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Faculty of Agricultural and Natural Resource Science

Resistance of faba beans to Ascochyta blight

Uyek Malik Yakop

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Department of Plant Science
Waite Agricultural Research Institute
Glen Osmond, South Australia

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STATEMENT

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SUMMARY

Ascochyta blight, caused by *Ascohyta fabae*, is one of the most destructive diseases on faba bean and it is widely distributed in all states in Australia, particularly in the southeastern region. Cultivation of resistant cultivars may reduce the damage. The present study was conducted to investigate various aspects of genetic resistance to Ascochyta blight of several accessions with the objective of providing the information to implement an efficient breeding strategy for the long-term control of Ascochyta blight.

There was variation among putatively resistant accessions in their response to Australian isolates of *A. fabae*. Several accessions were resistant to all isolates, while the remainders were resistant to several isolates and susceptible to the others. The variance within several accessions was reduced when lines produced by single plant selection were tested, rather than bulk samples. The average reaction of accessions that were homogenous resistant did not differ between single plant selections and the bulk populations. Variability for virulence was also evident and some isolates produced symptoms on all accessions, while others infected only several accessions.

The genetic resistance of Acc 622, one of the components of the Australian cultivar Ascot was investigated. Ascot was crossed to the *A. fabae* susceptible Australian cultivar Icarus and the reactions of reciprocal F_1 hybrids and the F_2 and F_3 generations were tested for reaction isolate 331/91. Reactions of the three generations indicated that a single co-dominant, or partially recessive, gene conferred resistance to isolate 331/91, relative to the susceptible Icarus.

The specific resistance of resistant accessions, most of which were obtained from ICARDA, was compared with Acc 622. The resistant lines were crossed to Acc 622 and the F_2 generation of each cross was tested for reaction to isolate 331/91. The alternative parent was considered to carry the same resistance gene as Acc 622 when all F_2 progeny produced a resistant reaction. Transgressive segregation, indicated by the presence of susceptible F_2 plants, was observed in several crosses and it was concluded that the *A. fabae* resistance of the alternative parent was conferred by a different gene to that present in Acc 622. Eight accessions (Acc 295, 297, 303, 484, 496, 668, 680 and 975, derived from BPL460, BPL465, BPL472, BPL74, BPL365, L83120, BPL2485 and L83125, respectively) were identified to carry the same resistance gene as Acc 622 and six accessions (Acc 299, 674, 712, 948, 970 and 1046, derived from BPL471, L83124, L82003, Quasar, ILB752 and BPL646, respectively) were different. The relationship between these alternative sources of resistance was not determined. Results for four populations were inconclusive due to a high variance of one of the parents.

AFLP analysis was utilised to identify the genetic distance among 20 resistant and 2 susceptible accessions. Three primer combinations (*Pst*ACA-*Mse*CAG, *Pst*ACA-*Mse*CCA and *Pst* ACA-*Mse*CGA) revealed a high level of polymorphisms. The average of genetic distance over all accessions was 0.34 with the pair-wise range from 0.09 to 0.53. The phylogenetic tree divided the 22 accessions into two major groups and several groups of two and three. The analysis was inconclusive when the genetic control of resistance to *A. fabae*, the region of origin and the source population were compared to the genetic distances among the accessions.

Resistance to *A. fabae* in the well adapted Australian cultivar Ascot is under simple genetic control therefore it should be relatively straightforward to transfer this resistance to other high yielding, but susceptible, lines either through simple crossing or backcrossing. As resistance is either partially recessive or co-dominant selection would be effective in the early generations. Identification of alternative resistance genes to the one in use in Australian agriculture should enable a long-term strategy for the development and deployment of resistant cultivars to be implemented.

Chapter 1. Introduction

Faba bean (*Vicia faba* L.) is a cool-season grain legume with the major areas of production being China, North Africa, West Asia, Europe and Australia. China accounts for more than 50 % of the world's production and faba bean is the major food legume in this country (Li-Juan et al., 1993; Yang and Luo, 1995). Australia is the fifth largest producer in the world, with 140,000 tonnes from 130,000 ha in 1997/98 (FAO, 1997; Australian Grain, 1998), and the second largest exporter of faba beans.

The Australian faba bean industry started in South Australia in 1980 with the release of the cultivar Fiord (Knight, 1994). Since then, production has expanded to the higher rainfall zones (generally > 450 mm per annum) of other states, Western Australia, New South Wales and Victoria in the latitude range 20 - 40°S. Faba bean is now one of the major pulse crops in Australia along with lupins (*Lupinus angustifolius*), fieldpea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*).

Faba bean is capable of high yields but is sensitive to biotic and abiotic stress (Bond et al., 1994). Ascochyta blight, caused by the pathogen *Ascochyta fabae* Speg., is one of the most destructive diseases on faba beans. It infects leaves, stems and pods. Severe outbreaks can result in total yield loss, depending on the degree of susceptibility of cultivars (Pritchard et al., 1989). Moreover, the pathogen can be seed borne and this results in infection of subsequent crops, reduced product quality and problems in providing seed certification for export targets with strict quarantine regulations (Bond and Pope, 1980; Jellis and Punithalingam, 1991).

The disease spreads rapidly under wet and cool conditions, which occur in Northern Europe, Canada, the Middle East and North Africa, as well as in Australia and New Zealand. In Australia, *A. fabae* is an important disease in all states and possibly the most significant disease problem for growers in the south-eastern region (Piggin and Lack, 1994).

Methods for controlling the disease include the application of fungicides, sowing clean seed and cultural techniques. Problems associated with the use of fungicides include the cost and time required for application, the increased risk of the pathogen developing resistance to the fungicide, and the potential for environmental damage (Robinson, 1996).

Although certified or disease-free seed may contribute to practical crop protection by preventing transmission of the pathogen from the seed to seedlings, this may not always be effective because infection can occur from infected plant debris and volunteer plants. Another problem with relying only on clean seed for disease control is that it can be difficult to produce grain of high quality in wet seasons (Jellis et al., 1985). Cultural control can also inhibit the spread of *A. fabae* to some extent.

Incorporation or burning crop residues to reduce inoculum, planting in rotation with cereals to allow residues to break down, late sowing, and using Ascochyta blight resistant cultivars are methods which have been widely used due to their success in limiting both disease development and environmental damage.

In order for disease resistance to be utilised as a long-term strategy, it is necessary to understand the interaction between the plant and the pathogen and variation within both the pathogen and the plant species. This will assist decision making in breeding programs and developing strategies for breeding disease resistance. Experimentation on this topic has been widely conducted overseas (Rashid

and Bernier, 1985; Hanounik and Robertson, 1989; Rashid et al., 1991a), but little work has been reported from Australia.

In Australia, the expansion in planting has slowed recently, as a result of outbreaks of the leaf diseases chocolate spot and Ascochyta blight. To address this problem, the National Faba Bean Breeding Program situated in Adelaide, South Australia, has produced resistant cultivars to chocolate spot and Ascochyta blight, including Icarus and Ascot, respectively. Ascot is a composite of two Ascochyta blight resistant selections from the cultivar Fiord and was commercially released in 1995. Cultivation of Ascot has led to reductions in the damage to crops caused by *A. fabae.*

One strategy employed in breeding Ascochyta blight resistant varieties is to utilise germplasm collections introduced from genetic resource centres, such as ICARDA. These collections may be used to improve the resistance to diseases by identifying and combining different resistance genes into one genome. A number of resistance genes to *A. fabae* have been identified (Rashid et al., 1991b) and sources of resistance are readily available (Bond et al, 1994). However, there is no information on the resistance of these lines relative to Ascot. It is necessary to establish the relationship between Ascot and other resistant lines in order to identify alternative resistance genes that could be used if the resistance of Ascot is overcome by a change in *A. fabae*. To achieve this it is necessary to characterise the genetic divergence of the various sources of resistance. A combination of conventional genetic and molecular methods could be used to identify the relationship between the sources of resistance to *A. fabae* and the two methods could provide a cross check for each other.

The aims of this study were (1) to understand the interaction between Australian isolates of the pathogen *A. fabae* and eight faba bean accessions, (2) to identify the genetic control of resistance to *A. fabae* found in the Australian cultivar Ascot, and (3) to identify the relationship between selected *A. fabae* resistant faba bean accessions and Ascot, as these may be used as alternative sources of resistance.

Chapter 2. Literature Review

2.1 Introduction

This review of literature discusses aspects relating to resistance of faba bean to *Ascochyta fabae,* the cause of Ascochyta blight.

This begins with description of the teleomorph and anamorph of *A. fabae,* followed by the symptoms of the disease on leaves, stems and pods of faba bean, the impact of blight on yield and ways to control the disease. In addition, it reviews the epidemiology and the variability of the pathogen.

In the genetics of resistance of faba bean to *A. fabae*, this review focuses on differences in the terminology and the types of resistance and the nature and variability of resistance in faba bean germplasm. It also notes the selection of resistance to Ascochyta blight with concentration on sources of heritable resistance, the development of suitable screening techniques, the assessment of resistance, and factors affecting successful inoculation.

The final section of this chapter reviews the effect of variability within faba bean genotypes on determining the type and number of genes carrying resistance and AFLP markers as a means to determine genetic variability of crops, including faba beans.

2.2 Ascochyta blight on faba bean

The genus *Ascochyta* was named by Link in 1883 (reviewed by Punithalingam, 1979). The pathogen causing Ascochyta blight has been ascribed to *Ascochyta pisi* Lib., *Ascochyta fabae* Speg., *A. pisi* Lib. var. *fabae* Speg., but all the descriptions refer to the same disease (Beaumont, 1950), which was first recorded in England by Carruthers and Spegazzini in 1899. It was not recorded in England

again until 1927, and has since been identified widely throughout the world (Beaumont, 1950).

2.2.1 The Anamorph of *Ascochyta fabae*

The anamorph, *Ascochyta fabae,* has been described as follows. Colonies growing on potato dextrose agar (PDA) are white or light grey with pycnidia scattered abundantly within the mycelium (Punithalingam and Holliday, 1975). However, on oat meal agar (OMA), colonies were described as yellowish and mycelia as hyaline to yellowish-brown (Jellis and Punithalingam, 1991). In culture, *A. fabae* grows between 8 and 33 ^{o}C, with an optimum range between 20 and 25 ^{o}C (Yu, 1947; Wallen and Galway, 1977).

Pycnidial dimensions of *A. fabae* varied from 172 - 178 µm (Yu, 1947); 150 - 220 µm (Omar, 1986), and 250 - 360 x 300 - 420 µm with ostioles 35 - 36 µm (Jellis and Punithalingam 1991). Pycnidiospore dimensions were 14 - 21 x 2.9 - 5.4 µm (Omar, 1986), 17.9 x 5.9 µm (Yu, 1947), and 16 - 19 x 3.5 - 4.5 µm (Punithalingam and Holliday, 1975). The optimum temperature for pycnidiospores to germinate was 22 ^{o}C, but the spores germinated well from 14 - 32 ^{o}C (Yu, 1947). The fungus is closely related to *A. pisi* but is distinguished by the larger size of its spores (Beaumont, 1950).

2.2.2 The Teleomorph of *Ascochyta fabae*

The teleomorph of *A. fabae* was reported for the first time in Cambridge, UK, by Jellis and Punithalingam (1991) and was identified as *Didymella fabae* Jellis & Punith. It was detected on infected stems, which had been gathered and put in net bags laid on a grassy bank close to a field trial. Immature ascomata were observed on the stems three months later but were not found on the pods, seeds or leaves. It was stated by these authors that *D. fabae* was also identified in Australia and may be

widespread in over-wintering bean straw. The teleomorph of the *D. fabae* was described as follows in Jellis and Punithalingam (1991). *Ascomata arranged in rows on bean straw, immersed, becoming partially erumpent, dark brown to blackish brown, subglobose, single, separate, sometimes in groups, 180 - 240 x 130 - 150 µm, with short necks, ostilate. Ostiole nearly circular, 35 - 50 µm wide, surrounded by dark brown cells. Asci arranged in relatively flat layer, hyaline, cylindrical to subclavate, bitunicate, eight-spored, 55 - 70 x 10 - 14 µm, usually constricted near the base to form a distinct foot. Ascospores irregularly distichorus, hyaline, smooth, slightly biconic, broadly ellipsoid, two-celled, constricted at the septum, with the upper cell broader than the lower cell, 15 - 18 x 5.5 - 6.5 µm. Naturally discharged ascospores on bean straw later turn yellowish brown to dark brown and sometimes three-septate* .

2.2.3 Symptoms of Ascochyta blight

A. fabae infects aerial parts of plants: leaves, stems and pods. Under ideal conditions, the disease is easily identified by the presence of pycnidia within the lesion, although in dry and warm conditions it may be confused with chocolate spot, another major disease of faba bean (Gaunt, 1983). Lesions on foliage caused by *A. fabae* are primarily circular and chocolate brown, although sometimes paler in the centre and slightly sunken with a clear margin (Punithalingam and Holliday, 1975). As they develop, the lesions become circular or elliptical of 2 - 22 mm x 2 - 16 mm, or less regular shapes, with a dark-brown or chocolate-brown edge (Li-Juan et al., 1993). At a later stage, many of the spots coalesce, forming irregular patches and turning almost black (Beaumont, 1950).

On stems, the spots are usually smaller, less abundant, deeply sunken into the host tissue, and darker than the leaf lesions (Punithalingam and Holliday, 1975; Gaunt, 1983; Li-Juan et al., 1993). In a severe attack, the stems can break at the site of infection and young plants may die (Gaunt, 1983).

Lesions on pods are similar to those on leaves, being circular or oval, dark brown with black margins and often sunken deeply into the host (Li-Juan et al., 1993). The infection may lead to the whole pod becoming necrotic; seed does not set and the pod aborts and falls off (Gaunt, 1983). Infected seeds normally do not germinate well (Li-Juan et al., 1993).

The differences between the reactions of susceptible and resistant cultivars to infection of *Ascochyta fabae* were described by Maurin et al. (1993). In the susceptible lines, a lesion consisted of a central necrotic region where the fungus invaded intercellular spaces between the epidermis and the mesophyll. Many cells collapsed even though no fungal hyphae existed in the surrounding region. In the resistant line, a hypersensitive reaction occurred and caused flecking but the fungus failed to develop further after penetration. The host cells underlying the flecks were orange-brown, and granal lamella of chloroplasts of the adjacent mesophyll cells were swollen. Although some necrotic lesions appeared, the development of these lesions was limited.

2.2.4 Yield losses

Ascochyta blight may cause loss of yield and also reduce the quality of faba beans through seed infection. The level of yield depression due to this disease varies, depending on the severity of the attack. On susceptible plants, lesions develop very quickly, particularly on younger leaves, and the yield loss can be high (Pritchard et al., 1989). Estimates of the yield loss resulting from Ascochyta blight vary. Madeira et al (1988) found 15% loss for a comparison between a prophylactic fungicide treatment and untreated control. Hampton (1980) found 35% loss for green biomass yield and 34 % for grain yield. Yield losses as high as 50% were reported in New Zealand for winter-sown beans (Gaunt and Liew, 1981), while Gaunt (1983) confirmed that complete loss could occur if there were local outbreaks. Similarly, Hanounik (1980) reported that infection levels on Giza-4, a highly susceptible

cultivar, were severe when inoculated with *A. fabae*, and yield decreased by 90 % compared with that of plants treated with the fungicide mancozeb.

2.2.5 Control of the disease

There are four main methods to control Ascochyta blight: with fungicides, disease-free seed, cultural methods, resistant varieties.

Fungicides can be applied either as seed treatments or foliar spray (Rahat et al., 1993). Of nine fungicides tested *in vitro* against *A. fabae*, captan was the only one which was able to inhibit fungal growth completely at 50 ppm (Rahat et al., 1993). However, Wallen and Galway (1977) reported that neither captan, benomyl nor thiram as a seed treatment significantly reduced the infection of seedlings or adult crops by *A. fabae*. Although fungicides as foliar sprays reduced plant damage and suppressed disease spread (Tivoli et al., 1987), they were not able to control *A. fabae* entirely, even when sprayed at weekly intervals (Hanounik, 1980).

A. fabae can transmit to seedlings through infected seed if the seed source carries 2 - 15 % of infected seed (Hewett, 1973). Use of clean or disease-free certified seed has been successful to some extent in controlling Ascochyta blight in Manitoba, Canada (Rashid et al., 1991a). In the UK, the seed health standards are 0.1 % infection in pre-basic seed, 0.2 % in basic seed, 0.4 % in first generation, and 1 % in seed generation certified seed (Jellis and Punithalingam, 1991). A major problem with such a strict regulation is that it is difficult to produce seed of these standards, particularly in wet seasons (Jellis et al., 1985).

