{-1}$. Fragments of DNA were visualized and photographed under transmitted ultra-violet light in black and white prints in a Multimage$^{TM}$ Light Cabinet (Alpha Innotech Corporation).

#### 4.2.2.3 Primer screening

Fifty-five random primers (Appendix 8) were screened using a sub-sample of the *A. rabiei* populations tested. Only 19% of the DNA fragments amplified were polymorphic even though 38 of the primers screened were selected because they were polymorphic in other populations of *A. rabiei* in laboratories around the world. Eighteen primers, which produced 50 potentially repeatable polymorphic bands, were tested on the population of 60 isolates (Table 4.2). Assuming that each RAPD primer detected 0.2% of the *A. rabiei* genome (Fischer et al., 1995), then 3.6% of the genome was covered in the present study assuming amplified bands did not overlap. Low levels of polymorphism were found from primers that had previously been used to classify the 1998 population of *A. rabiei* used in this study by Chongo et al. (2004).

#### 4.2.2.4 Data analysis

For each isolate and primer combination, a binary number system was used to manually score the bands, with 0 indicating the absence and 1 the presence of a band, regardless of band intensity. Amplification reaction failure or smearing of a particular band was scored as missing data (coded by 3). The binomial data matrix was used to construct a genetic similarity matrix using Dice's genetic similarity coefficient (Dice, 1945) by the SIMQUAL command in NTSYS-PC program (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1994). The resulting similarity data was analyzed by cluster analysis using unweighted pair-group method with arithmetic means (UPGMA) of the SAHN program in NTSYS-PC (Rohlf, 1994). The cophenetic correlation coefficient between the dendrograms and the Dice's similarity matrices were calculated and Mental's t-test for the correlation was tested with 1000 random permutations.

**Table 4.2:** Primers selected for RAPD fingerprinting on *Ascochyta rabiei* from 55 primers screened

| Primer | Primer Sequence | Total Loci Amplified | Polymorphic Loci Amplified |
|---|---|---|---|
| BioBasic S68[1] | 5'-TGGACCGGTG-3' | 7 | 4 |
| OP A9*[2] | 5'-GGGTAACGCC-3' | 10 | 4 |
| OP B17[2] | 5'-AGGGAACGAG-3' | 10 | 5 |
| OP C01[3] | 5'-TTCGAGCCAG-3' | 6 | 2 |
| OP E05[1] | 5'-TCAGGGAGGT-3' | 3 | 1 |
| OP I18[2] | 5'-TGCCCAGCCT-3' | 4 | 3 |
| OP J15[4] | 5'-TGTAGCAGGG-3' | 9 | 4 |
| OP K08[2] | 5'-GAACACTGGG-3' | 8 | 4 |
| OP L08[2, 4] | 5'-AGCAGGTGGA-3' | 11 | 5 |
| OP L15[2] | 5'-AAGAGAGGGG-3' | 9 | 2 |
| OP L18[1] | 5'-ACCACCCACC-3' | 5 | 1 |
| OP M03[1] | 5'-GGGGGATGAG-3' | 8 | 4 |
| OP N02[2] | 5'-ACCAGGGGCA-3' | 6 | 4 |
| UBC-702**[5] | 5'-GGGAGAAGGG-3' | 8 | 1 |
| UBC-708[5] | 5'-GGGTTGTGGG-3' | 8 | 1 |
| UBC 726[5] | 5'-GGTGTGGGTG-3' | 6 | 1 |
| UBC-727[5, 6] | 5'-GGGTGTGGTG-3' | 5 | 1 |
| UBC 756[6] | 5'-CCCTCCTCCT-3' | 4 | 3 |
| Total | | 127 | 50 |

[1] Primers selected randomly, [2] Primers previous used by Lichtenzveig et al. (2002), [3] Primers previous used by Navas-Cortés et al. (1998), [4] Primers previous used by Udupa et al. (1998), [5] Primers previous used by Chongo et al. (2004), [6] Primers previous used by Santra et al. (2001)

*Source: Operon Primers (OP), Operon Technologies Inc. (Alameda, CA), **Source: University of British Columbia (UBC), University of British Columbia (Vancouver, BC)

Genetic diversity indices were estimated and analysis of molecular variances was performed with Arlequin ver. 2.0 (Schneider et al., 2000). A permutation procedure using the euclidean square distances matrix, based on the pair-wise difference method was performed in order to provide significance tests for each variance component. A pair-wise genetic distance matrix was calculated for each pair of species using the parameter $F_{st}$ (Reynolds et al., 1983). The significance of the $F_{st}$ values was tested with 1000 permutations (Excoffier et al., 1992). Gene flow was

estimated from the $F_{st}$ value with the following formula: $0.5(1- F_{st})/ F_{st}$ (Schneider et al., 2000). Also using Arlequin ver 2.0 (Schneider et al., 2000), linkage disequilibrium was calculated.

### 4.2.3 Mating-type distribution

#### 4.2.3.1 Isolate selection

From stock DNA solutions of 191 isolates of *A. rabiei* populations collected in 1998, 2001 and 2002, MAT was determined using the MAT-specific PCR (Barve et al., 2003). Tested isolates included 35 collected in 1998, 30 collected in 2001 and 126 collected in 2002.

#### 4.2.3.2 Amplification and electrophoresis

Reactions were made up of 30 ng DNA template, 400 nM of each primer (Tail 5, Com1 and SP21), 2.5µL 10X Tris-Acetate (TAE) buffer (Invitrogen Life Technologies), 1.5 mM $MgCl_2$ (Invitrogen Life Technologies), 25 µM dNTPs each (Invitrogen Life Technologies), 1 U Taq DNA polymerase (Invitrogen Life Technologies) and were adjusted to a volume of 25 µL with sterile double de-ionized $H_2O$. Amplifications were performed with a PTC-200 Peltier Thermal Cycler (MJ Research) with an initial denaturation step of 95$^o$C for min, followed by 44 cycles of 95$^o$C for 30 s, 60$^o$C for 30 s each and 72$^o$C for 1 min, with a final extension of 72$^o$C for 5 min. Amplified products were separated by electrophoresis on a 1.2% agarose gel (Invitrogen Life Technologies) as described in section 4.2.2.3.

#### 4.2.3.3 Data analysis

Chi-square test values were calculated to test for a 1:1 ratio of mating type frequencies in all field samples, from all years and on 30 random samples from each year. The probability of a greater chi-square value under the null hypothesis of a 1:1 ratio was also calculated. When only two classes were tested for goodness of fit with small sample sizes (ie. less than 200), the probability of a Type I error (the probability of rejecting the null hypothesis when it is true) is greater (Bowley, 1999). To accommodate the lower sample sizes from each population, the test statistics in this

study were adjusted with Yates correction of continuity so the test statistic would more closely approximate the Chi-square distribution, thus reducing the chance of a Type I error.

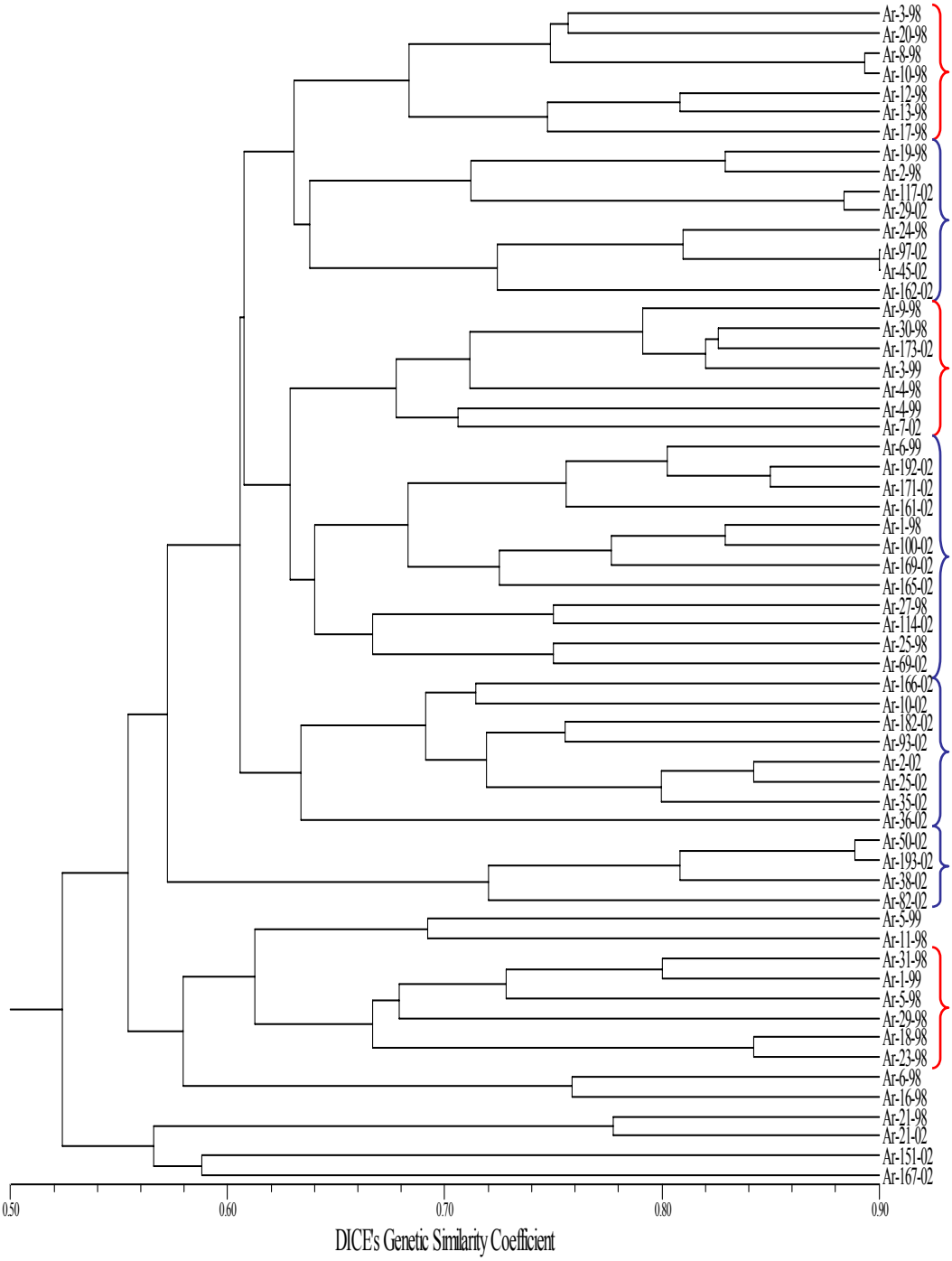
## 4.3 Results

### 4.3.1 Similarity of isolates of *Ascochyta rabiei*

The dendrograms constructed by the UPGMA cluster analysis based on Dice's genetic similarity coefficient is given in Figure 4.1. The cophenetic correlation coefficient between the dendrogram and Dice's similarity matrices was 0.55. The approximate Mental's t-test for the correlation was significant ($P<0.01$).

Similarity levels among all isolates ranged from 52 to 90%. Four isolates (167-02, 151-02, 21-02, 21-98) were most distinct with 52% similarity to all other isolates. None of the isolates tested with RAPD primers had identical banding patterns. One pair of isolates had 90% similarity (97-02/45-02) and 3 pairs of isolates were about 89% similar, one pair from 1998 (8-98/10-98) with the other 2 pairs from 2002 (29-02/117-02 and 50-02/193-02). The pair of isolates collected in 1998 with similar genotypes were collected approximately 20 km apart, but the pairs collected in 2002 were collected 225, 160 and 130 km apart, respectively. Isolates collected from the same field clustered at various levels of similarity. For example, isolates collected from the same field clustered at 52% to 62% similarity (2-98/3-98, 6-98/21-98, 29-98/30-98/31-98, 93-02/97-02, 114-02/117-02,161-02/162-02, 165-02/166-02/167-02/169-02, 171-02/172-02 and 192-02/193-02), while 3 isolates collected in 1998 from the same area clustered at 60% similarity (9-98/10-98/19-98). Only 2 pairs of isolates collected from the same field were more than 62% similar (8-98/20-98 at 75% similarity and 3-99/4-99 at 66% similarity). Isolates collected in different years from the same area of the province clustered at less than 60% similarity (45-02/50-02/25-98 and 16-02/93-02/97-02/100-02). Isolates collected in 2002 from different fields within the same area also shared 55 to 60% similarity (45-02/50-02 and 93-02/97-02/100-02).

**Figure 4.1**: Dendrogram of similarity of 30 isolates of *Ascochyta rabiei* collected in 1998 and 30 Isolates collected in 2002




*Red indicates isolates from the 1998 population and blue indicates isolates from the 2002 population

At 52% similarity (Figure 4.1) two major clusters composed of 46 and 10 isolates existed with the larger cluster having 27 isolates from the 2002 population and 19 isolates representing the 1998 population. The smaller cluster of 10 isolates was comprised entirely of 1998 isolates. Isolates of *A. rabiei* tended to group together primarily with isolates from the same year at 64% similarity. These groups of isolates from 1998 were found and 4 distinct groups primarily composed of isolates mostly from the 2002 population were evident. A group of 6 isolates all from 1998 was found at 66% similarity. This was the largest group at the highest similarity level of isolates from the same population.