Cultural control of Ascochyta blight is frequently effective, especially when combined with chemicals and certified seed. In dry environments crop residue persists from one season to the next, and this can provide inoculum to infect subsequent crops. Rotation or inter cropping with cereals is recommended in China (Li-Juan et al., 1993). In Australia, late sowing has been used in some areas to reduce disease severity on faba bean. Compared with early sowing, late sowing might reduce

expenditure on fungicides by 60%, although the yield potential is lower (Knight, 1994).

The best means of controlling Ascochyta blight in the long term is the use of resistant cultivars (Jellis and Punithalingam, 1991; Hanounik et al., 1993). Ascochyta resistant cultivars reduced seed infection and thereby contributed to seed certification in the UK, and have resulted in fewer crops being rejected on the basis of poor seed quality (Bond and Pope, 1980).

2.3 Epidemiology

2.3.1 Sources of infection

There are three major ways that *A. fabae* survives between crops: in infected seeds, on volunteer plants, and in debris from previous infected faba bean crops (Gaunt, 1983; Bond and Pope, 1980).

A. fabae can survive well over 12 months on stored seed under normal laboratory conditions (Gaunt, 1983). As little as 1 % infected seed in winter-sown beans has been shown to produce severe epidemics and severe yield loss (Gaunt and Liew, 1981). It has been suggested that planting infected seeds is the main cause of crop infection in China (Li-Juan et al., 1993).

Volunteer plants could be an important source of infection if they grow in or immediately adjacent to new crops (Gaunt, 1983). In the UK, volunteer plants could be a more important carryover mechanism than diseased seeds (Bond and Pope, 1980).

Crop debris is also a major source of infection, particularly in dry areas where faba beans are planted in short rotations and crop debris is not degraded between seasons (Rashid et al., 1991a). In Australia, crop debris is probably the main source of infection of *A. fabae* (Geard, 1962). As the teleomorph has been found on bean-straw debris, the dispersal of the pathogen from the infected debris could be very important (Jellis and Punithalingam, 1991). Pycnidia and pycnidiospores are

able to survive on faba bean stem and pod debris during winter or summer in China (Li-Juan et al., 1993). However, *A. fabae* was not able to survive over winter in field plots in Canada (Wallen and Galway, 1977).

2.3.2. Pathogen dispersal

Several mechanisms have been proposed to account for the dispersal of *A. fabae* within and between crops. Li-Juan et al. (1993) stated that when the weather became warm and humid, numerous pycnidiospores are released from the pycnidia through ostioles to infect lower leaves. Gaunt (1983) suggested that the spread of the disease caused by *A. fabae* within crops after initial infection could be through splash dispersal. Hewett (1973) suggested that *A. fabae* spreads around 10 m, whereas Bond and Pope (1980) indicated that the spread from volunteer plants could be as far as 200 m in winter grown crops. Punithalingam (1993) concluded that as infection occurs over long distances, it is possible that conidia are dispersed by wind.

There is no evidence for systemic infection of the whole plant, but it has been suggested that spread within a plant occurs by physical contact and transfer of pycnidiospores (Pritchard et al., 1989).

2.4 Variability in *Ascochyta fabae*

A high potential for variation within populations of fungal pathogens has been reported. One of the reasons for this is to sustain their existence. As simple organisms, fungi are able to evolve rapidly and adapt easily to different genotypes of hosts when they are modified (Clutterbuck, 1995). There are four major ways by which fungal pathogens change: (1) mutation in somatic cells; (2) sexual reproduction and recombination of nuclear genes; (3) somatic recombination in which haploid nuclei, carrying different genetic material, fuse to form diploid nuclei, (4)

extra chromosomal variation which usually occurs when there is mutation in cytoplasmic genes (Russell, 1978).

Kharbanda and Bernier (1980) reported considerable variability in morphological characteristics (granulation, size of conidia, production and pigmentation of pycnidia) of *A. fabae* isolates from several regions of the world. However, variation in these characteristics did not correlate with differences in pathogenicity or to the geographic origin of the isolates.

There is some controversy amongst different authors about the nature of pathogenic variability, particularly in relation to the concept of physiological races. "Race is a term commonly accepted in biology to denote a group of individuals possessing common features which distinguish them from other groups of the same kind within formally recognised species or subspecies" (Caten, 1987). Allard (1960) concluded that races are produced by sexual reproduction and recombination of genes which determine virulence and avirulence. Although the term race, abbreviated from physiological race, has been widely used, many authors have criticised the concept and its value (Caten, 1987).

Vanderplank (1982) argued that the concept of races is absurd. Due to the way races are identified based on their reaction on different cultivars, it would be impossible to determine the potential number of races, particularly if the host carries many different resistance genes. The author illustrated that approximately one billion races would exist for only 30 different resistance genes in the host. Moreover, races are not fixed and as new differential cultivars are added, previously identical isolates can be separated into different races (Vanderplank, 1982)

Parlevliet (1995) stated that races can only be identified in pathosystems which are characterised by major resistance genes, mostly of a dominant nature, and not by durable resistance. However, Caten (1987) concluded that physiological races should not be totally abandoned as they can still provide useful codes for particularly important pathogen genotypes.

Studies of pathogenic variability in *A. fabae*, leading to the identification of physiological races, have been carried out by several researchers. Kharbanda and Bernier (1980) reported that there was considerable pathogenic variability in *A. fabae*, with every isolate revealing a different reaction on each of the three cultivars tested; however, they were not able to identify physiological races by using host differentials. Another study conducted by Hanounik and Robertson (1989) showed that it was possible to classify eight isolates of *A. fabae* collected from Syria into four races based on their interaction on two cultivars tested. In a like manner, Rashid and Bernier (1985) found a large variation in the degree of pathogenicity of 10 isolates and a significant interaction between isolates and individual lines of faba bean in Canada. From this study it was possible to classify the fungi into 10 races based on their interaction on 15 lines tested. Another investigation by Rashid et al., (1991a) demonstrated that there were seven races of *A. fabae* based on the interaction between eight inbred lines of faba bean inoculated separately with ten isolates of *A. fabae*. Morphological variation of *A. fabae* has been identified among Australian isolates, but there was some uncertainty about the number of races that were distinguished (Lawsawadsiri, 1994).

2.5 Genetics of Resistance

2.5.1 The terminology of resistance to disease

There are many definitions of resistance to disease, and differences between definitions might result in confusion in interpreting results of resistance studies. Robinson (1969), for example, defined resistance as "the ability of the host to hinder the pathogen", while Day (1974) stated that resistance is displayed by "a host plant which does not become invaded and is not diseased". Russell (1978) broadened Robinson's definition and defined resistance as "any inherited characteristic of a host plant which lessens the effect of parasitism".

2.5.2 Types of resistance to disease

Based on the mechanisms of resistance, the mode of inheritance or epidemiology, different authors express the type of resistance in various ways. Day (1974) and Heat (1981, 1991) distinguished resistance into two major types: host resistance and non-host resistance. The former is the host being able to limit the growth of the pathogen as a result of genetic modification (Day, 1974). It is characterised by a parasite-specific reaction and controlled by a single or few genes with gene-for gene interactions (Heat, 1991). Non-host resistance, which is also called basic resistance, is multi-component, genetically complex and characterised by non-specific resistance. As a result, this resistance is highly effective, durable and not easily overcome through changes in the pathogen (Heat, 1991). According to Day (1974), it is likely that the resistance mechanism of non-host resistance is similar to that of host resistance. In practice, identifying non-host resistance is not easy.

Day (1974) divided genetic resistance into three categories, (1) oligogenic, (2) polygenic, and (3) cytoplasmic.

Oligogenic resistance is controlled by one or few genes. Russell (1978), however, was more specific and divided this type of resistance into monogenic, controlled by one gene, and oligogenic by a few genes. Such genes are often called major genes. According to Manners (1993), oligogenic resistance is most often dominant, conferring complete resistance or even immunity, Mendelian ratios are obtained in crosses, and the complete nature of the resistance results in unambiguous classification.

Polygenic resistance, however, is determined by many genes which individually have only a small effect on the expression of resistance (Day, 1974). These genes have frequently been called minor genes (Russell, 1978). This type of resistance is less common than oligogenic resistance and Mendelian ratios are not obtained in crosses (Manners, 1993).

Cytoplasmic resistance is less well known, particularly in higher plants, even though the cytoplasm and its organelles play an important role in heredity (Manners, 1993).

Vanderplank (1963) introduced two epidemiological terms for resistance: horizontal and vertical resistance. The former is defined to describe the many-gene resistance, and the latter to describe the single-gene resistance. Robinson (1969), however, synonymised vertical resistance as race-specific resistance characterised by the presence of genetic interaction between host and pathogen genotypes, and horizontal resistance as race non-specific resistance characterised by the absence of such genetic interaction. Vanderplank (1982) stated that vertical resistance always breaks down, while horizontal resistance is durable. However, Parlevliet (1995) argued against this concept, because it has been shown that monogenic resistance can be durable. Similarly, Mainers (1981) found that much of the resistance in edible legumes is race-specific and shows long lasting efficacy, with only a few cases where resistance has been overcome by pathogens.

Other important types of resistance are defined as field and adult resistance (Manners, 1993). The former is characteristically effective under natural conditions in the field, but is difficult to detect in laboratory or glasshouse tests (Russell, 1978). Adult-plant resistance occurs if the degree of resistance increases in adult plants. Conversely, seedling resistance can be identified in very young plants; sometimes the resistance can also occur in adult plants (Russell, 1978). Adult plant resistance might occur when genes carrying resistance are partially expressed in seedlings but confer complete vertical resistance in adult plants (Vanderplank, 1984).

2.5.3 Nature of the resistance of faba bean to *Ascochyta fabae*

Genetic differences in the reactions of faba beans to *A. fabae* have been reported by many authors. Hanounik and Robertson (1989) found that several lines carried either minor or major resistance genes to *A. fabae*. In a study of the reaction

of genotypes over a wide range of environments, lines BPL 471 and BPL 2485 were found to carry genes for broad-based general resistance, while BPL 818 and ILB 1814 were resistant to prevalent isolates in some regions, but susceptible in others (Hanounik and Robertson, 1989)

Resistance to *A. fabae* might be controlled by one or more resistance gene(s). Lawsawadsiri (1994) suggested that the Australian accession Acc 970 derived from ILB 752, carries a single dominant gene relative to an Australian isolate of *A. fabae*, designed as A26, while Ramsey et al. (1995) concluded the resistance of Ascot was conferred by a single recessive gene. However, Jellis and Vassie (1995) suggested that the low frequencies of homozygous resistant families in F_3 populations between the resistant 29H and either Toret or CH 170 were due to segregation at more than one gene.

Anti-nutritional factors (ANFs) such as tannin and vicine/convicine, have been proposed to play a role in inducing resistance to *A. fabae.* Fagbola and Jellis (1994) reported that low tannin is associated with the white-flowered character in faba beans, and that these were more susceptible to *A. fabae* than faba beans with coloured flowers in inoculated trials. Conversely, Helsper et al. (1994) found that condensed tannins do not contribute to resistance toward *A. fabae*, as leaves, stems, pods and seeds of tannin-free lines showed similar levels of damage due to *A. fabae* as the tannin-containing lines. Jellis and Vassie (1995) identified *A. fabae* resistant low tannin genotypes indicating that the previously described association between susceptibility to *A. fabae* and low tannin genotypes was probably due to a chance association between these traits in the lines tested rather than a physiological effect. Jellis and Vassie (1995) also reported linkage between the gene for zero vicine/convicine and the gene(s) for resistance to Ascochyta blight.

Faba beans with the *ti-1* gene, which induces a determinate growth habit and reduced straw length, are more susceptible to *A. fabae* than indeterminate plants with long straw (Jellis et al., 1985). This might be because the long straw results in lower humidity in the canopy and this environment is less conducive for development of *A.*

fabae than that of wetter conditions in short straw plants (Jellis et al., 1985). A relationship has also been observed between flowering date and resistance to *A. fabae* (Lockwood et al., 1985), as earlier flowering plants were more susceptible than later flowering plants. This may have been related to growth habit, where determinate plants tend to flower earlier.

2.6 Selection of resistance of faba bean to *Ascochyta fabae*

The development of disease resistant varieties requires integration between the areas of plant breeding and plant pathology and there are at least three prerequisite steps. These are (1) availability of useable sources of heritable resistance, (2) development of a suitable screening technique, and (3) a suitable selection procedure (Innes, 1992; Parlevliet, 1995).

2.6.1 Sources of heritable resistance

Information concerning the availability of useable sources of resistance will contribute to the effectiveness in developing varieties with a reliable level of disease resistance. Resistance to some diseases is poorly documented while resistance to others is readily available (Johnson, 1992). When desired genes are rare or not available in the current breeding material, they might be obtained by several methods including plant collections, induced mutation and gene transfer from related species (Russell, 1978; Lawes et al, 1983; Innes, 1992, Parlevliet, 1995).

A large number of plant collections, including sources of disease resistance, have been established in many centres in the world. ICARDA, Aleppo, Syria has the largest collection of faba beans, with around 3300 accessions. Other centres with faba bean collections include ZGK, Gatersleben, Germany; SVP, Wageningen, the Netherlands, and the Germplasm Institute, Bari, Italy (Jana and Singh, 1993; Lawes et. al. 1983).

Resistance to Ascochyta blight has been identified in germplasm from many countries, principally in WANA and Europe. These sources of resistance were summarised by Bond et al. (1994) as BPL 74 from Iraq; BPL 230 and 365 from Morocco; BPL 460, 465, 471 and 472 from Lebanon; BPL 818 from Ethiopia; BPL 266 from Greece; BPL 646, 2485, ILB 752, L83118, L83120, L83124, L83125, L83127, L83129, L83136, L83142, L83149, L83151, L83155, L83156, L82001 and L831818-1 selected by ICARDA; Quasar and Line 224 from UK; and 29H from France.

Accessions may be introduced by breeders on the basis of overall performance, because they carry a specific trait such as disease resistance, due to similarities between the region of origin and the target environment, or for widespread evaluation when there is little information on sources of the desired trait. At the Waite Agricultural Research Institute, for example, over a thousand faba bean accessions have been introduced from many countries and centres. Many of these were introduced from ICARDA as sources of disease resistance, including accessions that are resistant to Ascochyta blight. Ascochyta blight resistant accessions that have been introduced from ICARDA include BPL 74, 230, 365, 460, 465, 471, 472, 646, 818 and 2485, and ILB 752, L83120, L83124, L83125 and L83126 (Knight and Paull, pers. comm.) and these are considered as important inbred sources (Robertson and Saxena, 1993). Many of these lines were utilised in the present study.

Mutagenesis may be used to generate genetic variation for a trait if the variation does not occur in the crop gene pool. Mutagens, such those resulting from ionising radiation and chemical mutagenesis, have been used on faba bean and in general have produced morphological variation. They also usually result in a decrease in seed production, seedling germination, seedling vigour, and pollen fertility (Lawes et al., 1983). As resistance to *A. fabae* occurs within the faba bean gene pool, there is no requirement to attempt to induce mutations for this trait.

Another way to enhance the genetic variability of disease resistance is by the transfer of resistance genes from related species. Intensive backcrossing is often required for this process (Innes, 1992), to minimise the number and effect of undesirable characters from the alien source (Parlevliet, 1995). Developments in biotechnology leading to technology enabling the transfer of single genes might overcome these problems for traits controlled by major genes. This technology is likely to have a far lower impact on quantitative traits conditioned by many genes of minor effect.

High levels of resistance to diseases are found among relatives of *V. faba*, for example resistance of *Vicia narbonensis* to *A. fabae*. However, attempts to develop inter-specific hybrids between *V. faba* and its relatives have been unsuccessful, thus restricting the possibility of transferring resistance to *V. faba* (Lawes et al., 1983).

2.6.2. Development of a suitable screening technique

The methods for selecting disease resistance can be divided into three major categories: (1) field, (2) glasshouse (or growth chamber), and (3) laboratory methods (Hanounik and Maliha, 1984; Wolfe and Gessler, 1992; Parlevliet, 1995).

Screening tests for disease resistance conducted in the field are sometimes preferable, not only because large populations can be used and the process is under natural conditions (Russell, 1978), but also because breeders are able to select quantitative resistance, particularly when it is combined with other desirable agronomic characters (Wolfe and Gessler, 1992). It is easier to screen for resistance in the field if the epidemic of the fungal disease causes damage in most years, but if there is irregular occurrence of the pathogen in time and space, it might cause loss of a complete season of evaluation (Parlevliet, 1995).