### 4.3.2 Molecular variability

Analysis of molecular variance (AMOVA) showed that inter-population variability accounted for more than 90% of the total variation, whereas variability between the 1998 and 2002 populations was very low and accounted for only 6.7% of the variability observed (Table 4.3). The corresponding $F_{st}$ value was 0.067 ($P<0.001$) and the coancestry coefficient was 3.48 ($P<0.001$). A coancestry coefficient value of greater than or equal to 1.0 indicates there is little differentiation and the populations are prevented from diverging due to genetic drift (Phan et al., 2003b). By calculating the mean number of pairwise differences within both populations, it was shown that the genotypic variability of each population was equal between 1998 and 2002 (Table 4.4) and the corrected average pairwise difference value between the populations was 1.07 ($P<0.001$). The average gene diversity over loci was equal between the two populations as gene diversity is not affected by recombination (Milgroom, 1996; McDonald, 1997). Linkage disequilibrium was calculated for 1998 and 2001 populations and was found to be very low at 8.16% and 10.94% over 46 and 50 loci respectively ($P=0.05$). The values obtained for gene diversity in this study (Table 4.4) were very close to gene diversity values obtained by Phan et al. (2003b), suggesting adequate levels of genetic variation were detected to make accurate conclusions in this study.

**Table 4.3:** Analysis of molecular variance for RAPD analysis of *Ascochyta rabiei* populations from 1998 and 2002

| Source of Variation | DF | Sum of Squares | Variance Components | % Total Variation |
|---|---|---|---|---|
| Among Populations | 1 | 22.97 | 0.54 | 6.75 |
| Within Populations | 56 | 415.48 | 7.42 | 93.25 |
| Total | 57 | 438.45 | 7.96 | |

* $F_{st}$ = 0.067, P <0.001

**Table 4.4:** Mean number of pairwise differences and average gene diversity over loci for RADP polymorphisms of 1998 and 2002 populations of *Ascochyta rabiei*

| Population | 1998 | 2002 |
|---|---|---|
| Mean number of pairwise differences | 13.50 ± 6.24 | 16.27 ± 7.48 |
| Average gene diversity over loci | 0.27 ± 0.14 | 0.33 ± 0.17 |

### 4.3.4 Mating-type distribution

The MAT frequency among isolates was similar in the populations of 1998 and 2001, whereas the MAT frequency among isolates collected in 2002 showed a significant shift towards a higher frequency of *MAT1-1* in the population (Table 4.5). Skewing of the ratio due to the larger population size tested for 2002 was evaluated by randomly sub-sampling (and re-testing) 30 isolates from that year as well as from 1998 and 2001 (Appendices 1 and 6) and it was found that there was no significant difference from 1:1 of both MATs (Table 4.6).

**Table 4.5:** Mating type ratios in field samples from 1998, 2001 and 2002 of *Ascochyta rabiei* determined with MAT-specific PCR

| Sample[a] | N[b] | Ratio[c] | $\chi^{2d}$ | P[e] |
|---|---|---|---|---|
| 1998 | 35 | 16:19 | 0.114 | 0.735 |
| 2001 | 30 | 14:16 | 0.033 | 0.855 |
| 2002 | 126 | 77:49 | 5.786 | 0.016 |
| All Years | 191 | 107:84 | 2.534 | 0.111 |

[a] Samples obtained from commercial chickpea fields in respective years, [b] Number of isolates analyzed [c] *MAT1-1:MAT1-2*, [d] $\chi^2$ value for test of 1:1 ratio with Yates correction of continuity, [e] Probability of a greater $\chi^2$ value under the null hypothesis of 1:1 ratio

**Table 4.6:** Mating type ratios in 30 random samples each from 1998, 2001 and 2002 of *Ascochyta rabiei* determined with MAT-specific PCR

| Sample[a] | N[b] | Ratio[c] | $\chi^{2d}$ | P[e] |
|---|---|---|---|---|
| 1998 | 30 | 14:16 | 0.033 | 0.855 |
| 2001 | 30 | 14:16 | 0.033 | 0.855 |
| 2002 | 30 | 18:12 | 0.833 | 0.361 |
| All Years | 90 | 46:44 | 0.011 | 0.916 |

[a] Samples obtained from commercial chickpea fields in respective years, [b] Number of isolates analyzed [c] *MAT1-1:MAT1-2*, [d] $\chi^2$ value for test of 1:1 ratio with Yates correction of continuity, [e] Probability of a greater $\chi^2$ value under the null hypothesis of 1:1 ratio

## 4.4 Discussion

Results from both the cluster analysis (Figure 4.1) and analysis of molecular variance (Table 4.3) indicated that the 2002 population of *A. rabiei* in Saskatchewan was similar to that of 1998. Furthermore, genetic variation, as indicated by mean number of pairwise differences and average gene diversity over loci (Table 4.4), was equal between the two populations and both populations were in gametic equilibrium. When populations are undergoing regular random mating, it is expected that a 1:1 ratio of mating types and a low level of linkage disequilibrium are present (Milgroom, 1996). Furthermore, if a population is made up of recombined genotypes, there will be little or no phylogenetic consistency (Milgroom, 1996) as was observed in this study. All of the results from this study suggested the 1998 and 2002 populations of *A. rabiei* in Saskatchewan were mating randomly. This is supported by the presence of equilibrium in MAT ratios and equal diversity between the populations suggests.

It was thought that the population of *A. rabiei* in Saskatchewan was exposed to high selection pressure through widespread cultivation of the partially resistant variety 'Sanford' prior to the growing season in 2000. As a result the population was suggested to have changed in terms of its genetic variation and its aggressiveness. However, it is evident from the results of this study that a genetic shift between populations did not occur. Peever et al. (2004) observed a shift in the population of *A. rabiei* between historical and contemporary populations from the Pacific Northwestern US, but the historical population were from prior to 1990 and contemporary populations were from 1998 to 2000. Similarly, 33 of 35 isolates classified as Pathotype III by Udupa et al. (1998) were shown to cluster separately at 70% similarity compared to 12 isolates of Pathotype I and 2 isolates of Pathotype II. Since a shift in the population most likely occurs gradually over time, the distance between the 1998 and 2002 populations may not have been great enough to see the anticipated shift in this study.

Very few genetic population studies on *A. rabiei* have analyzed genetic diversity and linkage disequilibrium in populations, or connected MAT distribution with population structure and diversity. In fact, the objectives of most studies utilizing molecular markers were to compare isolates or populations from different lesions, plants, fields, regions of a country, continents or examine world-wide collections, whereas this study analyzed population diversity over a period of years. It is possible that high levels of genetic variation are present within each field, as Morjane et al. (1994) observed in field populations of *A. rabiei* in Tunisia. Field-level variation was not addressed in this study as the primary goal was to compare populations collected over the entire chickpea growing region of Saskatchewan in different years; still the analysis provided evidence for a high degree of genetic diversity within a single field.

Genetic similarity between populations calculated through cluster analysis has been utilized extensively. Navas-Cortés et al. (1998) found that 33 of 39 isolates from chickpea producing regions world-wide produced uniquely different patterns with only 5 RAPD primers. At about 90% similarity, the 39 isolates were grouped into 10 clusters mostly based on country of origin. Similarly, Fischer et al. (1995) used only 3 primers to distinguish 30 isolates from Italy of *A. rabiei* genetically, but found that the

phylogeny of the population could not be resolved. Santra et al. (2001) found 2 major clusters among 37 isolates from India with approximately 50% similarity between clusters, and the genetic similarity of most of the isolates were suggested to correlate with area of origin within the country.

No correlation of genetic similarity of the isolates with area of collection was evident in this study, as the most similar pair of isolates originated from locations more than 200 km apart. Furthermore, isolates collected from the same field usually clustered at 50% to 60% similarity with the maximum being 75% similarity found in only one pair of isolates. Isolates tested by Santra et al. (2001) were from a larger geographical area in India, a country which has a much longer history of chickpea production than Canada. Thus it was a population that was much more established and likely to be at equilibrium. Furthermore, differences in dispersion of air-borne ascospores, plant debris and use of seed infected with low levels of *A. rabiei* could play a large role in the mixing of local populations in Saskatchewan due to differing farming practices, climatic and geographical variables. For example, strong winds often prevail over the province without major geographic barriers to isolate chickpea growing regions. As well, plant debris is generally not incorporated into the soil due to the widespread muse of conservation tillage methods. Thus, debris and ascospores may be blown from the field of origin.

Phan et al. (2003b) found that 5 isolates of the 1998 *A. rabiei* population used in this study (Appendix 6) had greater gene diversity than isolates from Syria, the USA or different regions of Australia when analyzed with 19 STMS primer pairs which amplified 20 loci with a total of 76 alleles. Populations that have evolved over a longer time at one location are expected to have greater gene diversity (McDonald, 1997), in which case populations from northern Syria and south-eastern Turkey should have the largest gene diversity as chickpea is native to these areas (VanDerMaesen, 1987). The proportionally larger gene diversity in the Canadian isolates tested by Phan et al. (2003b) may suggest that *A. rabiei* was introduced from various sources of diversity. Chickpea has only been cultivated in Canada since the early 1990s, which most likely was insufficient time for the generation of new alleles through mutation, or accumulation of new alleles at frequencies that can be detected (McDonald, 1997).

Furthermore, since there was no change in gene diversity from 1998 to 2002 it may be assumed that genetically different isolates have not been introduced to the population during that time. Therefore, a recent introduction of more aggressive isolates of the disease from different countries is unlikely the reason for increased aggressiveness in the population.

Although not significant, the average gene pairwise differences between isolates was greater in 2002 suggesting genotypic variability may be increasing, due to sexual recombination. Peever et al. (2004) found that a single multilocus genotype of *A. rabiei* was found in the Pacific Northwest of the USA in a sample of isolates from 1983, yet by 1987 all alleles currently found in the population of *A. rabiei* were present. The Saskatchewan isolates tested by Phan et al. (2003b) were most closely related to a population of 6 isolates from Washington State in the USA. A possible explanation is that cultivars developed for the Pacific Northwest of the USA were grown in Saskatchewan before cultivars specifically adapted to the Canadian prairies were developed, and there is frequent exchange of material between breeding programs from the Pacific Northwest and Saskatchewan and commercial seed has been imported to Canada from this region thus providing a potential source of *A. rabiei* through seed.

Similar to Phan et al. (2003b), Santra et al. (2001) found that isolates from different countries (Syria, Pakistan and the USA) clustered separately from the Indian population, with the population from the USA being the most different from that of India based on RAPD primer analysis. Geistlinger et al. (1997b) also found that isolates from the USA clustered separately from isolates from the 'old world' (Morocco, Tunisia, Syria and Pakistan) at about 65% similarity when 268 polymorphic microsatellite markers were derived from 10 microsatellite oligonucleotide probes in combination with 2 enzyme digestions. In the same study, isolates from Pakistan and Morocco branched apart at 70% similarity and at about 74% similarity most of the Syrian isolates could be separated from Tunisian isolates. However, only a few isolates from each region were used in that study as the primary objective was to find maximum genetic diversity to cross isolates for mapping purposes. Overall, most genetic diversity studies performed on *A. rabiei* populations

to date have problems associated with the sampling methods. In order to properly assess genetic diversities within populations, random, equal, unbiased selection strategies with appropriate number of isolates should be used to ensure results are not skewed and are meaningful or significant.

Milgroom (1996) suggests that population sizes in many studies are not nearly as high as they should be, especially for detecting gametic disequilibrium in fungi. McDonald (1997) recommended that sample sizes for populations of fungi should be 30 to 100 individuals to obtain reliable information. McDonald and Martinez (1990) found that 50 to 60 isolates are needed from a single field to detect maximum genetic variation in population studies on *Magnaporthe graminicola*, thus the number of isolates from each population used in this study may not have been large enough to draw valid conclusions. Testing for linkage or gametic disequilibrium generally requires unlinked genetic markers, large sample sizes from a population of hundreds or thousands of isolates, and an appropriate sampling methodology. Any violation of these assumptions can cause a population in equilibrium to appear as one that is not (Milgroom, 1996; McDonald, 1997). An alternative to estimating linkage disequilibrium in small population sizes is determining Index of Association ($I_A$). The $I_A$ is a test for associations among loci that indicates the proportion of the population derived through sexual recombination *versus* asexual reproduction. Peever et al. (2004) suggested that $I_A$ might be a more precise and appropriate method of testing for recombination and equilibrium with small sample sizes than linkage disequilibrium analysis as frequencies of rare alleles and alleles near fixation are known to affect linkage disequilibrium estimates.

The loci scored in the current study seemed to be unlinked although the sampling methodology of the isolates tested for linkage disequilibrium may not have been appropriate as there were no inter-field isolate populations of significant size. Population sizes larger than what was tested may have resulted in population differentiation as was hypothesized. A large sample size from the population is needed so that clones will not be over represented (McDonald, 1997), although no identical haplotypes occurred in the populations tested in this study. It is more likely that clones would have been detected in the present study if more sampling occurred at

the field and plant level. In order to properly determine the degree of variability contributed to by sexual recombination in Saskatchewan populations, sampling on a much smaller spatial scale would have to have been employed. Furthermore, analyzing progeny from naturally occurring pseudothecia for segregation would give a further indication of the degree of variability due to recombination (Milgroom, 1996).

Determining gene diversity between different regions world-wide with appropriate population sample sizes would be an interesting indication of variability in the world-wide population of *A. rabiei* as different countries have different histories of growing chickpeas. Population structures of *A. rabiei* are affected by the length of time the pathogen has been established, the use of different sources of resistance genes and the genotypes of isolates contaminating seed imported for germplasm development in breeding programs.

The 2002 population had a higher frequency of *MAT1-1* as the null hypothesis of a 1:1 ratio of mating types was rejected ($P<0.01$). Populations ranging in size from 145 isolates to as low as 5 isolates have been considered adequate to determine mating type ratio in populations by Kaiser and Küsmenoglu (1997) and Navas Cortés (1998), respectively. When the Turkish mating type ratios (Kaiser and Küsmenoglu, 1997) were re-analyzed (by this author) to adjust for low sample numbers, the null hypothesis was rejected ($P<0.05$). This suggests that higher numbers of isolates need to be sampled in order to estimate the true mating type ratio, especially since several random sub-samples of 30 isolates of the 2002 population failed to reject a null hypothesis of a 1:1 mating type ratio (data not shown).

A bias in frequency towards one *MAT* could be an indication of the population changing. Alternatively, it may reflect that *MAT1-1* has a competitive advantage over *MAT1-2*, or it could indicate that the mating type gene is linked to a gene (or group of genes) that does. Unfortunately, no studies have been conducted to date that have investigated the correlation between MAT and aggressiveness of *A. rabiei*. The unequal frequency in mating types also suggests that there is less sexual recombination occurring in the population. Linkage disequilibrium increased slightly from 1998 to 2002 (8.2% and 10.9%, respectively) which may suggest there is less sexual production of ascospores in more recent populations. Re-testing of genetic

diversity and mating type frequencies in Saskatchewan populations of isolates in the future may confirm the effect of uneven mating type distributions on population diversity or dynamics, possibly using different tests for outbreeding such as $I_A$.

Overall, in countries where both mating-types have been discovered, there is little evidence of a clonal population structure suggesting that random mating occurs, as was seen in this research. One of the only major chickpea growing countries in the world where only one mating-type has been found is Australia where Phan et al. (2003b) found very low levels of diversity as compared to other populations throughout the world. However, this could be short-lived as *D. rabiei* has recently been discovered on chickpea stubble in Australia suggesting sexual reproduction and thus that the second mating-type most likely is present in the population and could lead to recombination resulting in more aggressive isolates of the disease (Galloway and MacLeod, 2003). Additionally, Phan et al. (2003b) found that there was no departure from equilibrium and that gene flow was very high in 36 isolates from 4 different regions in Australia, suggesting that the sexual cycle of *A. rabiei* occurs frequently. It takes relatively low frequencies of recombination to produce a population structure that has the appearance of random mating (Milgroom, 1996), thus low levels of the alternate mating type may be present in Australia, although not discovered yet.

## CHAPTER 5

### GENERAL DISCUSSION

The population of *A. rabiei* in Saskatchewan was shown to have increased in aggressiveness from 1998 to the 2001/2002 growing season, but no genetic shift was detected. Isolates of 2001 and 2002 caused significantly more disease on differentials used in pathogenicity testing, most notably on the cultivar 'Sanford' which was considered a resistant cultivar prior to epidemic levels of ascochyta blight encountered in the 1999 growing season by chickpea producers in Saskatchewan. Genetic similarity and molecular variation suggest that populations of *A. rabiei* collected in 1998 and 2002 were similar in genetic diversity with very little indication of genetic divergence between the populations. MAT ratios of each population was 1:1 when an equal number of isolates were chosen at random from each population. This implies the population is out-breeding, and therefore has the potential to generate genetic diversity through sexual recombination.

Interpretation of results from the molecular diversity portion of this project were difficult due to small sample sizes and low numbers of polymorphic loci. These factors resulted in a poor correlation between data and dendrogram results. Discovery of more polymorphic loci would have increased the dependability of the results, although levels of variability reported in populations tested with specific RAPD primers used in other molecular diversity studies on *A. rabiei* could not be reproduced. There is a good possibility that many reported studies on *A. rabiei* populations did not have stringent enough PCR conditions resulting in complex banding patterns that may not have reflected true genetic variability in populations. For example, several studies had annealing temperatures more than 10$^{o}$C lower than the annealing temperature used in the present study. However, it is also possible that RAPD primers used in other

studies detected more polymorphism as there was more genetic diversity within the populations studied.

A larger population sample for the molecular diversity study (ie. more than 100 isolates from each year) would have increased the reliability of the results from this study. As indicated by the difference in results on the 2002 population mating type study, a population of 30 random isolates was not large enough to reflect true ratios of loci occurring in the overall population. However, testing an appropriate sample size from the 1998 population was not possible as there were only 44 isolates available, many of which could not be re-isolated at the onset of the pathogenicity study of this project. Future populations of *A. rabiei* in Saskatchewan should be collected with the objective of acquiring more than 200 isolates per season representing the population.

Although no change in genetic variation was observed between the 1998 and 2002 populations, a comparison between 2001 and 2002 with larger population sizes may have revealed the degree of change between subsequent growing seasons and indicated how rapidly the population changes in response to agronomic practices currently employed. Through studying a large population in the future and comparing it to the 2002 population, the effect of an uneven mating type ratio on the genetic structure of the population might be determined. Furthermore, with larger population sizes, variability at a smaller spatial scale could be determined in order to properly determine the intra- and inter-field variation. By studying the population at this scale, a more accurate indication of the degree of variability derived from sexual reproduction occurring in the field would be found and would allow a more accurate estimate of other aspects of populations such as gene flow between fields and regions. Variability due to recombination would also be reflected by analyzing the genetic diversity in isolates derived from ascospores by collection of mature pseudothecia in early spring or from crosses made between isolates under laboratory conditions.

Determining gene diversity between different chickpea growing regions world-wide with appropriate population sample sizes would provide an interesting indication of the variability present in the global population of *A. rabiei* as different countries have different histories of growing chickpeas. It would be especially interesting to compare gene frequencies in Saskatchewan with Syria as it is the country of origin for

chickpea. This would indicate if precautions should be taken to avoid further importation of infected seed that may contain *A. rabiei* isolates with alleles not currently present in Saskatchewan. It is likely that gene diversity was also generated through mutation, although the rate at which mutation occurs in *A. rabiei* has not been documented.

The use of microsatellite markers, such as STMS used by Geistlinger et al. (2000), Phan (2003b) and Peever et al. (2004) seems to provide more accurate, reproducible results for molecular analysis than the RAPD markers that were used in this study. Thus, future studies on molecular diversity in populations of *A. rabiei* should consider using this fingerprinting method. Another marker system, Inter-Simple Sequence Repeats (ISSR), has no recorded use in *A. rabiei*. It is possible that using this marker technique with common microsatellite motifs found in the genome of *A. rabiei* as primers could detect more genetic variation. This would be advantageous as electrophoresis with this marker system is technically easier than STMS markers. This study also showed that the rapid fungal DNA extraction method resulted in less reliable banding patterns, thus in future studies, DNA should be extracted from *A. rabiei* using a CTAB method. In fact, all 40 isolates from the 1998 population had DNA extracted with the rapid extraction method and only 30 of those isolates produced banding patterns clear enough to be scored.

Populations of *A. rabiei* are often classified as pathotypes, although results from this study suggest this classification system may be misleading when studying the interaction between *A. rabiei* and *C. arietinum*. The only instance when it would be appropriate to classify aggressive races is when massive clonal propagation results in one or a few genotypes at high frequency, thus introducing discontinuities into the population (Caten, 1987). As shown by both the pathogenic and genetic results of this study, it was evident that the populations of *A. rabiei* were heterogeneous in both phenotype and genotype and have been since 1998. Additionally, frequency distribution of AUDPC over all isolates studied and each individual year were continuous with normal or near normal distribution supporting the hypothesis of multigenic control of resistance to the disease. Very few population studies on *A. rabiei* have used as large a sample size as in the pathogenicity study of this project.

Often through sampling errors, discontinuities may arise and falsely suggest the existence of discrete groups, races or pathotypes, yet larger sample sizes will reveal a continuous nature of the disease (Caten, 1987). Thus, smaller sample sizes used in other studies on populations of *A. rabiei* may have indicated a bimodal distribution although it was not detected in this study. Furthermore, *A. rabiei* does not produce symptoms that allow discernment of physiologic races. Usually a clear compatible or incompatible reaction must occur in the host tissue, such as a hypersensitive reaction (Caten, 1987).

There was very little evidence of specific pathogen-genotype interaction even when the interaction effects were calculated. This means that isolates of *A. rabiei* in the studied populations are more appropriately described in terms of differing aggressiveness rather than belonging to a particular pathotype, race or virulence form. As described by VanDerPlank (1982), if most of the variability is due to the host cultivars in an ANOVA, resistance can be considered race-non-specific and if the pathogen isolates account for a high degree of variability, the isolates can be considered to differ in aggressiveness in a race-non-specific resistance system. In contrast, if in ANOVA the interaction between the isolates and the cultivars account for the largest portion of the variance, race-specific resistance can be assumed and isolates can be referred to by their virulence or avirulence and classified into races or pathotypes. In pathosystems exhibiting race-specific resistance, it is most likely that the gene-for-gene hypothesis applies.

A pathogen population inappropriately studied for occurrence of physiological races may not detect important information regarding the frequencies of isolates with a particular aggressiveness (Caten, 1987). This may be the case with the three tiered pathotype system currently employed by many breeding programs worldwide for isolate classification and resistance gene mapping purposes. Moreover, host differential sets required for physiologic race classification may be of no practical relevance if they do not reflect the resistance factors in use in the field (Caten, 1987).

Testing the 54 isolates representing the 2002 population for pathogenicity in a smaller experiment under more controlled conditions with the set of differentials used to determine Pathotypes I, II and III (Udupa et al., 1998; Jamil et al., 2000; and Chen

et al., 2004) may have revealed more specific host-pathogen interactions. However, judging by the literature on previous studies of this pathogen and on the nature of the necrotropic fungi-host interaction, the results would most likely strengthen the argument against a pathotype classification system for populations of *A. rabiei*. The 3-tiered pathotype system appears to be robust in literature, but the author of this study believes it is due to biased population selection resulting in discontinuous distribution frequency as isolates are selected from only extreme levels of aggressiveness.

Conclusions from this project may have been stronger if various aspects of the design had been changed. The rating scale used in the pathogenicity testing was a mixed qualitative and quantitative scale that was poorly defined, very ambiguous and difficult to interpret and normalize. The Horsfall-Barratt (1945) scale may have been more appropriate emphasizing the quantitative nature of disease resistance to ascochyta blight should have been used. In order to properly assess pathogenic differences among populations, random samples from each population would have been suitable. Nonetheless, lack of significant differences between the stratified sample and the entire 1998 population suggest the results would not have been affected by sampling approaches.

Currently, it is unknown what chickpea growing region in the world contains the most aggressive population of *A. rabiei*. Through the exchange of breeding material and the use of disease nurseries with international entries, the relative disease potential can only be estimated. It was assumed that the differentials Amit and CDC Frontier would represent the most resistant sources of germplasm worldwide, yet significant disease was caused on both cultivars by several isolates from the 2002 population. This suggests that isolates with aggressiveness corresponding to 'pathotype III', the most aggressive isolates, may be present in the current population of *A. rabiei* in Saskatchewan. Knowing the relative disease-causing potential of major populations could be useful to breeders for the purpose of germplasm exchange for disease resistance. A random collection of equal numbers of isolates from each major chickpea growing region (Saskatchewan, the Pacific North-Western US, Australia, Syria, Turkey, India and Italy) tested in the same experiment under controlled conditions would answer this question. It is expected that Syria, Turkey and India

would have populations with the greatest aggressiveness, as chickpeas originated in Syria and Turkey and have been grown extensively in India.

If aggressiveness in *A. rabiei* is truly a quantitative trait controlled by many genes of individually small effect or polygenic control, the resulting progeny of crosses between isolates of differing aggressiveness should have a continuous distribution of levels of aggressiveness (Caten, 1987). Several $F_1$ populations of crosses between isolates tested for aggressiveness and mating type in this study have been made in the Pulse Crop Pathology Laboratory and await testing for aggressiveness on cultivars with different levels of resistance to test this hypothesis.

Conceptually, the gene-for-gene model could be extended to pathosystems involving multiple host-specific toxins where both quantitative and qualitative aspects of diseases are taken into consideration. A necrotropic host-parasite relationship has been described by Lamari et al. (2003) and Strelkov and Lamari (2003) for *Pyrenophora tritici-repentis* causing tan spot in wheat. Single dominant independently inherited genes in the host control sensitivity to related toxins. Three toxins in isolates of *P. tritici-repentis* have been identified, one which causes necrosis and is encoded by a single copy of that particular gene in the pathogen, and another 2 toxins which cause chlorosis on different cultivars and are controlled by two different corresponding genes. Recognition of the toxin by the host receptor leads to compatibility (susceptibility) rather than recognition leading to incompatibility (resistance) as in the traditional gene-for-gene system. This model extends the gene-for-gene concept to pathosystems involving multiple host-specific toxins and proposes that compatibility results from the unique interaction between pathogen-produced toxins and receptors in the host.

No correlation between symptomology (such as lesion type, size, placement on plant, amount of pycnidial development associated with toxins), in response to specific toxins produced by *A. rabiei* have been identified. However, a specific relationship between the amount of toxin produced by *A. rabiei* and aggressiveness of isolates has been documented (Latif et al., 1993; Kaur, 1995). It is a possibility that a similar model to tan spot on wheat may apply to this pathosystem as heterogeneity in toxin profiles of different isolates has been identified (Latif et al., 1993). By analyzing

isolates with differing aggressiveness on cultivars with different levels of resistance using High Pressure Liquid Chromatography (HPLC) and other biochemical profiling techniques, different solanpyrones and/or other toxins might be detected and potentially a relationship between pathogen aggressiveness and cultivar resistance could be established. If specific toxins are produced by particular isolates, it is possible that genes controlling toxin production in *A. rabiei* could be identified by crossing different isolates and analyzing toxin production in sexual progeny and subsequent asexual generations. Most research on resistance in chickpea to toxins produced by *A. rabiei* have hypothesized that resistance occurs through mechanisms suppressing toxin production (Platerosanz and Fuchs, 1978; Höhl et al., 1991) or differing toxin thresholds and detoxification mechanisms (Johal and Briggs, 1992). However, the possibility of toxin receptors being a source of susceptibility has not been explored. If physiological mechanisms associated with detoxification could be identified, the search for resistance to *A. rabiei* in chickpea could utilize a functional genomics. This would allow for identification of specific toxin receptor genes, and therefore marker genes could be identified and used for screening breeding lines for potential resistance.