Natural infection of faba beans by Ascochyta blight is often too uneven for satisfactory disease resistance evaluation (Van Breukelen, 1985). Uniform exposure to Ascochyta blight in the field usually requires artificial inoculation and maintaining

a high level of humidity by covering the entire plot overnight with a polyethylene sheet (Hanounik et al., 1993). Moreover, to improve spread of the pathogen, a susceptible genotype is planted within the trials. This method has been used at ICARDA (Hanounik and Maliha, 1984).

A two cycle screening technique was used by Hanounik and Maliha (1984) to screen a large number of plants for reaction to a wide range of pathogenic variability. The first cycle used a mixed inoculum of *A. fabae*, collected from different regions, to identify resistant cultivars on which only a few lesion appeared. In the second cycle, isolates believed to be virulent phenotypes, collected from the lesions on resistant plants of the first cycle, were then used for inoculum to screen the selected progenies. This technique succeeded in detecting reliable sources of resistance to *A. fabae* in the germplasm of ICARDA.

Another screening technique which is less costly, easy to carry out, and representative is the use of artificial screening methods conducted in a growth chamber or glasshouse (Parlevliet, 1995). This method is excellent for screening progeny with different races (Innes, 1992), and allows testing to be conducted throughout the year (Hanounik et al., 1993). This technique tends to identify and select major resistance genes that might be race specific which might easily be broken down. Therefore, it is only capable of detecting qualitative resistance and not suitable for screening for quantitative resistance (Parlevliet, 1995). Zakrzewska (1988) compared the results of screening for resistance to Ascochyta blight in the glasshouse with those of field trials and concluded that, during two years of screening, the intensity of disease was lower in the greenhouse than in the field, and variability in resistance was lower in field trials than in the greenhouse. However, Tivoli et al. (1987) reported that the growth chamber test was as effective as field screening in ranking the susceptibility of faba bean genotypes to Ascochyta blight during two years of cultivation. It has been suggested, therefore, that screening in the glasshouse can be used efficiently by breeders (Tivoli et al., 1987; Rashid et al., 1991a).

In many pathosystems, screening systems that use germinating seeds, seedlings or even plant parts are able to discriminate between resistance and susceptibility of many plants (Parlevliet, 1995). In faba beans, seedling screening techniques are particularly effective where selected plants can subsequently be isolated from insect pollinators to ensure self pollination (Hawtin, 1982). Van Breukelen (1985) suggested that the glasshouse test is not only suitable for discrimination between genotypes with small differences in resistance, but can also be utilised to select lines with uniform resistance to Ascochyta blight, and so it can reduce the cost of testing by limiting the amount of material to be tested in the field. In the current project, all experiments were conducted in a glasshouse as this has been found to be reliable, inexpensive and it enabled experiments to be conducted throughout the year.

Field and glasshouse based screening techniques rely on either naturally occurring or artificially induced disease epidemics. Plants are then rated on the basis of symptom expression, or their phenotype. Potential problems and limitations with these methods include uneven disease development resulting in misclassification, time taken for the assay, space required to grow plants and the limited number of traits which can be measured on each plant. Artificial screening methods conducted in a laboratory have the potential to overcome many of these limitations, and such methods may employ germinating seeds, very young plants, or even plant parts (Parlevliet, 1995). These screening methods generally identify major resistance genes and are unsuitable to screen for quantitative resistance (Parlevliet, 1995).

The detached leaf or stem test is one example of a laboratory based screening method. This method was used to distinguish 19 strains of bacteria *Pseudomonas syringae* pv. *syringae* on pear into 4 virulence groups (Yessad et al., 1992). The detached leaf test has also been used for screening faba beans for resistance to chocolate spot (Dennis, 1991), and to Ascochyta blight, where Lawsawadsiri (1994) differentiated between the *A. fabae* resistant line, Acc 970, and the susceptible line, Acc 508.

2.6.3 The Assessment of resistance

Generally, the growth and development of the pathogen will be less on resistant than susceptible plants. However, it is usually not possible to estimate the amount of pathogen present and evaluation of a crop reaction to disease has been based on estimation of the damage (Parlevliet, 1995). There are two ways to measure the amount of disease: disease incidence which is the proportion of plants infected; and disease severity, which is the proportion of plant tissue infected (Parlevliet, 1995).

The degree of attack can be estimated for each plant or group of plants and assigning a numerical score (Russell, 1978). A 0-5 scale (Kharbanda and Bernier, 1980) and a 1-9 rating scale (Hanounik and Robertson, 1989) have been used to classify *A. fabae* reaction on faba bean stems and leaves. Faba bean does not express a defined resistant reaction to *A fabae* , therefore the classification to reaction types, such as moderately susceptible or moderately resistant, is often arbitrary.

2.7 Factors affecting successful inoculation

Environmental conditions for inoculation and incubation, inoculum concentration and plant age are all major factors affecting inoculation success (Russell, 1978). Pritchard et al. (1989) reported that covering plants for 24 hours after inoculation to induce high humidity was sufficient for high infection. Van Breukelen (1985) concluded that symptom development was not significantly different between temperatures of 15 ^{o}C and 20 ^{o}C. Long incubation periods at high humidity increased the number of lesions, and covering plants for 72 hours after inoculation was sufficient for effective infection.

The age of faba bean plants affects the development of Ascochyta blight infection and younger tissue is more susceptible than old tissue (Pritchard et al., 1989). Lesions developed more rapidly on seedlings at the two leaf stage than at the

three leaf stage (Van Breukelen, 1989). Inoculation at three weeks (Rashid et al., 1991a) and four weeks (Lawsawadsiri, 1995) after sowing have both resulted in effective infection and discrimination between resistant and susceptible plants.

Inoculum concentration plays an important role in achieving the most satisfactory results. Van Breukelen (1985) applied inoculum of *A. fabae* to faba bean at a concentration between 6 x 10^5 and 12 x 10^5 spores per ml with 1ml of inoculum per plant. Rashid et al. (1991b) used a concentration of 1 x 10^6 with a sprayer and 3 - 4 ml per plant. Lawsawadsiri (1995) used a lower concentration of 3 - 5 x 10^5 spores/ml to inoculate seedlings of faba bean in a glasshouse, and this resulted in successful development of lesions on susceptible plants. This concentration was also applied in the present study.

2.8 Genotype variability within populations of faba bean

A study on the resistance of faba bean to disease requires an understanding of the variation within populations of the crop. Faba bean is partially cross-pollinated at levels varying from 4 to 84% depending on the genotype used, the methodology of the experiment, and the number of insect pollinators (Lawes et al., 1983). Cross pollination of faba bean is mainly mediated by honeybees (Rashid and Bernier, 1993). Curry et al. (1990) reported that in caged plots, honeybees were more effective in pollinating faba bean than leafcutter bees. Honeybees were identified as the only pollen vectors of faba bean in South Australia and Western Victoria where the incidence of pollination, was on average, 80% (Stoddard, 1991).

A consequence of their partially cross-pollinated nature is that most faba bean cultivars have levels of heterozygosity, heterogeneity and heterosis which are intermediate to those present in completely outcrossing and completely self-pollinating crop species (Lawes et al., 1983). Thus, there is often variation within cultivars or populations for traits such as resistance to disease, and single plant and

mass selection techniques can be applied to populations of faba bean as long as the trait is not subject to over-dominance.

It is important to develop uniform genetic material prior to undertaking genetic studies. Self pollination can reduce heterogeneity, and it was reported that after 4 cycles of selfing, inbred lines were homozygous for reaction to *A. fabae* (Rashid and Bernier, 1985). However, only one cycle of selfing, with progeny testing, was undertaken in developing genetic material for use in experiments described in this thesis due to limited time available.

2.9 AFLPs and genetic variability

Successful breeding programs depend on understanding of the amount and distribution of genetic variability present in the gene pool. This information may be used to select genotypes to cross for population development (dos Santos et al., 1994). Conventionally, identification of genetic variability relied on measurement of agronomical and morphological traits. However, this method is time consuming and problematical if the crop is heterogeneous with many overlapping morphological and physiological attributes (Paul et al., 1997). Recently, molecular techniques have been used to overcome this problem.

Molecular markers which have been used to characterise plant genetic variability include isozymes (Jana and Pietzrak, 1988), Restriction Fragment Length Polymorphisms (RFLPs) (dos Santos et al., 1994; Sharma et al., 1996), Random Amplified Polymorphic DNAs (RAPDs) (Williams et al, 1990; Vierling and Nguyen, 1992; dos Santos et al., 1994; Link et al., 1995), and Amplified Fragment Length Polymorphisms (AFLPs) (Cho et al., 1996; Sharma et al., 1996; Paul et al., 1997).

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (Vos et al., 1995). Similar to RAPD analysis, AFLP assays need no prior sequence knowledge, but detects greater number of loci than that of RAPDs (Maughan et al., 1996). Vos et al. (1995)

stated that AFLP is a combination of the reliability of the RFLP technique and the power of the PCR technique. Therefore, this technique is robust and reliable.

AFLPs have advantages for determining the degree of genetic variation among germplasm collections and reveal better results compared with the other molecular markers above. A major limitation of isozymes, for example, is the low levels of polymorphisms detectable, RFLP requires a larger amount of relatively pure DNA and as a result the extraction is laborious and uneconomical (Paul et al., 1997), while RAPDs are more sensitive to experimental conditions and the level of polymorphism is lower (Sharma et al., 1996; Paul et al., 1997). A comparison of some methods used in measuring genetic variation is listed in Table 2.1.

AFLP markers have been successfully employed to characterise genetic diversity in a wide array of plant species. Zhu et al. (1998) demonstrated that AFLP markers provided a wide coverage throughout the rice genome. This is important for biodiversity studies, as it minimises the risk of bias due to localised regions of high polymorphism. Sharma et al. (1996) reported that AFLP analysis reconstructed the phylogenetic history of the genus *Lens* better than that of other techniques. Similarly, Maughan et al. (1996) was able to determine the genetic relationship among soybean accessions by AFLP analysis. There are no published reports of the use of AFLPs to detect genetic variability among faba bean lines. Therefore, the present study adapted protocols for AFLP analysis to studying faba beans.

Table 2.1 Features of some currently methods currently employed in measuring genetic variation (after FAO, 1996)

Method	Variation Detected	Sample throughput	Loci analysed Per assay	Reproducibility between assay	Type of character analysed	Inheritance of character analysed	Technology level required
Morphology	low low	high	low no.	medium	phenotypic trait	qualitative/ quantitative	
Pedigree Analysis	medium	n.a	n.a	good	degree of coancestry	n.a	low
Isozymes	medium	medium	low no.	medium	proteins	co-dominant	medium
RFLP (low copy)	medium	low	low no. (specific)	good	DNA	co-dominant	high
RFLP (high copy)	high	low	high no. (specific)	good	DNA	dominant	high
RAPD	high to medium	high	high no. (random)	poor	DNA	dominant	medium
DNA Sequencing	high	low	low no. (specific)	good	DNA	co-dom./ dominant	high
Seq tag SSRs	high	high	medium no. (specific)	good	DNA	co-dominant	high
AFLPs	medium to high	high	high no. (random)	medium	DNA	dominant	high

A phylogenetic tree indicating the genetic distance among *A. fabae* resistant lines was constructed to enable a comparison of genetic distance per se and the genetic control of resistance to *A. fabae*. The objective of this study was to identify the most appropriate lines to be used to diversify the source of *A. fabae* resistance to be used in the Australian faba bean breeding program.

2.10 Conclusion

Evidence has been presented that *A. fabae* is one of the most destructive pathogens on faba bean and causes not only significant losses in production but also decreased quality. Chemical control can provide crop protection to some extent, but the combination of cultivating Ascochyta blight resistant cultivars integrated with

cultural methods would improve disease control and reduce fungicide use. Better understanding of the genetics of both the pathogen and the host is necessary in order for disease resistance to be utilised as a long term strategy.

Pathogenic variability in *A. fabae* is high. This variability has led some authors to identify physiological races of A. *fabae* (Kharbanda and Bernier, 1980; Rashid and Bernier, 1985; Hanounik and Robertson, 1989; Rashid et al., 1991a). They suggest that physiological races could be classified based on a partial interaction amongst pathogens and hosts. In Australia, little work has been carried out on identifying pathogenic variability of *A. fabae.* Therefore, part of the experimentation in this study was to investigate the variation of pathogenicity of *A. fabae* isolates collected from different regions in Australia.

There is also a great variation within faba bean accessions as a consequence of their partially cross-pollinated nature. This introduces complications to studies on *A. fabae* resistance of faba bean. To limit this complication, selfing of single plants together with progeny testing was used to minimise heterogeneity and heterozygosity in the present study.

An integrated investigation of resistance of faba bean to *A. fabae* was carried out using both laboratory and glasshouse methods. The laboratory methods focused on AFLP (amplified fragment length polymorphisms) with the aim of identifying the genetic differences among resistant lines of diverse origin. The glasshouse methods were utilised simultaneously to investigate the reaction of both parent and offspring lines of faba bean to *A. fabae.* The integration of both methods enabled a more robust explanation of resistance in faba bean.

Chapter 3. General Materials and Methods

3.1 Genetic Materials

Accessions of faba bean used for the present study are listed in Table 3.1. These lines were selected based on their reputation for resistance to *A. fabae* as reported by several authors and represent a range of geographic origins, although most are from the Mediterranean basin (Table 3.1).

The majority of the lines in this study were introduced from ICARDA. Two collections of faba beans are maintained at ICARDA and accessions in these two collections are of different genetic composition. The landrace collection, designated ILB (International Legume Faba Bean), comprises the original germplasm accessions which are usually highly heterogeneous and heterozygous. As a result, these accessions are maintained as populations. The Faba Bean Pure Line collection, designated BPL, consists of selections derived from the ILB collection following several rounds of single plant selection with selfing. ICARDA has also released accessions selected from hybridisation between two ILBs. For example, L 83120 was derived from a cross between ILB 284 and ILB 37 and this line was selection 120 at Lattakia in 1983.

Faba beans are partially cross-pollinated and therefore individual plants generally have a high level of heterozygosity. The duration of the project did not allow an extensive period of inbreeding and progeny testing to develop uniform homozygous lines. Therefore the alternative strategy of one round of selfing and limited progeny testing to establish the reaction of the original population to *A. fabae* was adopted. As many of the lines tested were the product of several cycles of single plant selection and selfing during the development of the BPLs, this compromise was

Table 3.1 Faba bean accessions used for the study

Waite Acc.	ICARDA Acc[a] Name	Original population/ pedigree	Origin	Reaction to *A. fabae*	References
290	BPL 230 A884	ILB 142	Morocco	Resistant	1, 5
295	BPL 460 A888	ILB 284	Lebanon	Resistant	1, 3, 5
297	BPL 465 A889	ILB 285	Lebanon	Resistant	1, 5
299	BPL 471 A8710	ILB 287	Lebanon	Resistant	1, 2, 3, 5
303	BPL 472 A8713	ILB 287	Lebanon	Resistant	1, 3, 5
342	Giza-4	-	Egypt	Susceptible	3, 5
484	BPL 74 A883	ILB 37	Iraq	Resistant	1, 3, 5
496	BPL 365 A886	ILB 227	Morocco	Resistant	1, 5
508	BPL 818 A8815	ILB 549	Ethiopia	Resistant	1, 2, 3, 5
622	Ascot component		Naxos	Resistant	4
668	L 83120 A882	BPL 460* ILB 37	ICARDA	Resistant	1
674	L 83124 A8824	ILB 37* BPL 2485	ICARDA	Resistant	1
680	BPL 2485 A88302	ILB 37	Iraq	Resistant	1, 2, 3, 5
683	L 83114 B8826	ILB 938* BPL1390	ICARDA	Resistant	7
712	L 82003 B8834	BPL 472*BPL 261	ICARDA	Resistant	7
722	L 82009 B8837	A2* ILB 938	ICARDA	Resistant	7
948	Quasar		UK	Resistant	6
970	ILB 752		Sweden	Resistant	1, 2, 5
975	L 83125	BPL 460*BPL2485	ICARDA	Resistant	1
976	L 83136	ILB 37*BPL 460	ICARDA	Resistant	7
1046	BPL 646	ILB 382	UK, USSR	Resistant	1, 2, 3, 5
Icarus	BPL 710	L81-248571	Equador	Susceptible	4

[a]) The ICARDA accession number includes the selection number of the line provided to the Waite faba bean breeding program

1 Bond et al. (1994)
2 Rashid et al. (1991a)
3 Hanounik and Robertson (1989)
4 Ramsey et al. (1995)
5 Hanounik and Maliha 1984)
6 Lockwood et al. (1985)
7 Robertson, L. pers comm.

considered acceptable. Nine plants of each accession were grown in 30 cm diameter pots with three seeds sown per pot. These plants were grown in a bee-proof glasshouse to prevent outcrossing. Three seeds from each selfed plant were tested for reaction to isolate 331/91 of *A. fabae.* Accessions with all progeny resistant were considered homozygous resistant. Where accessions segregated for reaction to *A. fabae,* seed for further study was obtained from plants which had all resistant progeny (Appendix 3.1). The low multiplication rate of faba bean prevented sufficient progeny being tested to enable the genotype of each individual to be identified, while leaving sufficient seed for further experiments. Details of numbers of seeds of accessions, parents, F_1 hybrids and F_2 and F_3 progeny are presented in individual chapters.