In the chickpea breeding program at the Crop Development Centre, breeding efforts continue to focus on multigenic control of the disease, as well as attempts to introgress resistance genes from wild cultivars into cultivated chickpea. Cultivars such as CDC Frontier are an example of an increased resistance resulting from the breeding program. An extensive project identifying sources of resistance in numerous breeding lines and resistance from novel sources in order to incorporate resistance and map resistance genes is currently underway (Warkentin et al., 2004). The chickpea breeding program has utilized the results of this study for resistance screening and breeding for ascochyta blight resistance. Disease resistance screening has been simplified based on the results of this project as only one aggressive isolate is now used to determine resistance levels in indoor studies.

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# APPENDICES

**Appendix 1:** Isolate numbers and names of *Ascochyta rabiei* collected in 1997, 1998, 1999, 2001 and 2002, location of origin, use in pathogenicity testing, RAPD analysis and mating-type studies

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] |
|---|---|---|---|---|---|---|---|
| 1997 | Ar-1-97 | Jan 9702 | Saskatoon | | | *MAT1-1* | |
| 1997 | Ar-2-97 | Feb 9710 | | | | *MAT1-1* | |
| 1997 | Ar-3-97 | Mar 9703 | Saskatoon | | | *MAT1-1* | |
| 1998 | Ar-1-98 | Jan 9803 | | | X | *MAT1-2* | X |
| 1998 | Ar-2-98 | Jan 9804 | Saskatoon | | X | *MAT1-1* | X |
| 1998 | Ar-3-98 | Jan 9805 | Saskatoon | 19 | X | *MAT1-1* | X |
| 1998 | Ar-4-98 | Feb 9803 | | | X | *MAT1-1* | X |
| 1998 | Ar-5-98 | May 9804 | Elrose | | X | *MAT1-2* | X |
| 1998 | Ar-6-98 | Jul 9803 | Swift Current | 81 | X | *MAT1-2* | X |
| 1998 | Ar-7-98 | Jul 9804 | Elrose | 13 | | *MAT1-1* | X |
| 1998 | Ar-8-98 | Jul 9805 | Goodale | 53 | X | *MAT1-1* | X |
| 1998 | Ar-9-98 | Jul 9806 | Preston | | X | *MAT1-1* | X |
| 1998 | Ar-10-98 | Jul 9807 | Preston | | X | *MAT1-1* | X |
| 1998 | Ar-11-98 | Jul 9808 | Bickleigh Rd | | X | *MAT1-2* | X |
| 1998 | Ar-12-98 | Jul 9809 | Plato | 46 | X | *MAT1-1* | X |
| 1998 | Ar-13-98 | Jul 9810 | Eston | 83 | X | *MAT1-2* | X |
| 1998 | Ar-14-98 | Jul 9811 | Kyle | | | *MAT1-2* | X |
| 1998 | Ar-15-98 | Jul 9812 | Kyle | 54 | | *MAT1-2* | X |
| 1998 | Ar-16-98 | Jul 9813 | Kyle | 24 | X | *MAT1-1* | X |
| 1998 | Ar-17-98 | Jul 9814 | Sanctuary | | X | *MAT1-2* | X |
| 1998 | Ar-18-98 | Jul 9818 | Brock | | X | *MAT1-2* | X |
| 1998 | Ar-19-98 | Aug 9803 | Preston | | X | *MAT1-1* | X |
| 1998 | Ar-20a-98 | Sep 9804 | Goodale | | X | *MAT1-1* | X |
| 1998 | Ar-20b-98 | Sep 9803 | | | | *MAT1-2* | X |
| 1998 | Ar-20c-98 | Sep 9805 | | | | *MAT1-1* | X |
| 1998 | Ar-21-98 | Sep 9806 | Swift Current | | X | *MAT1-2* | X |
| 1998 | Ar-22-98 | Oct 9804 | Vanscoy | 59 | | *MAT1-2* | X |
| 1998 | Ar-23-98 | Oct 9805 | Dinsmore | 75 | X | *MAT1-2* | X |
| 1998 | Ar-24-98 | Oct 9806 | Cabri | | X | *MAT1-1* | X |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 1998 | Ar-25-98 | Oct 9808 | Imperial | | X | *MAT1-2* | X | |
| 1998 | Ar-26-98 | Oct 9809 | Imperial | 61 | | | | |
| 1998 | Ar-27-98 | Nov 9803 | | | X | *MAT1-2* | X | |
| 1998 | Ar-28-98 | Nov 9805 | Demaine | | | *MAT1-2* | X | |
| 1998 | Ar-29-98 | Nov 9806 | Demaine | | X | *MAT1-1* | X | |
| 1998 | Ar-30-98 | Nov 9807 | Demaine | 65 | X | *MAT1-1* | X | |
| 1998 | Ar-31-98 | Nov 9808 | Demaine | | X | *MAT1-2* | X | |
| 1998 | Ar-32-98 | Dec 9804 | Kyle | | | | | |
| 1998 | Ar-33-98 | Oct 9811 | USDA+D328 | | | | | |
| 1999 | Ar-1-99 | Jan 9907 | | | X | *MAT1-1* | | |
| 1999 | Ar-2-99 | Feb 9914 | Elrose | | | *MAT1-2* | | |
| 1999 | Ar-3-99 | Feb 9923 | Simmie | 5 | X | *MAT1-1* | | |
| 1999 | Ar-4-99 | Feb 9924 | Simmie | | X | *MAT1-1* | | |
| 1999 | Ar-5-99 | Mar 9901 | Limerick | | X | *MAT1-1* | | |
| 1999 | Ar-6-99 | Mar 9912 | | | X | *MAT1-2* | | |
| 2001 | Ar-1-01 | Ar1S1P2 | Kincaid | | | *MAT1-1* | | |
| 2001 | Ar-2-01 | Ar1S1P3 | Kincaid | 17 | | *MAT1-2* | | |
| 2001 | Ar-3-01 | Ar1S1P5 | Kincaid | | | *MAT1-2* | X | |
| 2001 | Ar-4-01 | Ar1S3P2 | Kincaid | | | *MAT1-1* | | |
| 2001 | Ar-5-01 | Ar1S3P2-1 | Kincaid | | | *MAT1-1* | | same lesion* |
| 2001 | Ar-6-01 | Ar1S3P2-2 | Kincaid | 35 | | *MAT1-2* | X | same lesion |
| 2001 | Ar-7-01 | Ar1S3P2-3 | Kincaid | | | *MAT1-1* | | same lesion |
| 2001 | Ar-8-01 | Ar1S3P2-4 | Kincaid | 22 | | *MAT1-1* | | same lesion |
| 2001 | Ar-9-01 | Ar1S3P2-5 | Kincaid | | | *MAT1-2* | X | same lesion |
| 2001 | Ar-10-01 | Ar1S3P2-6 | Kincaid | | | *MAT1-1* | | same lesion |
| 2001 | Ar-11-01 | Ar1S3P2-7 | Kincaid | | | *MAT1-1* | | same lesion |
| 2001 | Ar-12-01 | Ar1S3P2-8 | Kincaid | | | *MAT1-2* | X | same lesion |
| 2001 | Ar-13-01 | Ar1S3P2-9 | Kincaid | | | *MAT1-2* | X | same lesion |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2001 | Ar-14-01 | Ar1S3P2-10 | Kincaid | | | *MAT1-1* | | same lesion |
| 2001 | Ar-15-01 | Ar1S3P4 | Kincaid | | | *MAT1-2* | | |
| 2001 | Ar-16-01 | Ar2S1P1 | Ponteix | | | *MAT1-2* | | |
| 2001 | Ar-17-01 | Ar2S1P3 | Ponteix | | | *MAT1-1* | | |
| 2001 | Ar-18-01 | Ar2S1P4 | Ponteix | | | *MAT1-1* | X | |
| 2001 | Ar-19-01 | Ar2S1P4-1 | Ponteix | | | *MAT1-1* | | same lesion |
| 2001 | Ar-20-01 | Ar2S1P4-2 | Ponteix | | | *MAT1-1* | X | same lesion |
| 2001 | Ar-21-01 | Ar2S1P4-3 | Ponteix | | | *MAT1-2* | | same lesion |
| 2001 | Ar-22-01 | Ar2S1P4-4 | Ponteix | | | *MAT1-1* | X | same lesion |
| 2001 | Ar-23-01 | Ar2S1P4-5 | Ponteix | | | *MAT1-1* | X | same lesion |
| 2001 | Ar-24-01 | Ar2S1P4-6 | Ponteix | 31 | | *MAT1-1* | | same lesion |
| 2001 | Ar-25-01 | Ar2S1P4-7 | Ponteix | 80 | | *MAT1-2* | | same lesion |
| 2001 | Ar-26-01 | Ar2S1P4-8 | Ponteix | | | *MAT1-1* | | same lesion |
| 2001 | Ar-27-01 | Ar2S1P4-9 | Ponteix | | | *MAT1-1* | | same lesion |
| 2001 | Ar-28-01 | Ar2S1P4-10 | Ponteix | | | *MAT1-1* | X | same lesion |
| 2001 | Ar-29-01 | Ar2S2P1 | Ponteix | | | *MAT1-2* | X | |
| 2001 | Ar-30-01 | Ar2S3P1 | Ponteix | | | *MAT1-2* | | |
| 2001 | Ar-31-01 | Ar2S3P2 | Ponteix | 39 | | *MAT1-1* | | |
| 2001 | Ar-32-01 | Ar2S3P3 | Ponteix | | | | | |
| 2001 | Ar-33-01 | Ar2S3P4 | Ponteix | | | *MAT1-2* | | |
| 2001 | Ar-34-01 | Ar2S3P5 | Ponteix | | | *MAT1-1* | X | |
| 2001 | Ar-35-01 | Ar3S1P2 | Moose Jaw | 7 | | *MAT1-1* | X | |
| 2001 | Ar-36-01 | Ar3S1P3 | Moose Jaw | | | *MAT1-1* | X | |
| 2001 | Ar-37-01 | Ar3S1P4 | Moose Jaw | | | *MAT1-2* | | |
| 2001 | Ar-38-01 | Ar3S1P4-1 | Moose Jaw | | | *MAT1-2* | | same lesion |
| 2001 | Ar-39-01 | Ar3S1P4-2 | Moose Jaw | 60 | | *MAT1-2* | | same lesion |
| 2001 | Ar-40-01 | Ar3S1P4-3 | Moose Jaw | | | | | same lesion |
| 2001 | Ar-41-01 | Ar3S1P4-4 | Moose Jaw | | | *MAT1-2* | | same lesion |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2001 | Ar-42-01 | Ar3S1P4-5 | Moose Jaw | 90 | | *MAT1-2* | | same lesion |
| 2001 | Ar-43-01 | Ar3S1P4-6 | Moose Jaw | | | *MAT1-2* | | same lesion |
| 2001 | Ar-44-01 | Ar3S1P4-7 | Moose Jaw | | | *MAT1-2* | | same lesion |
| 2001 | Ar-45-01 | Ar3S2P3 | Moose Jaw | | | *MAT1-2* | X | |
| 2001 | Ar-46-01 | Ar3S2P4 | Moose Jaw | | | *MAT1-2* | | |
| 2001 | Ar-47-01 | Ar3S2P5 | Moose Jaw | | | *MAT1-1* | | |
| 2001 | Ar-48-01 | Ar3S3P2 | Moose Jaw | 37 | | *MAT1-2* | | |
| 2001 | Ar-49-01 | Ar3S3P3 | Moose Jaw | | | *MAT1-2* | | |
| 2001 | Ar-50-01 | Ar3S3P5 | Moose Jaw | | | *MAT1-2* | | |
| 2001 | Ar-51-01 | Ar4S1P1 | Congress | 41 | | *MAT1-1* | | |
| 2001 | Ar-52-01 | Ar4S1P2 | Congress | | | *MAT1-2* | X | |
| 2001 | Ar-53-01 | Ar4S1P3 | Congress | | | *MAT1-2* | X | |
| 2001 | Ar-54-01 | Ar4S1P4 | Congress | | | | | |
| 2001 | Ar-55-01 | Ar4S1P5 | Congress | | | *MAT1-1* | | |
| 2001 | Ar-56-01 | Ar4S2P1 | Congress | | | *MAT1-1* | | |
| 2001 | Ar-57-01 | Ar4S2P2 | Congress | | | *MAT1-1* | X | |
| 2001 | Ar-58-01 | Ar4S2P3 | Congress | | | *MAT1-2* | X | |
| 2001 | Ar-59-01 | Ar4S2P4 | Congress | 4 | | *MAT1-1* | | |
| 2001 | Ar-60-01 | Ar4S2P5 | Congress | | | | | |
| 2001 | Ar-61-01 | Ar4S3P1 | Congress | 29 | | *MAT1-1* | | |
| 2001 | Ar-62-01 | Ar4S3P2 | Congress | 27 | | *MAT1-1* | | |
| 2001 | Ar-63-01 | Ar4S3P3 | Congress | | | | | |
| 2001 | Ar-64-01 | Ar4S3P5 | Congress | | | | | |
| 2001 | Ar-65-01 | Ar5S1P1 | Cupar | | | *MAT1-2* | | |
| 2001 | Ar-66-01 | Ar5S1P2 | Cupar | 99 | | *MAT1-1* | X | |
| 2001 | Ar-67-01 | Ar5S1P3 | Cupar | | | | | |
| 2001 | Ar-68-01 | Ar5S1P4 | Cupar | 94 | | | | |
| 2001 | Ar-69-01 | Ar5S1P5 | Cupar | | | *MAT1-1* | X | |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2001 | Ar-70-01 | Ar6S1 | Limerick | | | *MAT1-1* | X | |
| 2001 | Ar-71-01 | Ar6S2 | Limerick | 15 | | *MAT1-2* | | |
| 2001 | Ar-72-01 | Ar6S3 | Limerick | | | | | |
| 2001 | Ar-73-01 | Ar7S1 | Meyeronne | 68 | | *MAT1-2* | X | |
| 2001 | Ar-74-01 | Ar7S2 | Meyeronne | | | | | |
| 2001 | Ar-75-01 | Ar8S1 | Laflesche | 55 | | *MAT1-2* | X | |
| 2001 | Ar-76-01 | Ar9S1 | Craik | | | *MAT1-1* | X | |
| 2001 | Ar-77-01 | Ar9S2 | Craik | | | | | |
| 2001 | Ar-78-01 | Ar9S3 | Craik | 49 | | *MAT1-1* | X | |
| 2001 | Ar-79-01 | Ar10 | Craik | 62 | | | | |
| 2001 | Ar-80-01 | Ar11 | Cadillac | | | | | |
| 2001 | Ar-81-01 | Ar12S1 | Swift Current | | | | | |
| 2001 | Ar-82-01 | Ar12S3 | Swift Current | 57 | | *MAT1-2* | X | |
| 2001 | Ar-83-01 | Ar13S1 | Kyle | 86 | | | | |
| 2001 | Ar-84-01 | Ar13S2 | Kyle | | | *MAT1-2* | X | |
| 2001 | Ar-85-01 | Ar14S1 | Sun Valley | 2 | | | | |
| 2001 | Ar-86-01 | Ar14S2 | Sun Valley | | | | | |
| 2001 | Ar-87-01 | Ar15 | Markinch | | | | | |
| 2001 | Ar-88-01 | Ar16S1 | Outlook | 47 | | | | |
| 2001 | Ar-89-01 | Ar16S2 | Outlook | | | | | |
| 2001 | Ar-90-01 | Ar17 | Cabri | | | | | |
| 2001 | Ar-91-01 | Ar18 | Bounty | 89 | | | | |
| 2001 | Ar-92-01 | Ar19S1 | Sanctuary | | | | | |
| 2001 | Ar-93-01 | Ar19S2 | Sanctuary | 9 | | | | |
| 2001 | Ar-94-01 | Ar20 | Rosetown | 76 | | *MAT1-2* | X | |
| 2001 | Ar-95-01 | Ar21S1P1 | Outlook | | | | | |
| 2001 | Ar-96-01 | Ar21S1P2 | Outlook | | | | | |
| 2001 | Ar-97-01 | Ar21S1P3 | Outlook | 11 | | | | |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2001 | Ar-97-01 | Ar21S1P3 | Outlook | 32 | | | | |
| 2001 | Ar-98-01 | Ar21S1P4 | Outlook | | | *MAT1-2* | X | |
| 2001 | Ar-99-01 | Ar21S1P5 | Outlook | | | | | |
| 2001 | Ar-100-01 | Ar21S1P6 | Outlook | | | | | |
| 2001 | Ar-101-01 | Ar21S2P1 | Outlook | | | | | |
| 2001 | Ar-102-01 | Ar21S2P2 | Outlook | | | | | |
| 2001 | Ar-103-01 | Ar21S2P3 | Outlook | 26 | | | | |
| 2001 | Ar-104-01 | Ar21S2P4 | Outlook | | | | | |
| 2001 | Ar-105-01 | Ar21S2P5 | Outlook | | | | | |
| 2001 | Ar-106-01 | Ar21S2P6 | Outlook | 52 | | | | |
| 2001 | Ar-107-01 | Ar21S2P7 | Outlook | | | | | |
| 2001 | Ar-108-01 | Ar21S3P1 | Outlook | | | | | |
| 2001 | Ar-109-01 | Ar21S3P2 | Outlook | | | | | |
| 2002 | Ar-1-02 | Ar2-01S1 | Ponteix | | | *MAT1-1* | | Sanford** |
| 2002 | Ar-2-02 | Ar2-01S2 | Ponteix | | X | *MAT1-2* | X | Sanford |
| 2002 | Ar-3-02 | Ar2-01S3 | Ponteix | 16 | | | | Sanford |
| 2002 | Ar-4-02 | Ar2-02S1 | Aneroid | | | | | Kabuli |
| 2002 | Ar-5-02 | Ar2-02S2 | Aneroid | 73 | | | | Kabuli |
| 2002 | Ar-6-02 | Ar2-03S2 | Aneroid | | | *MAT1-2* | | Sanford |
| 2002 | Ar-7-02 | Ar2-03S3 | Aneroid | 33 | X | *MAT1-1* | X | Sanford |
| 2002 | Ar-8-02 | Ar2-03S4 | Aneroid | | | | | Sanford |
| 2002 | Ar-9-02 | Ar2-03S6 | Aneroid | | | *MAT1-2* | | Sanford |
| 2002 | Ar-10-02 | Ar2-04S1 | Kinkaid | | X | *MAT1-2* | X | Xena |
| 2002 | Ar-11-02 | Ar2-04S3 | Kinkaid | 18 | | *MAT1-2* | | Xena |
| 2002 | Ar-12-02 | Ar2-04S4 | Kinkaid | | | *MAT1-2* | | Xena |
| 2002 | Ar-13-02 | Ar2-04S5 | Kinkaid | | | *MAT1-2* | | Xena |