3.2 Culture of the pathogen

Isolates of *Ascochyta fabae* were supplied by South Australian Research and Development Institute (SARDI), and represented isolates collected from different faba bean growing areas in Australia. Single spore cultures were stored in sterile water at 4°C. Ten isolates (Table 3.2) were used for the experiment on the interaction between isolates and genotypes (Chapter 4). The isolate found to be the most aggressive in this experiment (331/91) was used for the rest of the study. Isolates were subcultured on 2% PDA and incubated at 25°C for two weeks. Pycnidiospores were collected by flooding the surface of the agar with sterile RO (Reverse Osmosis) water and scraping with a glass rod. The spore suspension was then poured onto a new petri dish containing 2% agar and covered by autoclaved cellophane. The cultures were incubated for another two weeks at 25°C. Pycnidiospores were harvested as above, and the concentration was adjusted to 3-5 x 10^5 spores/ml using a

Neubauer® haemocytometer. The inoculum was applied to plants with 2-3 drops of Tween-20 per litre.

3.3 Screening Methods

Screening was conducted in plastic covered humidity chambers (cabinets) within a glass house. The cabinet had fold-down flaps at the front and these were left open during initial establishment, but closed following inoculation to maintain high humidity. The cabinets contained automated overhead misters which were programmed to operate up to six times a day. Seeds were sown in 7 x 5 x 5 cm plastic punnets, containing Recycled Soil (RS) (Appendix 3.2) with one seed per punnet. The punnets were placed on plastic trays with each tray holding 16 punnets (Figure 3.1).

The trays were placed on benches in a humidity chamber with overhead misting adjusted to 1 minute, 2 times per day to maintain soil moisture. Seedlings were inoculated 4 weeks after sowing using a two-litre hand sprayer with sufficient inoculum applied to wet all plant surfaces. During incubation (4 days), the misters were adjusted to 1 minute, 5 times a day and the cover was closed to maintain high humidity. The temperature of the glasshouse was maintained at or below 20°C by evaporative airconditioning.

3.4 Rating Scale

A 1-9 scale was used to rate symptoms of Ascochyta blight (Table 3.3). The classifications of resistant and susceptible in Table 3.3 follow Hanounik and Robertson (1989), but the actual classifications in experiments described in

Figure 3.1 **A.** The humidity chamber used for screening for reaction to *A. fabae*

B. Close-up photo of the faba bean plants in punnets

A

B

this thesis varied according to the objectives of the particular experiment and the reaction of the this thesis varied according to the objectives of the particular experiment and the reaction of the control entries within the experiment. Disease severity was rated when the symptoms were spread evenly across the trial (approximately 14 days after inoculation) and the symptoms on the susceptible controls were either 7 or 9.

Table 3. 2 Australian isolates of *A. fabae* used in experiments, with dates collected and origin of the isolates

Isolate	Date collected	Place of collection
166/92	31/7/92	Hamley Bridge (SA)
219/92	2/8/92	Alma (SA)
252/92	4/9/92	Clare (SA)
260/92	8/9/92	Tumby Bay (SA)
331/91	4/12/91	Kaniva (Victoria)
493/92	5/11/92	Bordertown (SA)
526/92	16/11/92	Largerag (SA)
201/92	19/8/92	Bordertown (SA)
A26	22/4/92	Mintaro (SA)
A37	unknown	Bordertown (SA)

Table 3.3 Scale for rating plant reaction to Ascochyta blight (after Hanounik and Robertson, 1989)

Disease Rating	Host Status	Description
1	Highly resistant	No lesions or very small nonsporulating resistant flecks (less than 0.5 mm in diameter).
3	Resistant	Few small, discrete, dark, non-sporulating lesions on leaves (1-2 mm in diameter), sometimes with hypersensitive reaction.
5	Moderately resistant	Some discrete, circular lesions with a moderate number of pycnidia on leaves and pods.
7	Susceptible	Many large, coalesced irregular lesions with many pycnidia on leaves, pods, and stems.
9	Highly susceptible	Extensive, large, coalesced, irregular lesions with many pycnidia on leaves, pods, and stems, stem constriction and girdling with many plants dead.

3.5 Statistical Analysis

A randomized complete block design was used for experiments described in Chapter 4, 5 and 6. Data collected were analysed by Analysis of Variance and unpaired t test calculated using the program Super ANOVA version 1. 11 written by Abacus Concept, Inc. (1989-1991) on a Macintosh computer for the experiments reported in Chapters 4, 5, and Chi-square analysis for Chapters 5 and 6.

Chapter 4. Variation in the resistance of faba bean (*Vicia faba* L.) to Ascochyta blight in Australia

4.1 Introduction

A number of studies have shown that there is large variation among genotypes of faba bean in the response to infection by a range of isolates of *A. fabae* (Zakrzewska, 1988; Rashid et al., 1991a). A high degree of variability for virulence of *A. fabae* has also been identified, as well as a differential interaction between isolates of *A. fabae* and lines of faba beans (Hanounik and Robertson, 1989).

Resistant lines showed variable reaction to isolates of *Ascochyta fabae* from different regions of the world (Hanounik and Maliha, 1984; Hanounik and Robertson, 1989). For example, accessions BPL 472 and BPL 818, which were resistant to *A. fabae* when tested in many countries, were susceptible to French isolates. This indicated that these accessions have specific resistance compared with several other lines which were consistently resistant in all countries tested (Hanounik and Robertson, 1989).

The experiments reported in this chapter aimed to identify variation in the resistance of several faba bean accessions, most of which had been tested in many countries, to Australian isolates of *A. fabae*. The variability of virulence among isolates of *A. fabae* collected from several regions in Australia was also studied.

4.2 Materials and Methods

The experiment was conducted in two stages using two different seed samples, namely bulk samples and single plant derived pure lines. The bulk samples were used

as the experiment was conducted early in the project and the pure lines had not been developed.

In September 1995, a series of eight experiments was conducted based on eight isolates of *A. fabae* collected from different regions in Australia. The eight isolates were A37, A26, 201/92, 166/92, 219/92, 260/92, 331/91 and 526/92. Details of the date and the place the isolates were collected are listed in Table 3.2. Each individual isolate was tested against eight accessions (299, 303, 342, 508, 680, 948, 970 and Ascot) which were selected on the basis of the known range of resistance reported by several authors. Four accessions (299, 303, 508 and 680) had been tested in several countries by Hanounik and Robertson (1989). Acc 948 and 970 were resistant lines that originated from Europe (Lockwood et al. 1985; Bond et al. 1994), respectively. Ascot was the resistant cultivar adapted to the Australian environment reported by Ramsey et al. (1995), while Acc 342 (Giza-4) was selected as the susceptible control, since this accession was used in the host differential set studies by Hanounik and Robertson (1989). The accessions used in this experiment were obtained from bulk samples maintained by the Waite faba bean breeding program.

The experiment was arranged as a series of randomised complete block designs to determine the reaction of each accession separately for each isolate. This enabled the effect of each isolate on a range of accessions to be determined but not the interaction between isolates and accessions. The experiment was conducted as a series of randomised complete blocks, rather than a split plot design, to prevent cross-infection by the various isolates if all were included in the one block. The experiment contained four replications, each of which was four plants.

Seeds were sown in plastic punnet in trays using RS soil. The trays were placed in a humidity chamber with overhead misting for 1 minute, twice per day to maintain

humidity. Four weeks after sowing, plants in the individual experiments were inoculated separately with the eight isolates adjusted to 3-5 x 10^5 spores/ml. Rating was conducted 14 days later using a 1-9 scale. Details of the procedure are described in Chapter 3.

The second trial was carried out using seeds collected from pure resistant selections as described in Chapter 3. Using the same procedure as above (the first trial), eight accessions (Acc299, 303, 342, 508, 680, 948, 970 and 622) were tested with eight isolates (252/92, A26, 166/92, 493/92, 219/92, 260/92, 331/91 and 526/92). In this experiment the cultivar Ascot, which is a composite of two selections, was replaced by Acc 622, one of the accessions from which Ascot was derived. The isolates A37 and 201/92 used in the first trial were not included in the second trial, due to contamination during multiplication and insufficient production of spores. The trials were arranged as above. Data from the series of experiments were individually analysed by analysis of variance, as described in Chapter 3.

4.3 Results

4.3.1 Trial 1

Symptoms of disease developed 14 days after inoculation, particularly on Acc 342 and Acc 299. Lesions caused stems of susceptible plants to break, while symptoms did not develop on leaves of resistant plants. The eight accessions in each series of experiments showed variation in reaction to the eight isolates (Table 4.1).

Four accessions (Acc 303, 948, 970 and Ascot) displayed resistance to all isolates tested as the mean disease score was less than 3 of a 1-9 rating scale for all isolates. Although the reactions of Acc 508 and 680 to five isolates were resistant with score less than 3, these accessions had scores more than 3 in reaction to three isolates

(260/92, 331/91 and 526/92). Acc 299 appeared susceptible as it produced symptoms greater than 3 to all isolates tested, and was significantly more susceptible than the susceptible control (Acc 342) when tested with isolates 166/92 and 219/92 (LSD 5% = 1.1 and 0.9, respectively).

The virulence among isolates to eight accessions varied between experiments in the series. The overall average virulence of all individual isolates to all accession tested is presented in Figure 4.1. Three isolates, 260/92, 331/91 and 526/92, produced severe symptoms on Acc 299 and Acc 342, and these isolates also caused symptoms on Acc 303, 948, 970 and Ascot, whereas less symptoms developed on these accessions when inoculated with the other isolates (Table 4.1).

Table 4.1 Disease scores of eight accessions inoculated with eight different isolates of *A. fabae*, using seeds collected from bulk samples.

Accession	Disease score following inoculation with isolate							
	A37	A26	201/92	166/92	219/92	260/92	331/92	526/92
Acc 299	3.6	4.4	3.6	4.6	5.8	5.1	5.5	4.8
Acc 303	2.1	1.1	1.5	2.0	1.0	2.0	2.3	2.1
Acc 342	4.2	3.3	3.8	2.4	2.0	4.6	5.8	6.8
Acc 508	2.6	2.0	2.0	2.6	1.0	3.9	4.1	3.1
Acc 680	2.5	2.0	1.9	1.9	1.9	3.8	3.5	3.3
Acc 948	1.0	1.0	1.0	1.0	1.0	1.3	1.4	1.1
Acc 970	1.0	1.3	1.1	1.6	1.3	2.5	1.9	2.9
Ascot	1.3	1.5	1.3	1.5	1.3	1.6	2.4	1.9
LSD 5%	1.6	1.5	1.3	1.1	0.8	1.7	1.7	1.3

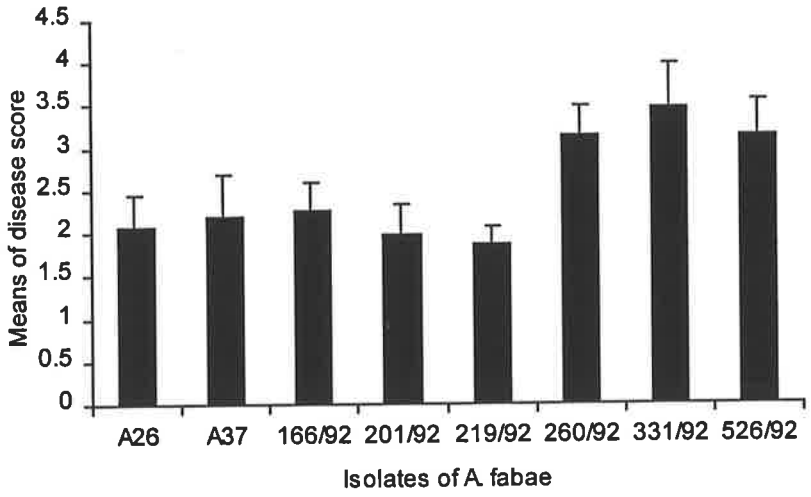


Figure 4.1 Variation in the virulence of eight isolates of *A. fabae* on eight faba bean accessions, using seeds collected from bulk samples. The bars represent the standard error of the mean.

4.3.2 Trial 2

Similar to the results of the first test, the reaction of the eight accessions from seeds derived from single self-pollinated plants varied when inoculated individually with eight isolates of *A. fabae* (Table 4.2). Acc 303, 948, 970 and 622 (substituted for Ascot) were resistant with scores less than 3. Acc 680 and 508 produced disease scores less than 3 to all isolates tested, with the sole exception of Acc 508 with isolate 331/91 which produced symptoms with a score higher than 3. Although having a score more than 3 in reaction to several isolates, Acc 299 was significantly more resistant to isolates A26, 166/92 and 219/92 compared to Acc 342 (Table 4.2).

As in the first trial, three isolates, 260/92, 331/91, and 526/92, produced the most severe symptoms and the mean score for 331/91 was greater than 3, while the other isolates were less virulent with the mean of symptoms less than 3 (Figure 4.2).

Table 4.1 Disease scores of eight accessions inoculated with eight different isolates of *A. fabae*, using seeds of pure lines.

Accession	Disease score following inoculation with isolate							
	252/92	A26	493/92	166/92	219/92	260/92	331/92	526/92
Acc 299	3.0	2.4	3.4	1.4	1.9	4.5	5.4	3.9
Acc 303	1.0	1.0	1.3	1.4	1.0	2.1	2.6	1.5
Acc 342	3.6	4.8	3.8	4.1	2.5	3.6	4.9	4.5
Acc 508	2.2	1.8	2.0	1.5	1.0	1.8	4.9	2.5
Acc 680	1.3	1.4	2.4	1.4	1.3	2.5	2.9	2.6
Acc 948	1.0	1.4	1.0	1.0	1.0	1.4	2.4	1.0
Acc 970	1.0	1.1	1.0	1.0	1.0	1.4	1.6	1.0
Ascot	1.3	1.0	1.0	1.4	1.0	1.1	1.9	1.6
LSD 5%	1.5	1.2	1.6	0.9	0.6	1.5	1.6	1.7

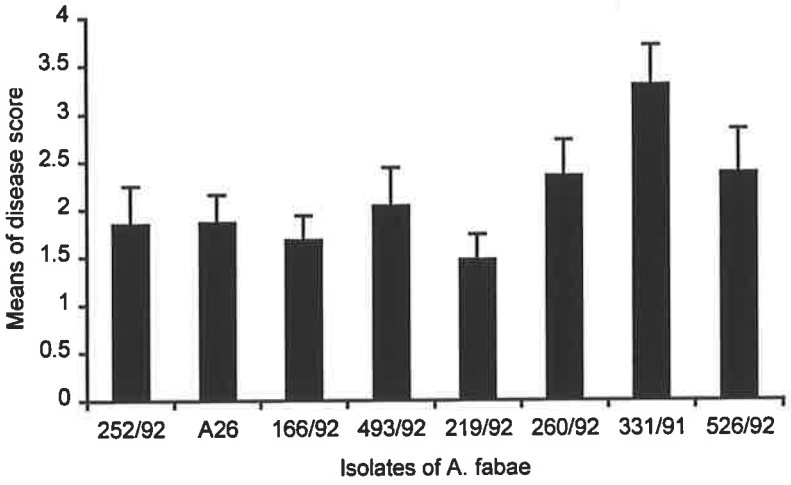


Figure 4.8 Variation in the virulence of eight isolates of *A. fabae* on eight faba bean accessions, using seeds collected from pure lines. The bars represent the standard error of the mean.

4.4 Discussion

These experiments demonstrated that lines developed by self-pollination and progeny testing were more homogeneous in resistance than the original accessions, particularly Acc 299, 508 and 680. Acc 303, 622 (Ascot), 948 and 970 appeared to be homogeneous for resistance to all isolates tested either using pure lines or bulk samples. As the bulk samples of several accessions were derived from several cycles of selfing and single plant selection (J. Paull, pers. com.), it is probable that these accessions were homozygous in resistance to *A. fabae*.