| 2002 | Ar-14-02 | Ar2-05S1 | Meyeronne | | | | | Unifoliate |
| 2002 | Ar-15-02 | Ar2-05S2 | Meyeronne | | | *MAT1-1* | | Unifoliate |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-16-02 | Ar2-05S3 | Meyeronne | 12 | | | | Unifoliate |
| 2002 | Ar-17-02 | Ar2-05S4 | Meyeronne | | | | | Unifoliate |
| 2002 | Ar-18-02 | Ar2-05S5 | Meyeronne | | | | | Unifoliate |
| 2002 | Ar-19-02 | Ar2-05S6 | Meyeronne | | | | | Unifoliate |
| 2002 | Ar-20-02 | Ar2-06S4 | Meyeronne | | | *MAT1-2* | | Chico |
| 2002 | Ar-21-02 | Ar2-06S5 | Meyeronne | 56 | X | *MAT1-1* | X | Chico |
| 2002 | Ar-22-02 | Ar2-08S1 | Woodrow | 44 | | | | B90 |
| 2002 | Ar-23-02 | Ar2-09S1 | Latkche | 34 | | | | Myles |
| 2002 | Ar-24-02 | Ar2-09S2 | Latkche | 8 | | | | Myles |
| 2002 | Ar-25-02 | Ar2-09S3 | Latkche | | X | *MAT1-2* | X | Myles |
| 2002 | Ar-26-02 | Ar2-10S1 | Melaval | 43 | | *MAT1-2* | X | Chico |
| 2002 | Ar-27-02 | Ar2-11S1 | Limerick | | | *MAT1-2* | | Chico |
| 2002 | Ar-28-02 | Ar2-11S2 | Limerick | 38 | | *MAT1-1* | | Chico |
| 2002 | Ar-29-02 | Ar2-11S3 | Limerick | | X | *MAT1-2* | X | Chico |
| 2002 | Ar-30-02 | Ar2-11S4 | Limerick | | | *MAT1-1* | | Chico |
| 2002 | Ar-31a-02 | Ar2-12S1 | Limerick | | | *MAT1-2* | | Chico |
| 2002 | Ar-31b-02 | Ar2-12S2 | Limerick | | | *MAT1-2* | | Chico |
| 2002 | Ar-32-02 | Ar2-12S3 | Limerick | | | | | Chico |
| 2002 | Ar-33-02 | Ar2-12S4 | Limerick | 98 | | | | Chico |
| 2002 | Ar-34-02 | Ar2-12S5 | Limerick | | | | | Chico |
| 2002 | Ar-35-02 | Ar2-13S4 | Mazenod | 6 | X | *MAT1-1* | | Chico |
| 2002 | Ar-36-02 | Ar2-15S1 | Gravelburg | 85 | X | *MAT1-1* | | Fern Kabuli |
| 2002 | Ar-37-02 | Ar2-15S2 | Gravelburg | | | | | Fern Kabuli |
| 2002 | Ar-38-02 | Ar2-16S1 | Sun Valley | | X | | | Unifoliate |
| 2002 | Ar-39-02 | Ar2-16S2 | Sun Valley | 67 | | *MAT1-1* | X | Unifoliate |
| 2002 | Ar-40-02 | Ar2-16S3 | Sun Valley | | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-41-02 | Ar2-17S1 | Imperial | | | | | Evans |
| 2002 | Ar-42-02 | Ar2-17S2 | Imperial | 58 | | *MAT1-1* | | Evans |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-43-02 | Ar2-17S3 | Imperial | | | | | Evans |
| 2002 | Ar-44-02 | Ar2-17S4 | Imperial | | | | | Evans |
| 2002 | Ar-45-02 | Ar2-17S5 | Imperial | | X | *MAT1-1* | X | Evans |
| 2002 | Ar-46-02 | Ar2-18S1 | Imperial | 51 | | | | Evans |
| 2002 | Ar-47-02 | Ar2-18S2 | Imperial | | | *MAT1-1* | | Evans |
| 2002 | Ar-48-02 | Ar2-18S3 | Imperial | | | *MAT1-1* | | Evans |
| 2002 | Ar-49-02 | Ar2-18S5 | Imperial | | | *MAT1-2* | | Evans |
| 2002 | Ar-50a-02 | Ar2-19S1 | Imperial | | X | *MAT1-1* | X | Evans/Xena |
| 2002 | Ar-50b-02 | Ar2-19S5 | Imperial | | | *MAT1-1* | | Evans/Xena |
| 2002 | Ar-51-02 | Ar2-19S5P1 | Imperial | 93 | | *MAT1-1* | | Evans/Xena |
| 2002 | Ar-52-02 | Ar2-19S5P5 | Imperial | | | *MAT1-1* | | Evans/Xena |
| 2002 | Ar-53-02 | Ar2-20S1 | Imperial | | | | | Myles |
| 2002 | Ar-54-02 | Ar2-20S2 | Imperial | | | *MAT1-1* | | Myles |
| 2002 | Ar-55-02 | Ar2-20S3 | Imperial | 3 | | | | Myles |
| 2002 | Ar-56-02 | Ar2-20S4 | Imperial | 77 | | *MAT1-1* | | Myles |
| 2002 | Ar-57-02 | Ar2-22S1 | Maidstone | | | *MAT1-2* | | Desi |
| 2002 | Ar-58-02 | Ar2-22S2 | Maidstone | | | *MAT1-2* | X | Desi |
| 2002 | Ar-59a-02 | Ar2-23S1 | Avonlea | | | *MAT1-2* | | Diva |
| 2002 | Ar-59b-02 | Ar2-23S2 | Avonlea | | | *MAT1-1* | | Diva |
| 2002 | Ar-60-02 | Ar2-23S3 | Avonlea | 69 | | | | Diva |
| 2002 | Ar-61-02 | Ar2-23S4 | Avonlea | | | | | Diva |
| 2002 | Ar-62-02 | Ar2-23S5 | Avonlea | | | *MAT1-2* | | Diva |
| 2002 | Ar-63-02 | Ar2-23S7 | Avonlea | | | | | Diva |
| 2002 | Ar-64-02 | Ar2-23S8 | Avonlea | | | *MAT1-1* | | Diva |
| 2002 | Ar-65-02 | Ar2.24S1 | Avonlea | | | *MAT1-2* | | Xena |
| 2002 | Ar-66-02 | Ar2-24S2 | Avonlea | 88 | | *MAT1-1* | | Xena |
| 2002 | Ar-67-02 | Ar2-24S3 | Avonlea | | | *MAT1-1* | | Xena |
| 2002 | Ar-68-02 | Ar2-24S4 | Avonlea | 70 | | *MAT1-2* | | Xena |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-69-02 | Ar2-24S5 | Avonlea | | X | *MAT1-2* | X | Xena |
| 2002 | Ar-70-02 | Ar2-25S2 | Avonlea | | | *MAT1-1* | | Xena |
| 2002 | Ar-71-02 | Ar2-25S4 | Avonlea | | | | | Xena? |
| 2002 | Ar-72-02 | Ar2-26S1 | Avonlea | 36 | | | | Xena? |
| 2002 | Ar-73-02 | Ar2-26S3 | Avonlea | | | *MAT1-1* | | Xena? |
| 2002 | Ar-74-02 | Ar2-26S4 | Avonlea | 14 | | | | Xena? |
| 2002 | Ar-75-02 | Ar2-27S5 | Avonlea | | | *MAT1-1* | | Xena? |
| 2002 | Ar-76-02 | Ar2-27S6 | Avonlea | 40 | | | | Xena? |
| 2002 | Ar-77-02 | Ar2-27S8 | Avonlea | | | | | Xena? |
| 2002 | Ar-78-02 | Ar2-28S1 | Madrid | | | *MAT1-1* | | Xena |
| 2002 | Ar-79-02 | Ar2-28S2 | Madrid | | | *MAT1-2* | | Xena |
| 2002 | Ar-80-02 | Ar2-28S3 | Madrid | | | *MAT1-1* | | Xena |
| 2002 | Ar-81-02 | Ar2-28S4 | Madrid | | | *MAT1-1* | | Xena |
| 2002 | Ar-82-02 | Ar2-28S5 | Madrid | | X | *MAT1-1* | X | Xena |
| 2002 | Ar-83-02 | Ar2-29S1 | Drinkwater | 87 | | *MAT1-2* | | Sanford |
| 2002 | Ar-84-02 | Ar2-29S2 | Drinkwater | | | | | Sanford |
| 2002 | Ar-85-02 | Ar2-29S3 | Drinkwater | | | *MAT1-1* | | Sanford |
| 2002 | Ar-86-02 | Ar2-29S4 | Drinkwater | | | *MAT1-1* | | Sanford |
| 2002 | Ar-87-02 | Ar2-29S5 | Drinkwater | 50 | | *MAT1-1* | X | Sanford |
| 2002 | Ar-88-02 | Ar2-30S4 | | | | *MAT1-1* | | |
| 2002 | Ar-89-02 | Ar2-31S1 | Rauleau | | | | | Xena |
| 2002 | Ar-90-02 | Ar2-31S4 | Rauleau | | | | | |
| 2002 | Ar-91-02 | Ar2-31S5 | Rauleau | | | | | Xena |
| 2002 | Ar-92-02 | Ar2-32S2 | | | | | | |
| 2002 | Ar-93-02 | Ar2-34S1 | Kyle | | X | *MAT1-1* | X | Unifoliate |
| 2002 | Ar-94-02 | Ar2-34S2 | Kyle | 23 | | | | Unifoliate |
| 2002 | Ar-95-02 | Ar2-34S4 | Kyle | | | | | Unifoliate |
| 2002 | Ar-96-02 | Ar2-34S5 | Kyle | 97 | | | | Unifoliate |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-97-02 | Ar2-34S6 | Kyle | | X | *MAT1-1* | X | Unifoliate |
| 2002 | Ar-98-02 | Ar2-35S1 | Kyle | | | *MAT1-1* | X | Unifoliate |
| 2002 | Ar-99-02 | Ar2-35S2 | Kyle | 82 | | | | Unifoliate |
| 2002 | Ar-100-02 | Ar2-35S3 | Kyle | | X | *MAT1-1* | | Unifoliate |
| 2002 | Ar-101-02 | Ar2-36S1 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-102-02 | Ar2-36S2 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-103-02 | Ar2-36S3 | Stewart Valley | 42 | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-104-02 | Ar2-36S4 | Stewart Valley | 91 | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-105-02 | Ar2-37S3 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-106-02 | Ar2-38S1 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-107-02 | Ar2-38S2 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-108-02 | Ar2-38S4 | Stewart Valley | | | | | Unifoliate |
| 2002 | Ar-109-02 | Ar2-39S1 | Stewart Valley | | | | | Fern Kabuli |
| 2002 | Ar-110-02 | Ar2-39S2 | Stewart Valley | | | | | Fern Kabuli |
| 2002 | Ar-111-02 | Ar2-39S3 | Stewart Valley | 1 | | *MAT1-1* | | Fern Kabuli |
| 2002 | Ar-112-02 | Ar2-39S4 | Stewart Valley | | | *MAT1-2* | | Fern Kabuli |
| 2002 | Ar-113-02 | Ar2-39S5 | Stewart Valley | 95 | | | | Fern Kabuli |
| 2002 | Ar-114-02 | Ar2-40S1 | Simmie | | X | *MAT1-1* | X | Sanford |
| 2002 | Ar-115a-02 | Ar2-40S2 | Simmie | 71 | | | | Sanford |
| 2002 | Ar-115b-02 | Ar2-40S3 | Simmie | | | *MAT1-2* | | Sanford |
| 2002 | Ar-116-02 | Ar2-41S3 | Simmie | | | *MAT1-1* | | Myles |
| 2002 | Ar-117-02 | Ar2-41S5 | Simmie | 79 | X | *MAT1-2* | X | Myles |
| 2002 | Ar-118-02 | Ar2-42S1 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-119-02 | Ar2-42S2 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-120-02 | Ar2-42S3 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-121-02 | Ar2-42S4 | Frontier | | | | | Sanford |
| 2002 | Ar-122-02 | Ar2-42S5 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-123-02 | Ar2-42S6 | Frontier | 84 | | *MAT1-1* | | Sanford |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-124-02 | Ar2-43S2 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-125-02 | Ar2-43S3 | Frontier | 48 | | | | Sanford |
| 2002 | Ar-126-02 | Ar2-43S4 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-127-02 | Ar2-44S1 | Frontier | | | *MAT1-2* | | Sanford |
| 2002 | Ar-128-02 | Ar2-44S2 | Frontier | | | *MAT1-2* | | Sanford |
| 2002 | Ar-129-02 | Ar2-44S3 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-130-02 | Ar2-44S4 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-131-02 | Ar2-45S1 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-132-02 | Ar2-45S2 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-133-02 | Ar2-45S4 | Frontier | 78 | | | | Sanford |
| 2002 | Ar-134-02 | Ar2-45S5 | Frontier | 74 | | | | Sanford |
| 2002 | Ar-135-02 | Ar2-45S6 | Frontier | | | | | Sanford |
| 2002 | Ar-136-02 | Ar2-46S1 | Frontier | | | *MAT1-2* | | Sanford |
| 2002 | Ar-137-02 | Ar2-46S3 | Frontier | | | *MAT1-1* | | Sanford |
| 2002 | Ar-138-02 | Ar2-46S5 | Frontier | | | | | Sanford |
| 2002 | Ar-139-02 | Ar2-46S7 | Frontier | 25 | | *MAT1-2* | | Sanford |
| 2002 | Ar-140-02 | Ar2-47S1 | Gravelburg | | | *MAT1-1* | | Sanford |
| 2002 | Ar-141-02 | Ar2-47S2 | Gravelburg | | | | | Sanford |
| 2002 | Ar-149-02 | Ar2-47S3 | Gravelburg | 66 | | *MAT1-1* | | Sanford |
| 2002 | Ar-150-02 | Ar2-47S4 | Gravelburg | | | | | Sanford |
| 2002 | Ar-151-02 | Ar2-50S1 | Swift Current | | X | *MAT1-2* | X | Myles |
| 2002 | Ar-152-02 | Ar2-51S1 | Cadillac | | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-153-02 | Ar2-51S2 | Cadillac | 92 | | *MAT1-2* | | Unifoliate |
| 2002 | Ar-154-02 | Ar2-51S3 | Cadillac | | | | | Unifoliate |
| 2002 | Ar-155-02 | Ar2-51S4 | Cadillac | 72 | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-156-02 | Ar2-51S5 | Cadillac | | | *MAT1-1* | | Unifoliate |
| 2002 | Ar-157-02 | Ar2-55S1 | Frontier | 30 | | *MAT1-2* | | 95NN-29 |
| 2002 | Ar-158-02 | Ar2-55S3 | Frontier | | | | | 95NN-29 |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-159-02 | Ar2-56S1 | Claydon | 10 | | | | Unifoliate |
| 2002 | Ar-160-02 | Ar2-56S2 | Claydon | | | *MAT1-2* | | Unifoliate |
| 2002 | Ar-161-02 | Ar2-57S1 | Kernan | | X | *MAT1-1* | X | |
| 2002 | Ar-162-02 | Ar2-57S2 | Kernan | 45 | X | *MAT1-2* | X | |
| 2002 | Ar-163-02 | Ar2-59S1 | Lisieux | | | *MAT1-1* | | B90 |
| 2002 | Ar-164-02 | Ar2-59S2 | Lisieux | | | | | B90 |
| 2002 | Ar-165-02 | Ar2-60S1 | Elrose | | X | *MAT1-1* | X | |
| 2002 | Ar-166-02 | Ar2-60S2 | Elrose | | X | *MAT1-1* | X | |
| 2002 | Ar-167-02 | Ar2-60S3 | Elrose | | X | *MAT1-1* | | |
| 2002 | Ar-168-02 | Ar2-60S4 | Elrose | 28 | | *MAT1-2* | | |
| 2002 | Ar-169-02 | Ar2-60S5 | Elrose | | X | *MAT1-2* | X | |
| 2002 | Ar-170a-02 | Ar2-60S6 | Elrose | | | *MAT1-2* | | |
| 2002 | Ar-170b-02 | Ar2-60S8 | Elrose | | | *MAT1-1* | | |
| 2002 | Ar-171-02 | Ar2-61S1 | Fiske | | X | *MAT1-2* | | Desi |
| 2002 | Ar-172-02 | Ar2-61S2 | Fiske | 100 | | *MAT1-2* | | Desi |
| 2002 | Ar-173-02 | Ar2-61S3 | Fiske | | X | *MAT1-1* | X | Desi |
| 2002 | Ar-174-02 | Ar2-61S4 | Fiske | 21 | | *MAT1-2* | | Desi |
| 2002 | Ar-175-02 | Ar2-61S8 | Fiske | | | *MAT1-2* | | Desi |
| 2002 | Ar-176-02 | Ar2-62S1 | Fiske | | | | | Desi |
| 2002 | Ar-177-02 | Ar2-62S2 | Fiske | | | | | Desi |
| 2002 | Ar-178-02 | Ar2-62S3 | Fiske | | | *MAT1-1* | | Desi |
| 2002 | Ar-179-02 | Ar2-62S4 | Fiske | | | | | Desi |
| 2002 | Ar-180-02 | Ar2-63S1 | Cabri | | | | | Xena |
| 2002 | Ar-181a-02 | Ar2-63S2 | Cabri | | | *MAT1-2* | | Xena |
| 2002 | Ar-181b-02 | Ar2-63S3 | Cabri | | | *MAT1-2* | | Xena |
| 2002 | Ar-182-02 | Ar2-63S4 | Cabri | | X | *MAT1-2* | | Xena |
| 2002 | Ar-183-02 | Ar2-63S5 | Cabri | 20 | | *MAT1-2* | | Xena |
| 2002 | Ar-184-02 | Ar2-63S6 | Cabri | | | | | Xena |

| Year[a] | Isolate Number[b] | Isolate Name[c] | Location[d] | PathNumber[e] | RAPD[f] | MAT[g] | MatSub[h] | Comments |
|---|---|---|---|---|---|---|---|---|
| 2002 | Ar-185-02 | Ar2-63S7 | Cabri | 64 | | | | Xena |
| 2002 | Ar-186-02 | Ar2-64S2 | Eatonia | | | | | Sanford |
| 2002 | Ar-187-02 | Ar2-64S4 | Eatonia | | X | *MAT1-1* | | Sanford |
| 2002 | Ar-188-02 | Ar2-64S5 | Eatonia | | | | | Sanford |
| 2002 | Ar-189-02 | Ar2-64S6 | Eatonia | | | *MAT1-2* | | Sanford |
| 2002 | Ar-190-02 | Ar2-64S7 | Eatonia | | | *MAT1-1* | X | Sanford |
| 2002 | Ar-191-02 | Ar2-64S9 | Eatonia | | | | | Sanford |
| 2002 | Ar-192-02 | Ar2-64S10 | Eatonia | | X | *MAT1-2* | X | Sanford |
| 2002 | Ar-194-02 | Ar2-65S3 | Birsay | | | | | Fern Kabuli |
| 2002 | Ar-195-02 | Ar2-65S5 | Birsay | 63 | | | | Fern Kabuli |
| 2002 | Ar-196a-02 | Ar2-65S4 | Birsay | | | *MAT1-1* | X | Fern Kabuli |
| 2002 | Ar-196b-02 | Ar2-65S6 | Birsay | | | *MAT1-1* | X | Fern Kabuli |
| 2002 | Ar-197-02 | Ar2-65S8 | Birsay | | | | | Fern Kabuli |

[a]Year of collection, [b]Number assigned to isolates, [c]Name given to isolate when collected, first number indicates field number where 2-number indicates isolates collected in 2002, S number indicates spot in field and P number indicates plant number , [d]Location of origin, [e]Random number given to isolates for pathogenicity testing, [f]Yes indicates RAPD primers used to detect genetic diversity, [g]Indicates mating-type, [h]Isolates used in a random sample from each year to compare mating -type ratios,

*All isolates from same plant collected from the same lesion, **Names indicate cultivar, possible cultivar or leaf type of cultivar which isolates were collected off in field

**Appendix 2:** One way analysis of variance of the stratified sample of 13 *Ascochyta rabiei* isolates compared to the total population of 40 isolates collected in the growing season of 1998

| Source | DF | Type III Sum of Squares | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Population | 1 | 0.70 | 0.70 | 1.05 | 0.3094 |

**Appendix 3:** 10 X 10 Triple Lattice Designs used as described by Cochran and Cox (1992)

| Block | Rep. I | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (1) | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** |
| (2) | **11** | **12** | **13** | **14** | **15** | **16** | **17** | **18** | **19** | **20** |
| (3) | **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** |
| (4) | **31** | **32** | **33** | **34** | **35** | **36** | **37** | **38** | **39** | **40** |
| (5) | **41** | **42** | **43** | **44** | **45** | **46** | **47** | **48** | **49** | **50** |
| (6) | **51** | **52** | **53** | **54** | **55** | **56** | **57** | **58** | **59** | **60** |
| (7) | **61** | **62** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **70** |
| (8) | **71** | **72** | **73** | **74** | **75** | **76** | **77** | **78** | **79** | **80** |
| (9) | **81** | **82** | **83** | **84** | **85** | **86** | **87** | **88** | **89** | **90** |
| (10) | **91** | **92** | **93** | **94** | **95** | **96** | **97** | **98** | **99** | **100** |

| Block | Rep. II | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (11) | **1** | **11** | **21** | **31** | **41** | **51** | **61** | **71** | **81** | **91** |
| (12) | **2** | **12** | **22** | **32** | **42** | **52** | **62** | **72** | **82** | **92** |
| (13) | **3** | **13** | **23** | **33** | **43** | **53** | **63** | **73** | **83** | **93** |
| (14) | **4** | **14** | **24** | **34** | **44** | **54** | **64** | **74** | **84** | **94** |
| (15) | **5** | **15** | **25** | **35** | **45** | **55** | **65** | **75** | **85** | **95** |
| (16) | **6** | **16** | **26** | **36** | **46** | **56** | **66** | **76** | **86** | **96** |
| (17) | **7** | **17** | **27** | **37** | **47** | **57** | **67** | **77** | **87** | **97** |
| (18) | **8** | **18** | **28** | **38** | **48** | **58** | **68** | **78** | **88** | **98** |
| (19) | **9** | **19** | **29** | **39** | **49** | **59** | **69** | **79** | **89** | **99** |
| (20) | **10** | **20** | **30** | **40** | **50** | **60** | **70** | **80** | **90** | **100** |