The two trials suggested that the reaction of pure lines of Acc 303, 680, 948, 970 and 622 or Ascot were resistant to all isolates tested, while Acc 299 and 508 were resistant to several isolates, but not all. Similar variation has been indicated in several reports. Hanounik and Robertson (1989) reported the results of testing a

number of faba bean lines in six geographically diverse countries. Several of these lines were included in the present study. BPL 2485 (Acc 680) was identified as resistant in the six countries and was considered to have a broad resistance to *A. fabae*. BPL 472 (Acc 303) and BPL 818 (Acc 508) were resistant in five countries, but susceptible in France. Lawsawadsiri (1994) also identified variation in the reaction of Acc 508 to a range of isolates of *A. fabae* and it was classified as resistant to seven Australian isolates, but it was susceptible to isolate A26. In the present trials, both the bulk population and a pure line of this accession were resistant to A26, but not to isolate 331/91. Acc 299 (BPL 471) was also studied by Lawsawadsiri (1994) and, as with the present trials, Acc 299 was susceptible to isolate A26. However, Hanounik and Robertson (1989) reported BPL 471 (Acc 299) was resistant in six countries.

Resistance of Ascot to a mixed inoculum of several isolates of *A. fabae* was reported by Ramsey et al. (1995) in two trials with ratings of 1.3 and 0.27 respectively on a 0-5 rating scale. Resistance of Acc 970 to isolate A26 was reported to be homogeneous when tested by Lawsawadsiri (1994), with a mean score of 0.1 on a 0-5 rating scale. The resistance of Quasar (Acc 948) was assessed by Jellis and Vassie (1995) who reported it had a mean score of 2.4 with the range of individual plants of 1-5 on a 1-9 rating scale.

Although it is not possible to identify a race structure of isolates of *A. fabae* in these trials, as the experimental design was not appropriate to investigate the differential interaction between accessions and isolates, the pathogenic variability in Australian isolates of *A. fabae* was apparent. This is based on the fact that some isolates caused more disease on all accessions (331/91 and 526/92), while other isolates caused disease on either most, or only a few accessions. This indicated that

the virulence of isolates depended on the resistant host used. For example, seven accessions were resistant to isolate 219/92 but the bulk of Acc 299 developed severe symptoms in reaction to this isolate. In regard to this accession, Hanounik and Robertson (1989) demonstrated that only 4% of isolates of *A. fabae* inoculum IA induced susceptible reactions on BPL 471 (Acc 299), but 20% of isolates induced susceptible reactions on ILB 1814. For inoculum IB, 8% and 84% of isolates induced susceptible reactions on BPL 471 and ILB 1814, respectively.

Isolate 331/91 resulted in the greatest discrimination between resistant and susceptible accessions and for this reason it was selected for genetic studies described in later chapters.

Chapter 5. Genetics of Resistance to Ascochyta blight in the faba bean cultivar Ascot

5.1 Introduction

Ascot is a composite of two *Ascochyta fabae* resistant selections from the cultivar Fiord. Fiord was developed from a landrace originating on the island of Naxos, Greece. Following evaluation in the 1970s, it was released in 1980, as the first faba bean cultivar in Australia (Ramsey et al., 1995). The considerations which led to the release of Fiord were its high yield and small seed (48g/100seeds) which enabled growers to sow and harvest the crop with conventional cereal seeders and harvesters (Lawsawadsiri, 1994).

During the period 1980 - 1985, the crop of Fiord was free from infection with Ascochyta blight, but since then the potential for damage from the disease has occurred every year and, as a result, fungicides have been used in every planting season in most districts (Lawsawadsiri, 1994).

Efforts to improve the resistance of Fiord were made by the National Faba Bean Breeding Program located in Adelaide, SA. Resistant plants were identified in a space-planted field of Fiord that was inoculated with *A. fabae*. Susceptible plants were rogued prior to flowering and the resistant plants were individually harvested. Following several generations of progeny testing to confirm resistance, and yield evaluation, Accessions 621 and 622, which originated from the same initial selection, were composited to form Ascot which was released in 1995.

As Ascot is well adapted to the faba bean growing regions of southern Australia it is potentially of great value as a source of resistance to *A. fabae* for the breeding program. Information on the genetic control of resistance would assist an efficient breeding and selection procedure. On the basis of the segregation of an F_2

population, Ramsey et al. (1995) suggested that resistance in Ascot is conferred by a single recessive gene relative to the susceptible cultivar Icarus. This conclusion was based on limited data with an arbitrary determination between the resistant and susceptible categories and there was no testing at the F_3 to identify the genotype of F_2 plants. This preliminary observation is investigated further in this chapter. The first experiment examined the response of the F_1 hybrid, relative to the parents, in order to provide information on degree of dominance of the genes conferring resistance to *A. fabae*. The segregation patterns of F_2 and F_3 populations were examined to estimate the number of genes conferring resistance to *A. fabae*.

The aim of the experiments described in this chapter was to investigate whether Ascot carries a single or more than one, recessive or dominant gene for resistance to *A. fabae*. Experiments were conducted on only one of the components of Ascot, namely Acc 622. It was considered probable that the results for Acc 622 would be representative of Ascot for two reasons (1) Ascot and Acc 622 showed the same reaction to a range of isolates of *A. fabae* (Chapter 4) and (2) both component lines of Ascot were derived from a single resistant plant.

5.2 Materials and Methods

5.2.1 Evaluation of F_1 hybrids

Acc 622 and the susceptible cultivar Icarus were crossed, including reciprocal combinations, by hand in a bee-proof glasshouse. Before crossing, both parents were selfed and progeny tested to ensure they were homozygous for reaction to *A. fabae*, as described in Chapter 3.

The reactions of F_1 hybrid plants were compared with those of the two parents using a Randomised Complete Block Design with four replicates. Sixteen seeds of each parent (obtained from the plants used for crossing) and of reciprocal F_1 hybrids (four seeds per replicate) were sown in plastic trays which were placed on benches in humidity chambers. Plants were inoculated with isolate 331/91 using the same inoculation procedure and scale for rating the disease as described in Chapter 3. The data for response of the F_1 hybrid relative to the parents was subjected to analysis of variance. In addition, an unpaired t-test was used to test the significance of the difference between reciprocal F_1 hybrids and both parents.

In order to confirm the identity of the plants (that is, that the F_1 hybrid plants were hybrids of the two parents and not the result of selfing of maternal parents) the F_1 hybrids and parents were compared by AFLP analysis. Leaves of all F_1 hybrid and parental plants were sampled for DNA extraction and AFLP analysis. Full details of the methods for AFLP analysis are described in Chapter 7.

5.2.2 Evaluation of the F_2 population and F_2 derived F_3 families

177 seeds of the F_2 generation of Acc 622 x Icarus and 80 F_2 derived F_3 families (12 seeds each), supplied by the Waite Institute Faba Bean Breeding Program, were tested with Isolate 331/91, using the same procedure as above. F_3 families were tested to distinguish the genotype of F_2 plants, which could not be identified by testing the F_2 population. The parents were included for both the F_2 and F_3 evaluation with two plants of each parent per tray for F_2 evaluation, and one plant of each parent per tray for F_3 evaluation as limited parental seeds were available. F_1 hybrid plants were included in the F_2 testing with 32 plants of the F_1 hybrid of Acc 622 x Icarus. Although 80 F_3

families of Acc 622 x Icarus were sown, 19 families were omitted from analysis due to poor germination resulting in insufficient plants to classify the families.

Two methods were used to estimate the number of genes responsible for resistance to *A. fabae* in Acc 622, namely (1) chi-square analysis of observed segregation patterns compared to the expected ratio for segregation at one locus, and (2) comparison of the observed variance of the F_2 population with the variance expected for segregation at one and two loci (Mather and Jinks, 1977). The second method was employed because there is not a distinct resistant reaction and separation into discrete categories for chi-square analysis, particularly at the F_2 generation, was based on arbitrary classifications.

The expected variance was calculated from the average of the variance components of the parents and the F_1 hybrids. The variance components of the F_2 generations were partitioned based on an additive dominance model which was described by Mather and Jinks (1977) as follows :

$$V_{F2} = 1/2\ D + 1/4\ H + E$$

Where V_{F2} is the expected variance of the F_2 population

D is the additive component of the variance, defined as d^2 for a single locus and $(d_a^2 + d_b^2)$ for two loci,

d is the departure of AA from the mid-point (m) of AA and aa for a single locus (Fig. 5.1),

d_a is the departure of AA from the mid-point of AA and aa, and d_b is the departure of BB from the mid-point of BB and bb, for two loci,

H is the dominance component of the variance, defined as h^2 for a single locus and $(h_a^2 + h_b^2)$ for two loci,

h is the departure of Aa from the mid point of the homozygotes AA and aa,

h_a and h_b are the departures from the mid point of the heterozygous genotypes AaBb, AaBB, AABb and aaBb and the homozygous intermediate aaBB and AAbb.

E is the environmental variance, calculated as :

$$E = 1/4\ VP_1 + 1/4\ VP_2 + 1/2\ VF_1$$

Where VP_1 and VP_2 are the variances of the parents, and

VF_1 is the variance of the F_1 hybrid

With the assumptions of no linkage and no epistasis, the equation for estimating the expected variance of an F_2 population in the case of one gene segregating is

$$VF_2 = 1/2\ d^2 + 1/4\ h^2 + E$$

and in the case of two genes segregating is

$$VF_2 = 1/2\ (d_a^2 + d_b^2) + 1/4\ (h_a^2 + h_b^2) + E$$

The confidence interval (p=0.05) of the observed variance was calculated as

$$(V_o \times df)/\chi^2 a \leq \text{Confidence interval} \leq (V_o \times df)/\chi^2 b$$

where V_o is the observed variance of the F_2 population

df is the degrees of freedom of n-1 (n is the number of plants of an F_2 population)

χ^2a and χ^2b are the lower and the upper level of chi-square values at the probability of 0.95 and degrees of freedom of n-1 (D. Pederson, pers. comm.).

If the expected variance was outside the range of the confidence interval (p=0.95) of the observed variance, the F_2 population was identified as deviating significantly from the expected variance for the particular model.

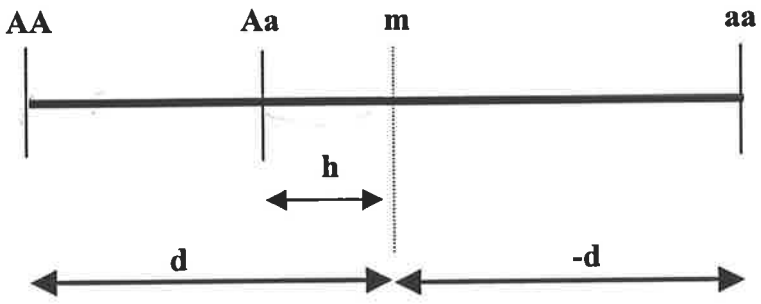


Figure 5.1 The d and h metrics of the allelic difference A-a. Deviations are measured from the mid-parent, m, midway between the two homozygous genotypes AA and aa. Aa may lie on either side of m and the sign of h will vary accordingly (Mather and Jinks, 1977)

F_2 derived F_3 population

An alternative method to estimate the number of genes conferring resistance to *A. fabae* was by progeny testing at the F_3 generation. As the cross did not deviate from the one gene model in the F_2 evaluation, the F_3 families were assigned to three

categories, homozygous resistant, segregating and homozygous susceptible. Families were classified as (1) homozygous resistant when the disease score of all plants was 1 or 3, (2) homozygous susceptible when the disease score of all plants ranged from 5 to 9, and (3) segregating when they included plants with scores of 3 or less and 5 or more.

Chi-square analysis was used for testing the goodness of fit of the observed segregation ratios of the F_3 families to values expected for the monogenic model of 1 homozygous resistant : 2 segregating : 1 homozygous susceptible.

5.3 Results

5.3.1 Reaction of F_1 hybrids to *A. fabae*

Evaluation of F_1 hybrids and parents confirmed a significant difference between parents in degree of resistance ($p<0.05$) (Figure 5.2). There was no significant difference between the reciprocal F_1 hybrids in resistance to *A. fabae*. The reciprocal F_1 hybrids were both significantly more susceptible than the resistant parent, Acc 622, while the F_1 hybrid of Acc 622 x Icarus was significantly more resistant than Icarus, but Icarus x Acc 622 was not significantly more resistant than Icarus. AFLP analysis of the F_1 hybrids and parents confirmed that all of the hybrid plants included polymorphic bands derived from both parents (Figure 5.1).

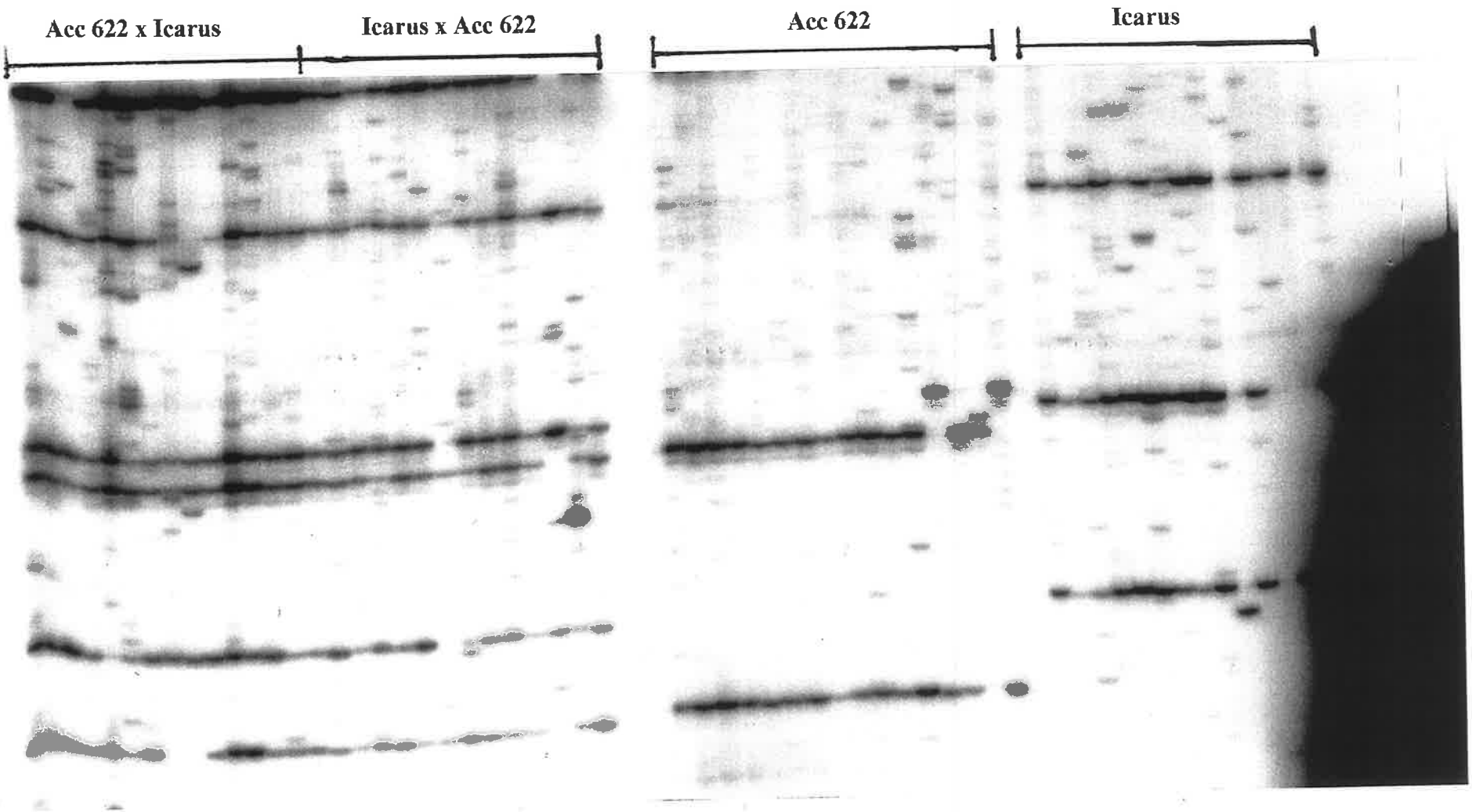


Figure 5.1 Acc 622, Icarus and reciprocal F_1 hybrids after AFLP analysis with primer combination *Pst* ACA - *Mse* CAG

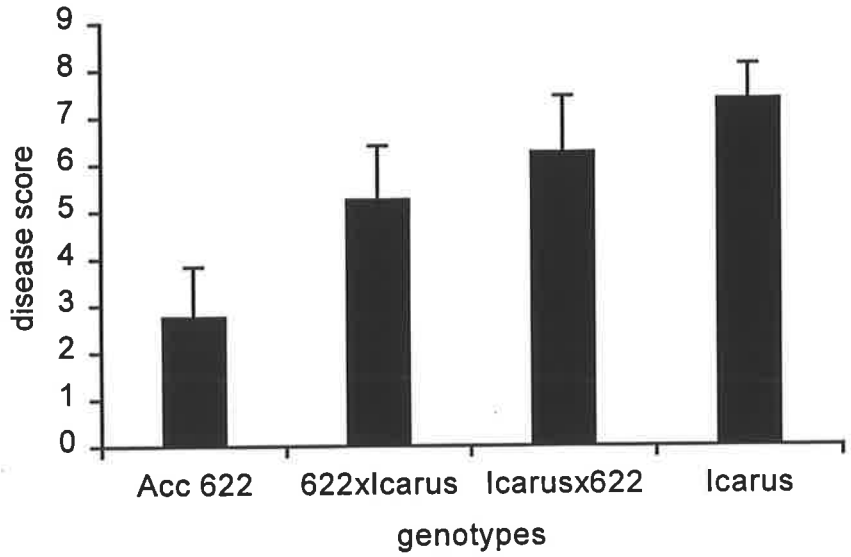


Figure 5.2 Responses of Acc 622, Icarus and reciprocal F_1 hybrids after inoculating with *A. fabae* isolate 331/91. The bars represent the standard error of the mean

5.3.2 Evaluation of the F_2 population of Acc 622 x Icarus

Acc 622, Icarus, the F_1 hybrid and F_2 population tested in this study differed in response to *A. fabae* and results confirmed previous observations with Ascot being resistant, Icarus susceptible and the F_1 hybrid intermediate to the two parents, although slightly higher than the F_2 mean. The variances of the two parents and the F_1 hybrid were similar to each other and much less than for the F_2 population (Table 5.1). The frequency distribution of the F_2 population did not indicate discrete resistant and susceptible categories and there was some overlap of the two parents (Figure

5.3). Therefore, chi-square analysis was not used to analyse the segregation pattern of the F_2 population. Rather, the number of genes controlling resistance to *A. fabae* was assessed in the F_2 generation by comparing the observed variance with the variances expected for segregation at one and two genes.

The minimum value for the confidence interval of the observed variance of the F_2 population was much greater than the expected variance for segregation at two genes and only slightly greater than the expected variance for a single gene model (Table 5.2). Thus, the reaction to *A. fabae* observed in the F_2 population of Acc 622 x Icarus is more likely to be due to segregation at a single gene than at two genes.

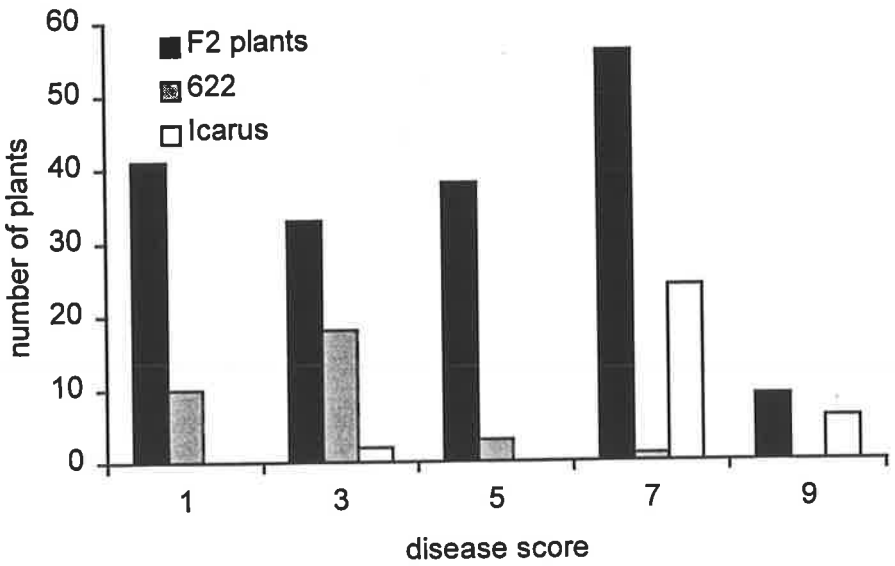


Figure 5.3 Frequency distributions of parents and the F_2 population for a cross between Acc 622 x Icarus when inoculated with *A. fabae*

Table 5.1 Number of plants and means and variances of disease scores for faba bean plants inoculated with *A. fabae*

Populations	Number of plants	Mean	Variance
Acc 622	32	2.69	2.09
Icarus	32	7.13	1.79
F1 hybrid	32	5.89	1.79
F2	177	4.54	6.35

Table 5.2 Observed variance of parents and the F_2 population and the expected variance of the F_2 population for segregation at one and two genes

Observed variance	Acc 622	2.09
	Icarus	1.79
	F_1 hybrids	1.79
	F_2 population	6.35
Estimated parameters	Single gene model	
	E	1.87
	m	4.91
	D	4.91
	H	0.98
	Two gene model	
	E	1.87
	m	4.91
	D	2.45
	H	0.49
Expected variance	1 gene	4.58
	2 genes	3.22
Confidence Interval of Observed F_2 variance		5.24 - 7.96

5.3.3 Evaluation of F_2 derived F_3 families

The F_3 families were classified into three categories, namely homozygous resistant, segregating and homozygous susceptible based on the response of individual plants within each family (Figure 5.4). Chi-square analysis supported the hypothesis of segregation at one gene for reaction to *A. fabae* in this cross (Table 5.3). While the chi-square analysis was not significant at the 5% confidence level, the deviation of the observed values from expected for segregation at a single gene was in the direction that would be expected if any plants escaped the disease, ie there was a surplus of resistant families.

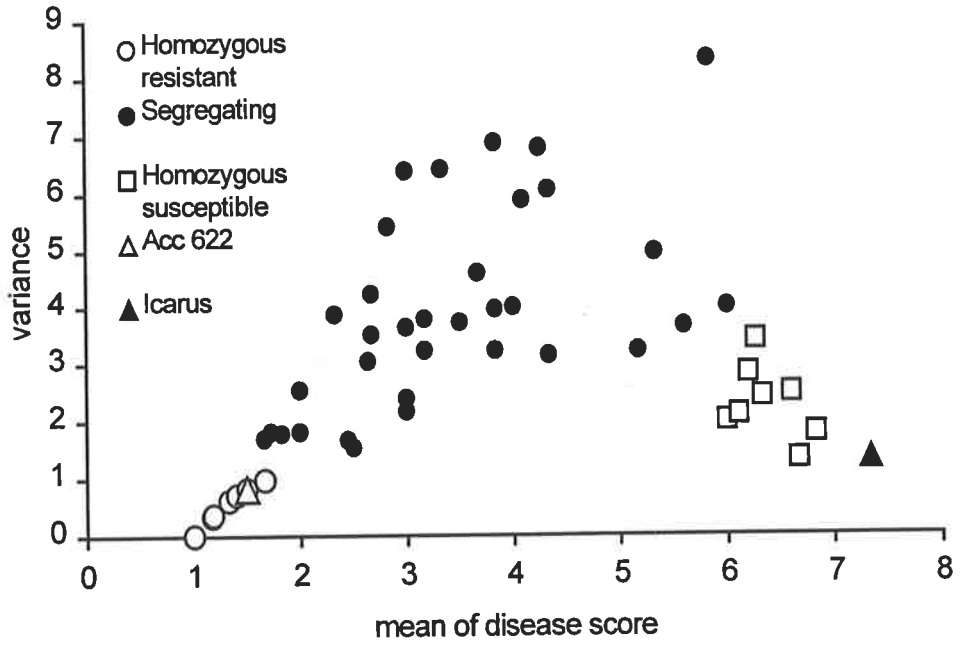


Figure 5.4 The mean disease scores and variances of F3 families derived from the cross between Acc 622 and Icarus.

Table 5.3 Chi-square calculations of the observed and expected segregation ratios of the F_3 families derived from the cross Icarus x Acc 622, for segregation at one gene

Number of F_3 families	Observed Ratio HR[a] : S[b] : HS[c]	Expected Ratio 1HR : 2S : 1HS	X^2	p-value
61	15 : 38 : 8	15.25 : 30.5 : 15.25	5.29	0.05 - 0.10

[a] Homozygous resistant, [b] Segregating, [c] Homozygous susceptible

5.4 Discussion

5.4.1 Evaluation of F_1 hybrids

The response of F_1 hybrids to *A. fabae* did not differ between reciprocals, both of which were significantly more susceptible than Acc 622, the resistant parent. However, the response of the reciprocal F_1 hybrids differed when compared to Icarus; Acc 622 x Icarus was significantly more resistant than Icarus, but there was no statistically significant difference in response between Icarus and Icarus x Acc 622. This result indicates resistance to *A. fabae* is either a partially recessive, or recessive, trait. Further information on the expression of resistance to *A. fabae* is provided by the reaction of F_2 and F_3 plants. The F_2 population mean was approximately the mid-point of the parental values, while the average scores of the segregating F_3 families were skewed in the direction of the resistant parent. In the case of the F_2 population, if resistance was a fully recessive trait, for a single gene it would be expected that the segregation would be 1 resistant : 3 susceptible with the F_2 population mean skewed to the susceptible parent value, rather than the mid-parent value as observed (Table 5.1). Similarly, the segregating F_3 families would tend to the susceptible parent value if

resistance was fully recessive but this was not observed; rather the mean values for the segregating families were intermediate to the two parents (Fig 5.4). Both of these observations support the hypothesis that resistance is either co-dominant or partially recessive, rather than a fully recessive trait.

Resistance to *A. fabae* has been described as co-dominant, dominant and recessive, depending on genetic combinations and isolates of *A. fabae*. For example Lawsawadsiri (1994) tested the F_1 and F_2 generations of the cross between Acc 970 (resistant to *A. fabae*) and Acc 811 (susceptible), inoculating with an Australian isolate of *A. fabae*, designated A 26. All F_1 hybrids were resistant and the F_2 population segregated with the ratio of 3 resistant : 1 susceptible plants, indicating that the resistance gene of Acc 970 to isolate A26 was dominant relative to Acc 811, and the resistance was due to a single dominant gene.

Rashid et al. (1991a) demonstrated that the expression of resistance differed between crosses, depending on the susceptible parents and isolates used. The F_1 hybrids between a resistant line, 15025-2 (derived from ILB 752, the source population of Acc 970), and susceptible lines Herz Freya-7 or Erfordia-3 were resistant to isolate A of *A. fabae*, but reacted as moderately susceptible to susceptible to the same isolate if crossed with the susceptible line 2N40. In addition, all F_1 hybrids tested were resistant when inoculated with isolates B, X and X^1, but some F_1 hybrids were resistant and others intermediate when inoculated with isolate Y^1, indicating the expression of resistance differed according to the isolate (Rashid et al., 1991a).

In Ascot, a recessive gene conferring resistance was reported by Ramsey et al. (1995), where it was inoculated by a mixture of isolates *A. fabae*, and the segregation ratio of the F_2 population of Icarus x Ascot fitted the ratio 1 resistant : 3 susceptible.

Robertson (1983) identified resistant plants in faba bean populations following several generation of selfing. This suggests that recessive resistant genes, which were masked as heterozygotes in open-pollinated populations, were expressed when homozygous.

5.4.2 Number of genes conferring resistance to *A. fabae*

In estimating the number of genes conferring the resistance to *A. fabae* of Ascot, relative to Icarus, chi-square analysis of the observed segregation pattern compared to ratios expected for alternative genetic models was considered not suitable to use in the F_2 generation, as resistant plants did not display a distinct resistant reaction and it was not possible to separate plants into discrete categories. Therefore, the observed variance of the F_2 population was compared with the variance expected for segregation at one and two loci. This analysis indicated that the number of genes in Acc 622 controlling resistance relative to Icarus was more probably one than two or more.

Evaluation of F_2 derived F_3 families enabled the genotypes of the parent F_2 plants to be identified within limits imposed by the size of each F_3 family. Thus X^2 analysis was based on the genotypes of the F_2 parents, whereas most other reports of the genetic control of resistance to *A. fabae* are based on F_2 phenotypes.

The F_2 derived F_3 families were assigned to three categories, homozygous resistant, segregating and homozygous susceptible. On the basis of resistance of Acc 622 being co-dominant or partially-recessive, as indicated by the reaction of F_1 hybrids, homozygous resistant families were those where the parent F_2 plants were of the genotype *aa*, segregating families were *Aa* and homozygous susceptible families were *AA*. The reaction of the F_3 families was consistent with the hypothesis of one gene segregating for reaction to *A. fabae* in the cross between Acc 622 and Icarus.

Other authors have reported either one or two genes controlling resistance to *A. fabae*. Jellis and Vasie (1995) indicated that the *A. fabae* resistant line 29H probably carried more than one gene to control resistance, although it was recognised that the experiment on which this conclusion was based was not designed to address this particular issue. Rashid et al. (1991a) suggested that of nineteen inbred lines tested by 5 different isolates of *A. fabae*, more than seven genes were identified to be responsible for resistance to *A. fabae*, and BPL 2485 and 471 might carry two genes for resistance to isolate Y^1 of *A. fabae*, one of which was heterozygous in the initial parent population

The results of the present experiments concur with Ramsey et al. (1995) who concluded that the resistance of Ascot to *A. fabae* was conferred by a single gene. As resistance is under major gene control, selection of resistant genotypes should be effective in early segregating generations of a breeding program.

Chapter 6. Genetics of Resistance of Faba Bean Accessions to *Ascochyta fabae*

6.1 Introduction

Successful breeding programs depend on an understanding of the amount and distribution of genetic variability present in the gene pool. Genetic variability is needed in breeding for disease resistance so that alternative genes are available to use in the event of breakdown of resistance. In addition, this information may be used to select genotypes to intercross in order to combine, or pyramid, different resistance genes, so that the longevity of resistance genes may increase and the possibility of the resistance breaking down may be minimised.

A number of faba bean accessions resistant to *A. fabae* are held in the faba bean collection at the Waite Institute. The lines were introduced from faba bean breeding programs throughout the world, particularly from ICARDA, Aleppo, Syria. Several authors have reported genetic variation among *A. fabae* resistant germplasm, either on the basis of segregation among the progeny of two resistant parents (Rashid et al., 1991) or differential response to a range of isolates (Hanounik and Maliha, 1984; Hanounik and Robertson, 1989). However, the reaction of many of the resistant accessions in the Waite Institute collection to *A. fabae* has not been characterised, and it is not known whether the resistance genes in the various accessions are the same or different. Crossing with an identified line and screening the progeny may enable the genotype of the parents to be identified. Where there is segregation in the F_2 population of a cross between two homozygous resistant lines, it can be hypothesised that the parents carry different resistance genes.

The Australian *A. fabae* resistant cultivar "Ascot" is a composite of two lines derived from a single plant. One of the two lines, Acc 622, in which resistance was

identified as a partially recessive or co-dominant trait conferred by a recessive gene (Chapter 5), was used as the check for crossing in the present experiment. The aims of this experiment were (1) to investigate the genetics of a number of resistant accessions relative to Acc 622 in order to identify alternative resistance genes to be used in the Australian faba bean breeding program and (2) to compare the resistance of accessions from different regions.

6.2 Materials and Methods

6.2.1 Genetic Material

Experiment 1

Accession 622, one of the component lines of Ascot, was crossed as the female parent with seventeen resistant lines and one susceptible line of geographically diverse origins, most of which were obtained from ICARDA. These lines are listed in Table 6.1 and full details are presented in Table 3.2. Before crossing, all parents except the susceptible check were selfed and progeny tested to identify homozygous resistant plants, as described in Chapter 3. Crossing and production of F_2 seeds were conducted in a bee-proof glasshouse. Seed obtained from plants used in crossing was included as controls when testing the F_2 generation.

Experiment 2

The second experiment further investigated four lines, two that appeared to carry the same resistance gene(s) as Ascot and two that were different from Ascot in the first experiment. The limitation of time prevented all accessions being included in this second study, thus the relationship among all accessions was not determined. Four lines were crossed to each other in all possible combinations, excluding reciprocals, to produce six crosses, namely 303 x 484, 303 x 680, 303 x 970, 484 x

680, 484 x 970 and 680 x 970 (female parent listed first for all crosses). Acc 303 and 484, were selected to represent the group with the same genes for resistance to *A. fabae* as Acc 622, while Acc 680 and 970 represented the group with different genes from Acc 622.