**Appendix 4:** Mixed model used in SAS to determine adjusted least squares mean AUDPC for each differential, isolate and each differential x isolate combination

```
proc mixed;
class r  iso rpt rep block diff;
model audpc = iso diff iso*diff;
random r rpt*block(rep) iso*rpt*block(rep);
lsmeans iso diff iso*diff;

estimate 'iso1 - iso2' iso 1 -1;
estimate 'iso1 - iso11' iso 1 0 0 0 0 0 0 0 0 0 -1;
estimate 'diff1 - diff2' diff 1 -1;
estimate 'diff3 - diff4' diff 0 0 1 -1;
estimate 'iso1-diff1 - iso1-diff2' diff 1 -1 iso*diff 1 -1;
estimate 'iso1-diff1 - iso2-diff1'iso 1 -1 iso*diff 1 0 0 0 0 0 0 -1;
estimate 'iso1-diff1 - iso11-diff1' iso 1 0 0 0 0 0 0 0 0 0 -1
          iso*diff 1 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                  -1 0 0 0 0 0 0;
estimate '(i1d1-i2d1)-(i1d2-i2d2)' iso*diff
                   1 -1 0 0 0 0 0
                  -1  1 0 0 0 0 0;
estimate '(i1d1-i11d1)-(i1d3-i11d3)' iso*diff
                   1 0 -1 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                   0 0 0 0 0 0 0
                  -1 0 1 0 0 0 0;
```

Where,

iso = *A. rabiei* isolate number (1 … 100 from Appendix 2)

rpt = repetition number (1=lattice 1, 2=lattice 2 from Cochran and Cox (1992) Plan 10.8)

rep = replication (1 or 2) within each repetition

blk = incomplete block (1 to 20) from Cochran and Cox (1992) Plan 10.8

NOTE: Original coding for blocks for repetition 2 was changed by subtracting 20 so the program can recognize block 21 as containing the same set of isolates as block 1 in repetition 1)

diff = chickpea differential (numbered 1 through 8)

audpc = area under the disease progress curve calculated from disease ratings at six times

**Appendix 5:** Mixed model used in SAS to analyze group (year) data based on adjusted means

```
data;
  input iso grp rep rpt block diff audpc;
  r = (rpt-1)*2+rep;
proc mixed;
  class r  iso grp rpt rep block diff;
  model audpc = grp diff grp*diff;
  random iso(grp) r rpt*block(rep) iso*rpt*block(grp rep);
  lsmeans grp diff grp*diff/tdiff;
```

Where,

grp = group of isolate (1=1998, 2=2001, 3=2002)

**Appendix 6:** Method of DNA extraction used for isolates of *Ascochyta rabiei* and use in fingerprinting procedures

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-104-01 | 2001 | CTAB | 1.09 | | X | | | |
| Ar-12-01 | 2001 | CTAB | 1.92 | | X | X | | |
| Ar-13-01 | 2001 | CTAB | 1.44 | | X | X | | |
| Ar-16-01 | 2001 | CTAB | 2.10 | | X | | | |
| Ar-18-01 | 2001 | CTAB | 1.49 | | X | X | | |
| Ar-20-01 | 2001 | CTAB | 1.90 | | X | X | | |
| Ar-22-01 | 2001 | CTAB | 1.92 | | X | X | | |
| Ar-23-01 | 2001 | CTAB | 1.71 | | X | X | | |
| Ar-28-01 | 2001 | CTAB | 1.89 | | X | X | | |
| Ar-29-01 | 2001 | CTAB | 2.14 | | X | X | | |
| Ar-3-01 | 2001 | CTAB | 1.92 | | X | X | | |
| Ar-34-01 | 2001 | CTAB | 1.85 | | X | X | | |
| Ar-35-01 | 2001 | CTAB | 1.71 | | X | X | | |
| Ar-36-01 | 2001 | CTAB | 1.78 | | X | X | | |
| Ar-37-01 | 2001 | CTAB | 1.07 | | X | | | |
| Ar-39-01 | 2001 | CTAB | 1.39 | | X | | | |
| Ar-45-01 | 2001 | CTAB | 1.96 | | X | X | | |
| Ar-52-01 | 2001 | CTAB | 2.05 | | X | X | | |
| Ar-53-01 | 2001 | CTAB | 1.28 | | X | X | | |
| Ar-57-01 | 2001 | CTAB | 2.05 | | X | X | | |
| Ar-58-01 | 2001 | CTAB | 1.46 | | X | X | | |
| Ar-6-01 | 2001 | CTAB | 1.94 | | X | X | | |
| Ar-66-01 | 2001 | CTAB | 1.32 | | X | X | | |
| Ar-67-01 | 2001 | CTAB | 1.37 | | X | | | |
| Ar-69-01 | 2001 | CTAB | 1.64 | | X | X | | |
| Ar-70-01 | 2001 | CTAB | 1.63 | | X | X | | |
| Ar-73-01 | 2001 | CTAB | 1.64 | | X | X | | |
| Ar-75-01 | 2001 | CTAB | 1.90 | | X | X | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-76-01 | 2001 | CTAB | 1.31 | | X | X | | |
| Ar-78-01 | 2001 | CTAB | 2.28 | | X | X | | |
| Ar-82-01 | 2001 | CTAB | 1.68 | | X | X | | |
| Ar-84-01 | 2001 | CTAB | 1.79 | | X | X | | |
| Ar-9-01 | 2001 | CTAB | 1.77 | | X | X | | |
| Ar-94-01 | 2001 | CTAB | 1.91 | | X | X | | |
| Ar-98-01 | 2001 | CTAB | 1.54 | | X | X | | |
| Ar-137-02 | 2002 | CTAB | 2.00 | | X | | | |
| Ar-1-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-2-02 | 2002 | CTAB | 1.85 | X | X | X | | |
| Ar-6-02 | 2002 | CTAB | 1.07 | | X | | | |
| Ar-7-02 | 2002 | CTAB | 1.41 | X | X | X | | |
| Ar-10-02 | 2002 | CTAB | 1.89 | X | X | X | | |
| Ar-11-02 | 2002 | CTAB | 2.10 | | X | | | |
| Ar-12-02 | 2002 | CTAB | 1.71 | | X | | | |
| Ar-13-02 | 2002 | CTAB | 1.82 | | X | | | |
| Ar-15-02 | 2002 | CTAB | 2.01 | | X | | | |
| Ar-18-02 | 2002 | CTAB | 1.01 | | X | | | |
| Ar-20-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-21-02 | 2002 | CTAB | 1.89 | X | X | X | | |
| Ar-25-02 | 2002 | CTAB | 1.69 | X | X | X | | |
| Ar-26-02 | 2002 | CTAB | 1.92 | X | X | X | | |
| Ar-27-02 | 2002 | CTAB | 2.05 | | X | | | |
| Ar-28-02 | 2002 | CTAB | 1.72 | | X | | | |
| Ar-29-02 | 2002 | CTAB | 1.58 | X | X | X | | |
| Ar-30-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-30b-02 | 2002 | CTAB | 1.61 | | X | | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-31-02 | 2002 | CTAB | 1.69 | | X | | | |
| Ar-35-02 | 2002 | CTAB | 1.67 | X | X | | | |
| Ar-36-02 | 2002 | CTAB | 1.80 | X | X | | | |
| Ar-39-02 | 2002 | CTAB | 1.80 | X | X | X | | |
| Ar-40-02 | 2002 | CTAB | 1.95 | | X | | | |
| Ar-42-02 | 2002 | CTAB | 1.49 | | X | | | |
| Ar-45-02 | 2002 | CTAB | 1.72 | X | X | X | | |
| Ar-47-02 | 2002 | CTAB | 1.84 | | X | | | |
| Ar-48-02 | 2002 | CTAB | 3.92 | | X | | | |
| Ar-49-02 | 2002 | CTAB | 1.52 | | X | | | |
| Ar-50-02 | 2002 | CTAB | 1.75 | X | X | X | | |
| Ar-50b-02 | 2002 | CTAB | 1.92 | | X | | | |
| Ar-50c-02 | 2002 | CTAB | 1.92 | | X | | | |
| Ar-51-02 | 2002 | CTAB | 1.86 | | X | | | |
| Ar-52-02 | 2002 | CTAB | 1.88 | | X | | | |
| Ar-54-02 | 2002 | CTAB | 1.61 | | X | | | |
| Ar-56-02 | 2002 | CTAB | 1.87 | | X | | | |
| Ar-57-02 | 2002 | CTAB | 1.81 | | X | | | |
| Ar-58-02 | 2002 | CTAB | 1.80 | X | X | X | | |
| Ar-59a-02 | 2002 | CTAB | 1.62 | | X | | | |
| Ar-59-02 | 2002 | CTAB | 1.79 | | X | | | |
| Ar-62-02 | 2002 | CTAB | 2.11 | | X | | | |
| Ar-64-02 | 2002 | CTAB | 1.91 | | X | | | |
| Ar-65-02 | 2002 | CTAB | 1.83 | | X | | | |
| Ar-66-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-67-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-68-02 | 2002 | CTAB | 2.00 | | X | | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-69-02 | 2002 | CTAB | 2.02 | X | X | X | | |
| Ar-70-02 | 2002 | CTAB | 1.64 | | X | | | |
| Ar-73-02 | 2002 | CTAB | 2.04 | | X | | | |
| Ar-75-02 | 2002 | CTAB | 1.81 | | X | | | |
| Ar-78-02 | 2002 | CTAB | 1.93 | | X | | | |
| Ar-79-02 | 2002 | CTAB | 1.79 | | X | | | |
| Ar-80-02 | 2002 | CTAB | 1.86 | | X | | | |
| Ar-80-02 | 2002 | CTAB | 1.79 | | X | | | |
| Ar-81-02 | 2002 | CTAB | 1.82 | | X | | | |
| Ar-82-02 | 2002 | CTAB | 1.74 | X | X | X | | |
| Ar-83-02 | 2002 | CTAB | 1.80 | | X | | | |
| Ar-85-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-86-02 | 2002 | CTAB | 1.72 | | X | | | |
| Ar-87-02 | 2002 | CTAB | 1.77 | X | X | X | | |
| Ar-88-02 | 2002 | CTAB | 1.73 | | X | | | |
| Ar-93-02 | 2002 | CTAB | 1.49 | X | X | X | | |
| Ar-97-02 | 2002 | CTAB | 1.47 | X | X | X | | |
| Ar-98-02 | 2002 | CTAB | 1.79 | X | X | X | | |
| Ar-100-02 | 2002 | CTAB | 1.74 | X | X | | | |
| Ar-103-02 | 2002 | CTAB | 1.84 | | X | | | |
| Ar-104-02 | 2002 | CTAB | 1.53 | | X | | | |
| Ar-111-02 | 2002 | CTAB | 1.95 | X | X | | | |
| Ar-112-02 | 2002 | CTAB | 1.85 | | X | | | |
| Ar-114-02 | 2002 | CTAB | 1.74 | X | X | X | | |
| Ar-116-02 | 2002 | CTAB | 1.74 | | X | | | |
| Ar-117-02 | 2002 | CTAB | 1.65 | X | X | X | | |
| Ar-118-02 | 2002 | CTAB | 1.98 | X | X | | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-119-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-120-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-122-02 | 2002 | CTAB | 1.76 | | X | | | |
| Ar-123-02 | 2002 | CTAB | 1.36 | | X | | | |
| Ar-124-02 | 2002 | CTAB | 2.01 | | X | | | |
| Ar-126-02 | 2002 | CTAB | 1.90 | | X | | | |
| Ar-127-02 | 2002 | CTAB | 1.85 | | X | | | |
| Ar-128-02 | 2002 | CTAB | 1.95 | | X | | | |
| Ar-129-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-130-02 | 2002 | CTAB | 2.07 | | X | | | |
| Ar-131-02 | 2002 | CTAB | 1.81 | | X | | | |
| Ar-132-02 | 2002 | CTAB | 1.66 | | X | | | |
| Ar-136-02 | 2002 | CTAB | 1.66 | | X | | | |
| Ar-139-02 | 2002 | CTAB | 6.74 | | X | | | |
| Ar-140-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-149-02 | 2002 | CTAB | 1.43 | | X | | | |
| Ar-151-02 | 2002 | CTAB | 1.72 | X | X | X | | |
| Ar-152-02 | 2002 | CTAB | 1.67 | | X | | | |
| Ar-153-02 | 2002 | CTAB | 2.05 | | X | | | |
| Ar-155-02 | 2002 | CTAB | 1.67 | X | X | | | |
| Ar-156-02 | 2002 | CTAB | 1.71 | | X | | | |
| Ar-157-02 | 2002 | CTAB | 1.85 | | X | | | |
| Ar-157-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-160-02 | 2002 | CTAB | 2.13 | | X | | | |
| Ar-161-02 | 2002 | CTAB | 1.71 | X | X | X | | |
| Ar-162-02 | 2002 | CTAB | 1.56 | X | X | X | | |
| Ar-163-02 | 2002 | CTAB | 1.81 | | X | | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-165-02 | 2002 | CTAB | 1.40 | X | X | X | | |
| Ar-166-02 | 2002 | CTAB | 1.56 | X | X | X | | |
| Ar-167-02 | 2002 | CTAB | 1.88 | X | X | | | |
| Ar-168-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-169-02 | 2002 | CTAB | 1.03 | X | X | X | | |
| Ar-170-02 | 2002 | CTAB | 1.87 | | X | | | |
| Ar-170b-02 | 2002 | CTAB | 2.09 | | X | | | |
| Ar-171-02 | 2002 | CTAB | 2.50 | X | X | | | |
| Ar-171-02 | 2002 | CTAB | 1.24 | | X | | | |
| Ar-172-02 | 2002 | CTAB | 1.99 | | X | | | |
| Ar-173-02 | 2002 | CTAB | 1.67 | X | X | X | | |
| Ar-174-02 | 2002 | CTAB | 1.89 | | X | | | |
| Ar-175-02 | 2002 | CTAB | 2.31 | | X | | | |
| Ar-178-02 | 2002 | CTAB | 2.53 | | X | | | |
| Ar-181-02 | 2002 | CTAB | 1.78 | | X | | | |
| Ar-181b-02 | 2002 | CTAB | 1.97 | | X | | | |
| Ar-182-02 | 2002 | CTAB | 1.78 | X | X | | | |
| Ar-183-02 | 2002 | CTAB | 2.21 | | X | | | |
| Ar-192-02 | 2002 | CTAB | 1.72 | X | X | X | | |
| Ar-187-02 | 2002 | CTAB | 2.00 | | X | | | |
| Ar-189-02 | 2002 | CTAB | 1.98 | | X | | | |
| Ar-190-02 | 2002 | CTAB | 1.80 | X | X | X | | |
| Ar-191-02 | 2002 | CTAB | 1.74 | | X | | | |
| Ar-196a-02 | 2002 | CTAB | 1.59 | X | X | X | | |
| Ar-193-02 | 2002 | CTAB | | X | | | | |
| Ar-196-02 | 2002 | CTAB | 1.78 | X | X | X | | |
| Ar-19-98 | 1998 | Rapid | 1.46 | X | X | X | | X |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-3-99 | 1999 | CTAB | 1.82 | | X | | | X |
| Ar-2-97 | 1997 | CTAB | 1.92 | X | X | | | X |
| Ar-2-97 | 1997 | CTAB | 2.02 | | X | | | X |
| Ar-4-98 | 1998 | Rapid | 1.48 | X | X | X | | X |
| Ar-3-99 | 1999 | CTAB | 1.66 | X | X | | | X |
| Ar-3-99 | 1999 | CTAB | 4.24 | | X | | | X |
| Ar-4-99 | 1999 | CTAB | 1.79 | X | X | | | |
| Ar-1-98 | 1998 | CTAB | 1.92 | | X | | X | X |
| Ar-1-98 | 1998 | CTAB | 1.91 | X | X | X | | X |
| Ar-2-98 | 1998 | Rapid | 1.63 | X | X | X | | X |
| Ar-3-98 | 1998 | CTAB | 1.63 | X | X | X | | X |
| Ar-3-98 | 1998 | CTAB | 1.91 | | X | | | X |
| Ar-1-99 | 1998 | Rapid | 1.48 | X | X | | | X |
| Ar-6-98 | 1998 | Rapid | 1.33 | X | X | X | | X |
| Ar-8-98 | 1998 | Rapid | 1.53 | X | X | X | | X |
| Ar-9-98 | 1998 | CTAB | 1.85 | X | X | X | | X |
| Ar-10-98 | 1998 | Rapid | 1.56 | X | X | X | X | X |
| Ar-11-98 | 1998 | Rapid | 1.31 | X | X | X | | X |
| Ar-12-98 | 1998 | Rapid | 1.56 | X | X | X | | X |
| Ar-13-98 | 1998 | Rapid | 1.64 | X | X | X | | X |
| Ar-14-98 | 1998 | Rapid | 1.27 | X | X | X | | X |
| Ar-16-98 | 1998 | Rapid | 1.49 | X | X | X | | X |
| Ar-17-98 | 1998 | CTAB | 1.71 | | X | X | | X |
| Ar-17-98 | 1998 | Rapid | 1.14 | X | X | | | X |
| Ar-18-98 | 1998 | Rapid | 1.38 | X | X | X | X | X |
| Ar-5-99 | 1999 | Rapid | 1.83 | X | X | | | |
| Ar-6-99 | 1999 | CTAB | 1.88 | X | X | | | |