6.2.2 Experimental procedure

The reactions of the F_2 populations in both experiments were compared with those of the two parents in humidity chambers using a Randomised Complete Block Design. The number of seeds of each parent and of the F_2 populations depended on the availability of seeds (Table 6.1), but the minimum requirement of seeds for identifying F_2 segregation was observed as suggested by Graybill and Kneebone (1959). Plants were inoculated with isolate 331/91 and rated with the 1 - 9 scale, as described in Chapter 3.

6.3.3 Data analysis

The F_2 progenies were divided into three categories: segregating, non-segregating and those that produced inconclusive results.

The population was classified as segregating when the parents were homozygous (*ie* all check plants with disease scores not greater than 5) but the F_2 segregated and included some F_2 plants with scores more than 5. For an F_2 population of 80 plants, the presence of one plant having a score 7 or 9 was considered indicative of segregation. With a population of 80 individuals the smaller category for a segregation ratio of 15:1 would have an expected value of 5, a number sufficient for chi-square analysis. The expected size of the smallest category for other

ratios possible for segregation at two genes, such as 9:7, 9:6:1, 13:2:1 and 13:3, would be 5 or greater and thus also sufficient for chi-square analysis.

Crosses were classified as non-segregating when the parents and all F_2 progenies were rated in the range from 1 to 5. Several crosses included at least one parent with some plants having a disease score of 7 or 9 indicating that the plant used for crossing was probably heterozygous. Interpretation of the reaction of the F_2 in these crosses was inconclusive.

Table 6.1 The number of seeds of parents and F_2 populations of faba bean accessions crossed to Acc 622 used in identifying their genetic resistance to *A. fabae*. The same number of plants of Acc 622 as the other parent was included for each cross.

Accession	Number of seeds	
	Parents	F_2 population
290	18	60
295	12	80
297	12	80
299	12	80
303	12	80
342	12	80
484	12	80
496	18	53
508	12	60
668	18	60
674	12	80
680	18	54
683	12	60
712	12	60
948	12	80
970	12	80
975	18	60
1046	12	80

6.3 Results

6.3.1 Reactions of parents

All plants of Acc 622 over all experiments were rated as 1, 3 or 5, with the exception of a single plant rated as 7 (Fig. 6. 1). No plant of Acc 622 was rated as 9.

The other parents reacted variously to *A. fabae* with both uniformly resistant and heterogeneous reactions. Parents which were classified as homozygous (disease score 1-5) included Acc 295, 297, 299, 303, 484, 668, 674, 680, 712, 948, 970, 975 and 1046 (Figure 6.2; 6.3), and heterozygous parents with some plants rated 7 or 9 were Acc 290, 342, 496, 508 and 683 (Figure 6.4).

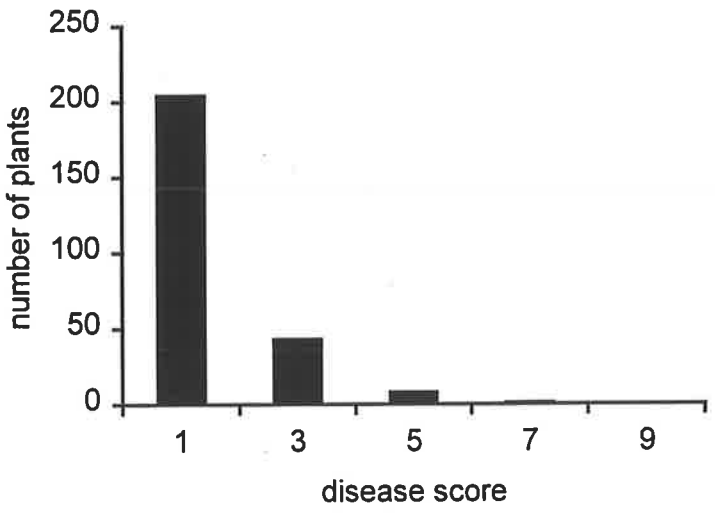


Figure 6.1 Frequency distribution of disease scores of Acc 622 from the results over all crosses between Acc 622 and the other accessions

6.3.2 Experiment I

Non-segregating populations

The F_2 populations classified as non-segregating were those between Acc 622 and Acc 295, 303, 484, 668, and 975 (Figure 6.2). The highest disease score of either parent or F_2 plants in these crosses was 5, thus the populations did not segregate and it can be hypothesised that both parents have the same resistance genes. The F_2 population of the cross between Acc 622 and 496 did not show evidence of segregation with all F_2 plants rated as 1 or 3, although one plant of each parent was rated as 7 (Figure 6.2). This population was also classified as non-segregating.

Segregating populations

There were eight crosses where all parental controls were classified as resistant (score of 5 or less), but segregation occurred among the progeny (Figure 6.3). These crosses were between Acc 622 and Acc 297, 299, 674, 680, 712, 948, 970 and 1046. X^2 analysis indicated that the observed segregation ratios were consistent with several segregation ratios that might be expected for segregation of two genes, for example (15R : 1S when the susceptible plants are homozygous recessive at two loci) (13R : 2 intermediate : 1 S for one dominant gene and one co-dominant gene where susceptible plants are homozygous recessive at two loci and the intermediate class is homozygous recessive at one locus and heterozygous at the co-dominant locus) or in one case (Acc 1046) (13R : 3S) (Table 6.2).

Figure 6.2 Frequency distributions of reactions of parents and F_2 populations of the crosses Acc 295 x 622, 303 x 622, 484 x 622, 975 x 622, 668 x 622 and 496 x 622 to *A. fabae*

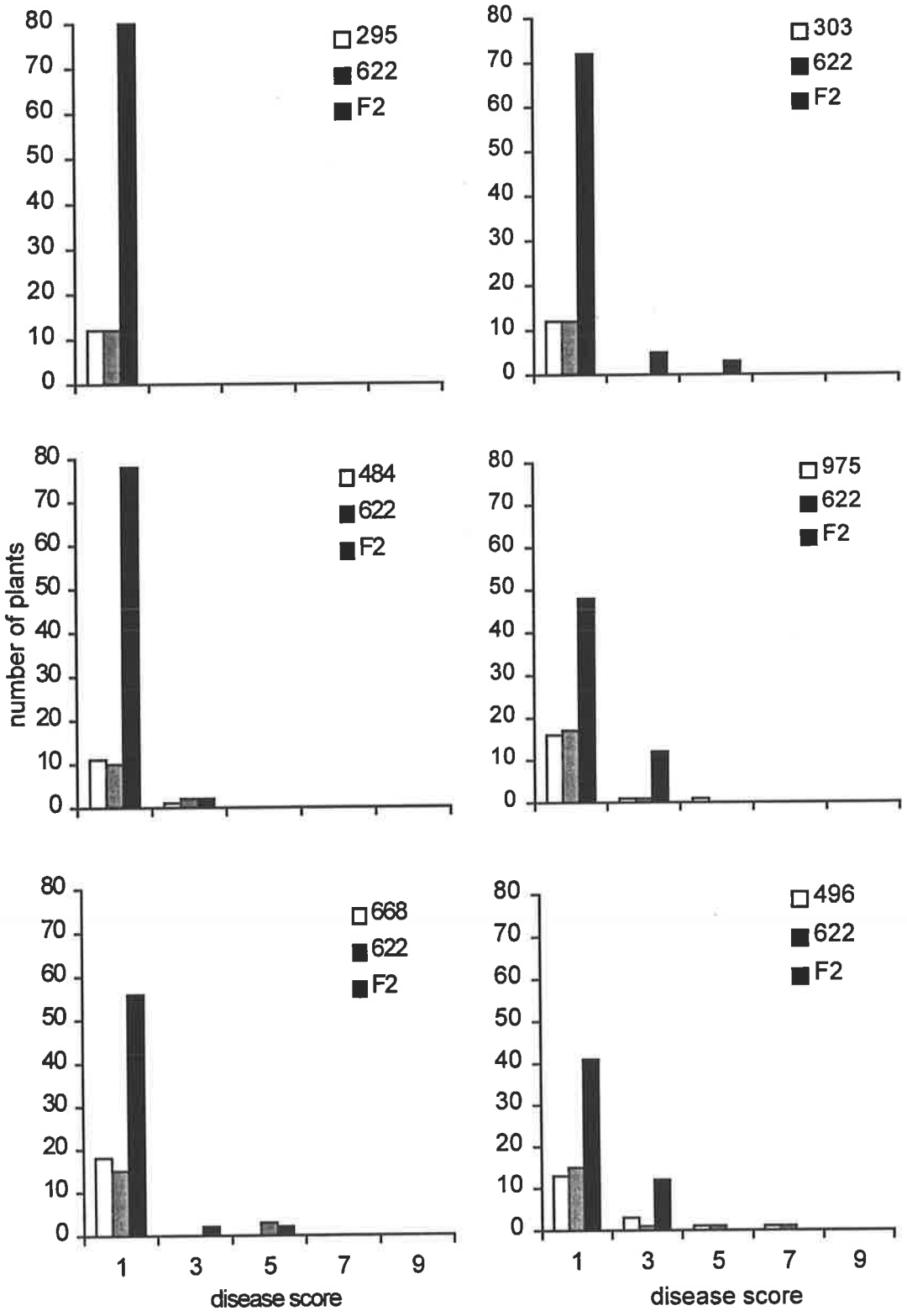

295
622
F2
303
622
F2
484
622
F2
975
622
F2
668
622
F2
496
622
F2
number of plants
disease score
disease score
0
10
20
30
40
50
60
70
80
1
3
5
7
9

Figure 6.3 Frequency distributions of reactions of parents and F_2 populations of the crosses Acc 674 x 622, 680 x 622, 712 x 622 and 297 x 622 to *A. fabae*

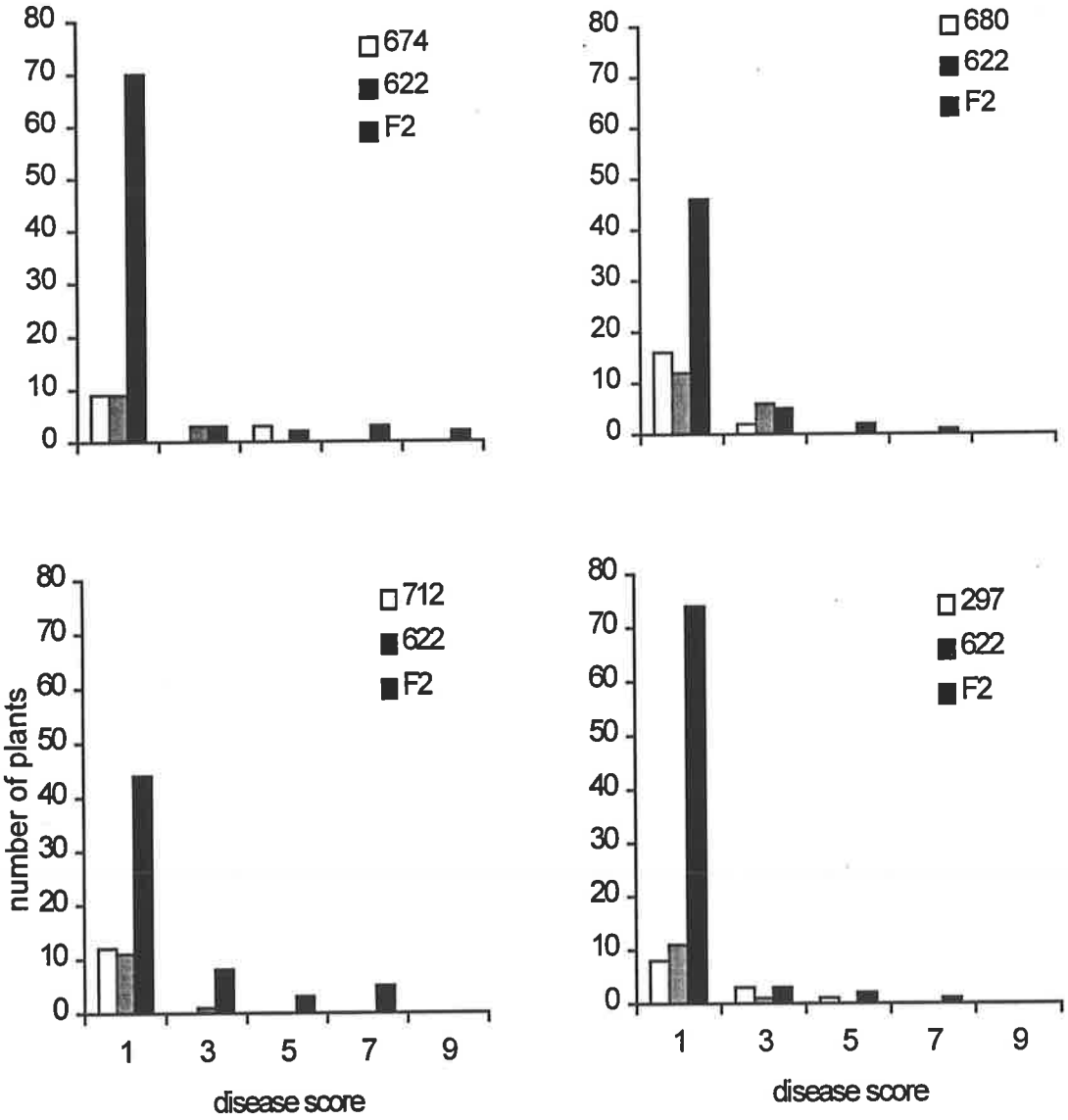
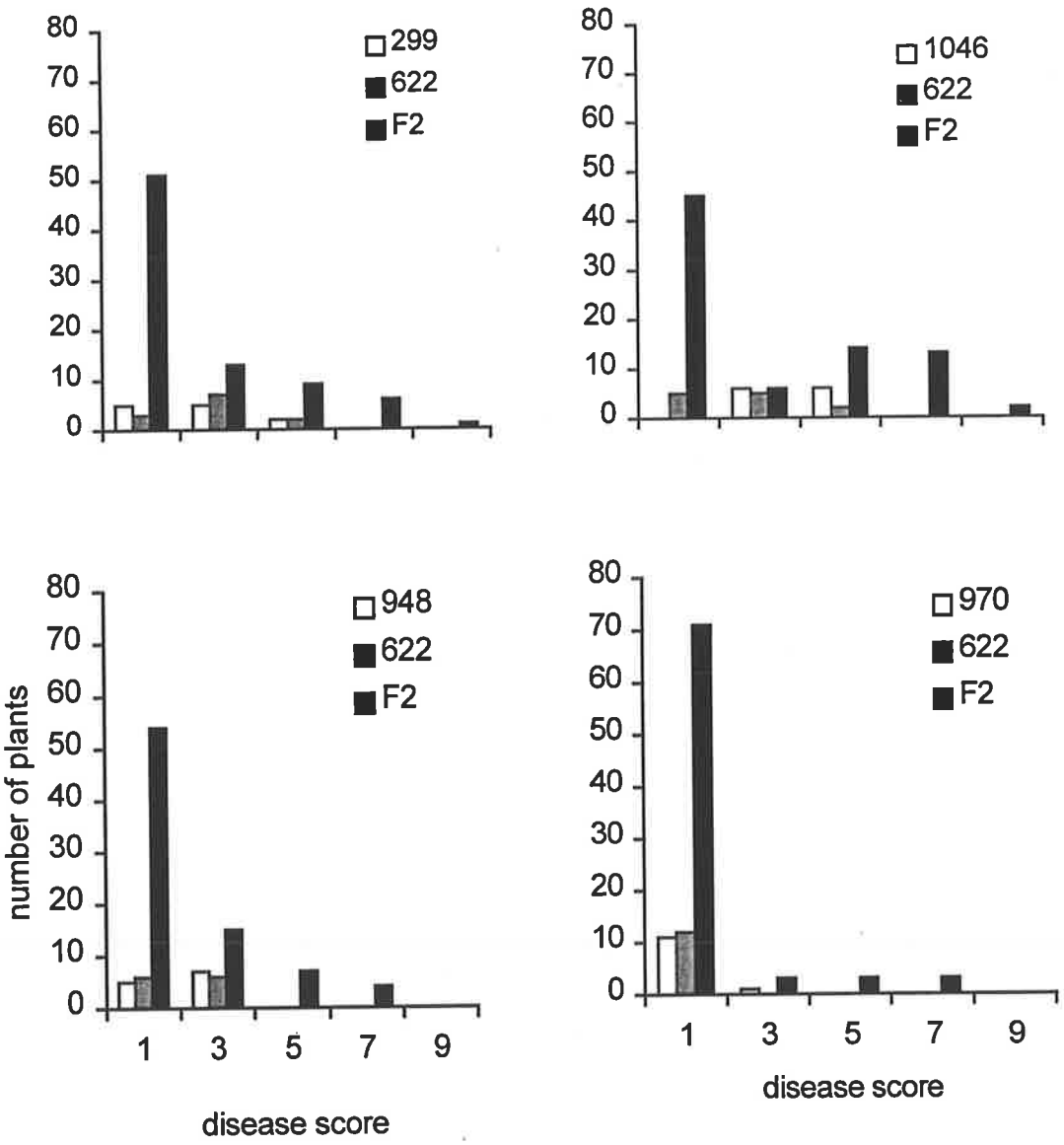

80
70
60
50
40
30
20
10
0
674
622
F2
680
622
F2
712
622
F2
297
622
F2
number of plants
1
3
5
7
9
disease score
disease score

Figure 6.3 (cont.) Frequency distributions of reactions of parents and F_2 populations of the crosses Acc 299 x 622, 1046 x 622, 948 x 622 and 970 x 622 to *A. fabae*


299
622
F2
1046
622
F2
948
622
F2
970
622
F2
number of plants
disease score
disease score
0
10
20
30
40
50
60
70
80
1
3
5
7
9

Inconclusive classification

There were four crosses where some parental controls expressed susceptible symptoms. These crosses were between Acc 622 and Acc 290, 342, 508 and 683 (Figure 6.4). There are two possible explanations for this observation, namely (1) experimental error, such as position in the humidity chamber, uneven distribution of inoculum or variation in rating of symptoms or (2) the initial plant selected for crossing was heterozygous and the progeny showed segregation. The second hypothesis is the more likely case for Acc 290 where there was a high frequency of susceptible plants, but the cause is less certain for the other three lines, where there were only one or two susceptible plants of each. As there was uncertainty about the status of the parents, the apparent segregation observed in the F_2 could not necessarily be attributed to a difference in genetic control of resistance to *A. fabae* between Acc 622 and the other line. These crosses were therefore classified as inconclusive.