| Isolate | Year | Extraction Method[a] | Ratio[b] ($OD_{260}/OD_{280}$) | RAPD[c] | MAT[d] | MAT2[e] | Phan et al. (2004)[f] | Chongo et al. (2004)[f] |
|---|---|---|---|---|---|---|---|---|
| Ar-5-98 | 1998 | Rapid | 1.70 | X | X | X | | X |
| Ar-27-98 | 1998 | Rapid | 1.73 | X | X | X | | X |
| Ar-28-98 | 1998 | Rapid | 1.49 | X | X | X | | X |
| Ar-29-98 | 1998 | Rapid | 1.54 | X | X | X | | X |
| Ar-30-98 | 1998 | CTAB | 1.71 | X | X | X | | X |
| Ar-30-98 | 1998 | CTAB | 1.45 | | X | | | X |
| Ar-31-98 | 1998 | Rapid | 1.54 | X | X | X | | X |
| Ar-31-98 | 1998 | Rapid | 1.50 | X | X | | | X |
| Ar-22-98 | 1998 | CTAB | 2.03 | | X | X | | X |
| Ar-23-98 | 1998 | Rapid | 1.59 | X | X | X | | X |
| Ar-24-98 | 1998 | Rapid | 1.62 | X | X | X | | X |
| Ar-25-98 | 1998 | Rapid | 1.71 | X | X | X | | X |
| Ar-20-98 | 1998 | CTAB | 1.57 | | X | | | X |
| Ar-20-98 | 1998 | CTAB | 1.35 | X | X | X | | X |
| Ar-21-98 | 1998 | Rapid | 1.04 | X | X | X | X | X |
| Sep9803 | 1998 | | | | X | X | | |
| Sep9805 | 1998 | | | | X | X | | |

[a] Cetyltrimethyl-ammonium bromide method (CTAB) or rapid fungal extraction method (Rapid), [b] Indicates purity of the nucleic acid extracted, [c] Used in RAPD marker study, [d] Mating-type of isolate determined, [e] 30 random isolates from each year tested, [f] Isolate also used in study

**Appendix 7:** Primers screened and used for RAPD fingerprinting

| Primer | Used[a] | Total Loci Amplified[b] | Polymorphic Loci Amplified[c] | Nucleotide Sequence[d] |
|---|---|---|---|---|
| BioBasic S68[1] | X | 7 | 4 | 5'-TGGACCGGTG-3' |
| OP A09 [2] | X | 10 | 4 | 5'-GGGTAACGCC-3' |
| OP A13[1]* | | 4 | 0 | 5'-CAGCACCCAC-3' |
| OP A18 [1] | | 4 | 0 | 5'-AGGTGACCGT-3' |
| OP B04[1] | | 0 | 0 | 5'-GGACTGGAGT-3' |
| OP B07[1] | | 5 | 0 | 5'-GGTGACGCAG-3' |
| OP B10[1] | | 4 | 0 | 5'-CTGCTGGGAC-3' |
| OP B17[2] | X | 10 | 5 | 5'-AGGGAACGAG-3' |
| OP B18[2, 7] | | 2 | 0 | 5'-CCACAGCAGT-3' |
| OP B20[2] | | 1 | 0 | 5'-GGACCCTTAC-3' |
| OP C01[3] | X | 6 | 2 | 5'-TTCGAGCCAG-3' |
| OP C13[7] | | 0 | 0 | 5'-AAGCCTCGTC-3' |
| OP C18 [5, 7] | | 5 | 0 | 5'-TGAGTGGGTG-3' |
| OP D03[1] | | 8 | 0 | 5'-GTCGCCGTCA-3' |
| OP D16[1] | | 7 | 0 | 5'-AGGGCGTAAG-3' |
| OP E05[1] | X | 3 | 1 | 5'-TCAGGGAGGT-3' |
| OP E15[2] | | 1 | 0 | 5'-ACGCACAACC-3' |
| OP I14[2] | | 7 | 0 | 5'-TGACGGCGGT-3' |
| OP I16[2, 4] | | 8 | 0 | 5'-TCTCCGCCCT-3' |
| OP I18[2] | X | 4 | 3 | 5'-TGCCCAGCCT-3' |
| OP J01[4, 6] | | 0 | 0 | 5'-CCCGGCATAA-3' |
| OP J15[4] | X | 9 | 4 | 5'-TGTAGCAGGG-3' |
| OP K08[2] | X | 8 | 4 | 5'-GAACACTGGG-3' |
| OP K09[4] | | 6 | 0 | 5'-CCCTACCGAC-3' |
| OP K12[4] | | 5 | 0 | 5'-TGGCCCTCAC-3' |
| OP K15[4] | | 5 | 0 | 5'-CTCCTGCCAA-3' |
| OP L05[4] | | 5 | 0 | 5'-ACGCAGGCAC-3' |
| OP L06[2] | | 5 | 0 | 5'-GAGGGAAGAG-3' |
| OP L08[2] | X | 11 | 5 | 5'-AGCAGGTGGA-3' |
| OP L15[2] | X | 9 | 2 | 5'-AAGAGAGGGG-3' |
| OP L18[1] | X | 5 | 1 | 5'-ACCACCCACC-3' |
| OP M03[1] | X | 8 | 4 | 5'-GGGGGATGAG-3' |
| OP M04[1] | | 6 | 0 | 5'-GGCGGTTGTC-3' |
| OP M16[2] | | 0 | 0 | 5'-GTAACCAGCC-3' |
| OP M19[1] | | 0 | 0 | 5'-CCTTCAGGCA-3' |
| OP N02[2] | X | 6 | 4 | 5'-ACCAGGGGCA-3' |

| Primer | Used[a] | Total Loci Amplified[b] | Polymorphic Loci Amplified[c] | Nucleotide Sequence[d] |
|---|---|---|---|---|
| OP O02[1] | | 0 | 0 | 5'-ACGTAGCGTC-3' |
| OP O16[1] | | 7 | 0 | 5'-TCGGCGGTTC-3' |
| OP O17[1] | | 0 | 0 | 5'-GGCTTATGCC-3' |
| OP X06[2] | | 4 | 0 | 5'-ACGCCAGAGG-3' |
| OP X09[2] | | 7 | 0 | 5'-GGTCTGGTTG-3' |
| OP X10[2] | | 0 | 0 | 5'-CCCTAGACTG-3' |
| OP X11[2] | | 5 | 0 | 5'-GGAGCCTCAG-3' |
| OP X15[2] | | 9 | 0 | 5'-CAGACAAGCC-3' |
| OP Y03[2] | | 3 | 0 | 5'-ACAGCCTGCT-3' |
| UBC-151 [6] ** | | 1 | 0 | 5'-GCTGTAGTGT-3' |
| UBC-702[5] | X | 8 | 1 | 5'-GGGAGAAGGG-3' |
| UBC-708[5] | X | 8 | 1 | 5'-GGGTTGTGGG-3' |
| UBC-726[5] | X | 6 | 1 | 5'-GGTGTGGGTG-3' |
| UBC-727[5, 6] | X | 5 | 1 | 5'-GGGTGTGGTG-3' |
| UBC-731[5] | | 2 | 0 | 5'-CCAACACCAC-3' |
| UBC-739[5] | | 6 | 0 | 5'-GGAGGGAGAG-3' |
| UBC-740[5] | | 5 | 0 | 5'-GGAGGGAGGG-3' |
| UBC-756[6] | X | 4 | 3 | 5'-CCCTCCTCCT-3' |
| **Total loci** | | **266** | **50** | |

[a] Indicates if primer was used in RAPD study, [b] Number of loci amplified on a random sample of 16 isolates of *A. rabiei*, [c] Number of polymorphic loci amplified in a random sample of 16 isolates of *A. rabiei*, [d] Sequence of primer

[1] Primers selected randomly, [2] Primers previous used by Lichtenzveig et al. (2002), [3] Primers previous used by Navas-Cortés et al. (1998), [4] Primers previous used by Udupa et al. (1998), [5] Primers previous used by Chongo et al. (2004), [6] Primers previous used by Santra et al. (2001), [7] Phan et al. (2003a)

*Source: Operon Primers (OP), Operon Technologies Inc. (Alameda, CA), **Source: University of British Columbia (UBC), University of British Columbia (Vancouver, BC)

**Appendix 8:** Nucleotide sequence of Primers used in *MAT*-specific PCR as described by Barve et al. (2003)

| Primer | Nucleotide Sequence | Comments |
|---|---|---|
| Com1 | 5'-GCATGCCATATCGCCAGT-3' | *MAT*-specific PCR-common flanking primer |
| SP21 | 5'-ACAGTGAGCCTGCACAGTTC-3' | *MAT1-1*-specific primer |
| Tail 5 | 5'-CGCTATTTTATCCAAGACACACC-3' | *MAT1-2*-specific primer |