6.3.3 Experiment II

Non-segregating populations

The F_2 populations with all plants rated from 1 to 5 and therefore considered as non-segregating were Acc 303 x 484, Acc 303 x 680, and Acc 484 x 680 (Figure 6.5). Acc 303 and 484 were identified as carrying the same gene(s) as Acc 622 in the first experiment. The absence of segregation in the F_2 of Acc 303 x 484 supports this hypothesis. Conversely, Acc 680, which was considered to carry a different gene from Acc 622, did not produce susceptible F_2 progeny when crossed with either Acc 303 or Acc 484. This suggests that the resistance of Acc 680 has the same genetic basis as Acc 303 and Acc 484, thus contradicting the initial hypothesis of Acc 680 being different from Acc 622 and similar lines.

Figure 6.4 Frequency distributions of reactions of parents and F_2 populations of the crosses Acc 683 x 622, 508 x 622, 290 x 622 and 342 x 622 to *A. fabae*

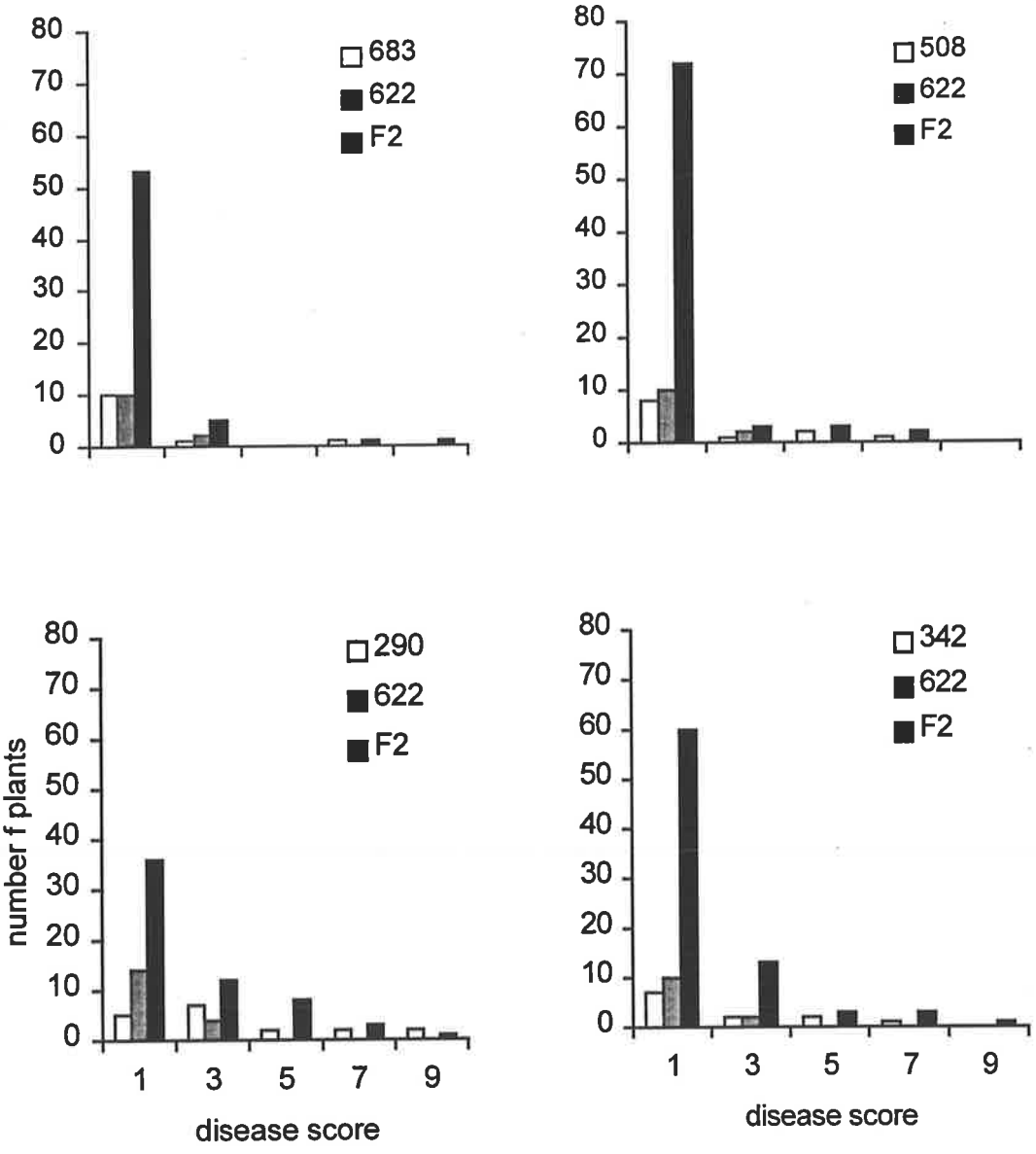
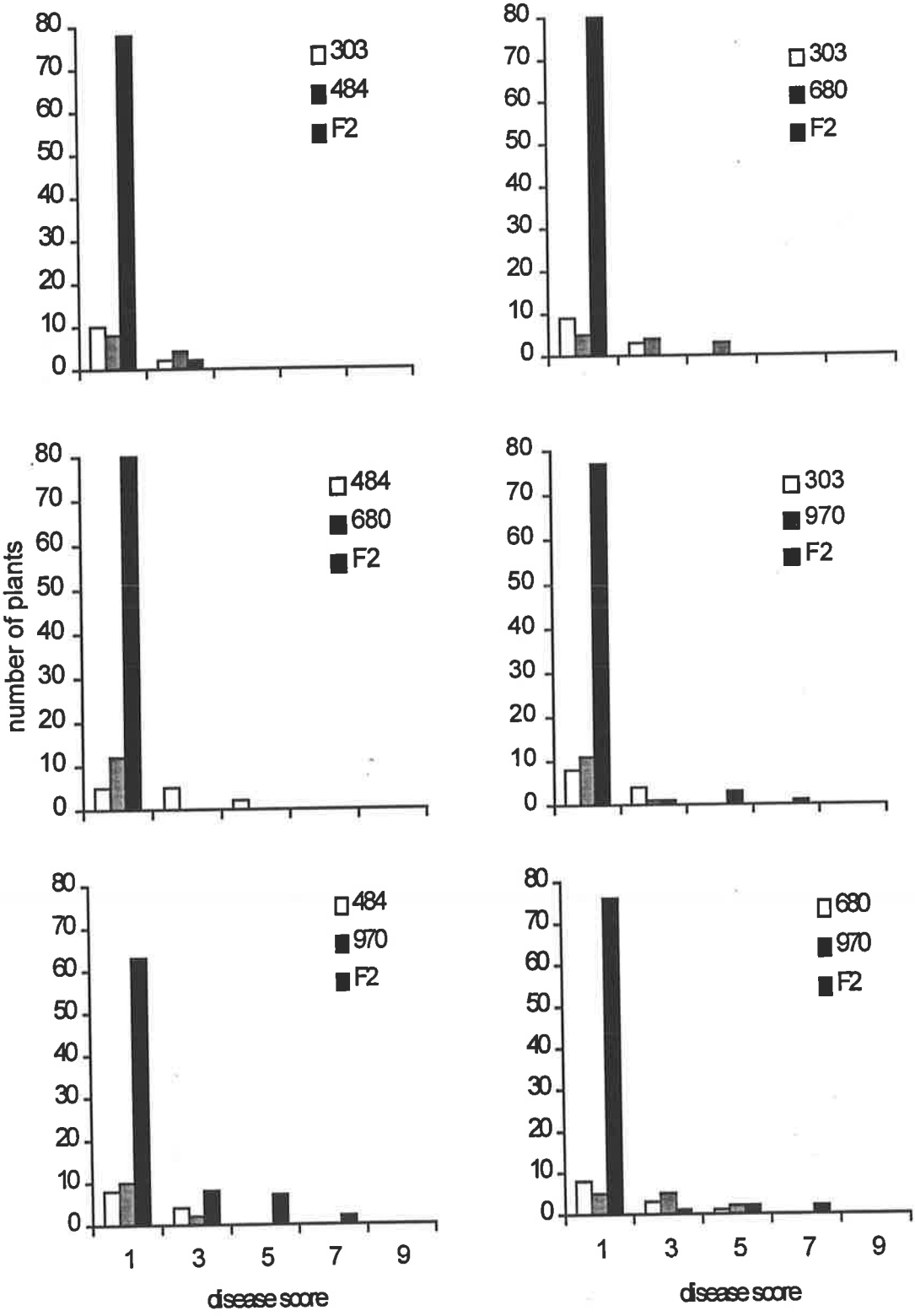

683
622
F2
508
622
F2
290
622
F2
342
622
F2
number f plants
disease score
disease score
0
10
20
30
40
50
60
70
80
1
3
5
7
9

Figure 6.5 Frequency distributions of reactions of parents and F_2 populations of the crosses Acc 303 x 484, 303 x 680, 484 x 680, 303 x 970, 484 x 970 and 680 x 970 to *A. fabae*


number of plants
80
70
60
50
40
30
20
10
0
303
484
F2
303
680
F2
484
680
F2
303
970
F2
484
970
F2
680
970
F2
1
3
5
7
9
disease score
disease score

Segregating populations

Segregation in reaction to *A. fabae* was identified in all F_2 populations with Acc 970 as one of the parents (Figure 6.5, Table 6.2). Segregation in the crosses between Acc 970 and Acc 303 and Acc 484, which are proposed to carry the same resistance gene(s) as Acc 622, supports the hypothesis that the resistance of Acc 970 and Acc 622 are conferred by different genes. Segregation in the F_2 population of Acc 680 x 970 also indicates that the two parents carry different resistance genes to *A. fabae*. This is consistent with the observation of no segregation in the progeny between 680 and 303 and 484.

Table 6.2 Chi-square calculations of the observed and expected segregation ratios of F_2 populations identified as segregating

F_2 population	Number of plants		Mean score		Exp. Ratio	X^2	p-values
	R[a]	S[b]	R	S			
297 x 622	79	1	1.2	7.0	15R : 1S	3.41	0.05 - 0.10
299 x 622	77	3	1.2	7.0	15R : 1S	0.85	0.30 - 0.50
674 x 622	75	5	1.2	7.8	15R : 1S	0	1.00
680 x 622	53	1	1.3	7.0	15R : 1S	1.78	0.10 - 0.20
712 x 622	55	5	1.5	7.0	15R : 1S	0.44	0.50 - 0.70
948 x 622	76	4	1.8	7.0	15R : 1S	0.21	0.50 - 0.70
970 x 622	73	7	1.9	7.3	15R : 1S	0.85	0.30 - 0.50
1046 x 622	65	15	2.0	7.3	15R: 1S	21.33	< 0.05
1046 x 622	65	15	2.0	7.3	13R: 3S	0	1.00
303 x 970	79	1	1.2	7.0	15R : 1S	3.41	0.05 - 0.10
484 x 970	78	2	1.5	7.0	15R : 1S	1.92	0.10 - 0.20
680 x 970	78	2	1.1	7.0	15R : 1S	1.92	0.10 - 0.20

[a]) R = resistant and indicates a score of 1, 3 or 5
[b]) S = susceptible and indicates a score of 7 or 9

Table 6.3 Accessions of faba beans having the same and different resistance genes as Acc 622, with region of origin

Accessions	Resistance genes compared to Acc 622	Origin
295	same	Lebanon
297	same	Lebanon
303	same	Lebanon
484	same	Iraq
496	same	Morocco
668	same	Lebanon, Iraq*
680	same	Iraq
975	same	Lebanon, Iraq*
299	different	Lebanon
674	different	Iraq
712	different	Lebanon, unknown*
948	different	UK
970	different	Sweden
1046	different	UK or USSR
290	inconclusive	Morocco
342	inconclusive	Egypt
508	inconclusive	Ethiopia
683	inconclusive	ICARDA(unknown)

* derived from crossing

6. 4 Discussion

The resistance of Acc 622 in this experiment was consistent with reactions observed in previous experiments (Chapter 4 and 5). The majority of plants of Acc 622 were scored as 1 or 3, while a few were scored 5 and only one as 7 over all crosses. No plant was scored 9. On the basis of the reaction of the Acc 622 control plants, scores of 1, 3 and 5 were considered as resistant, and 7 as susceptible and indicative of segregation when present in the F_2 population of a cross between Acc 622 and another homozygous resistant line.

All plants used for crossing to produce the F_2 populations were derived from plants that had been selfed and progeny tested for reaction to *A. fabae*. Inclusion of selfed progeny from the parental plants as checks when testing the F_2 populations revealed that the majority of parents were homozygous for resistance to *A. fabae*. However, several lines, including Acc 290, 342, 508, 683, 342 and 508, appeared to segregate which indicates misclassification in the initial selection of parents.

Based on the reaction of the F_2 populations, it was possible to identify six accessions (Acc 295, 303, 484, 496, 668, and 975) with the same genetic control of resistance to *A. fabae* as in Acc 622. There was no evidence of segregation in the F_2 with the disease scores of the parents and F_2 populations ranging from 1 to 5 (highly resistant to moderately resistant) in all crosses. Five of the six accessions originated from the Middle East with the other from Morocco (Table 6.3). Interestingly, Acc 975, which was derived from a cross between ILB 284 (Acc 295) and ILB 37 (Acc 484) showed the same reaction as both Acc 295 and 484. Therefore it could be suggested that the resistance(s) of Acc 975 originated from the same source as Acc 295 and 484.

Eight F_2 populations, from crosses between Acc 622 and 297, 299, 674, 680, 712, 948, 970 and 1046, showed segregation in reaction to *A. fabae* and included plants with scores of 7 or 9. In particular, the F_2 populations of crosses between Acc 622 and Acc 299, 674, 712, 948, 970 and 1046 included a far greater proportion of susceptible progeny than observed in parental controls, indicating the susceptible F_2 plants resulted from transgressive segregation rather than environmental effects. This experiment has therefore demonstrated that the resistance to *A. fabae* of a number of accessions is conferred by different resistant genes to those of Acc 622.

It is possible to speculate on the genetic control that would result in susceptible progeny between two resistant lines. The simplest explanation would be the case for resistance in two lines being controlled by single, but different, dominant genes. In this case, the F_2 progeny of Line 1 (genotype *AAbb*) x Line 2 (genotype *aaBB*) with the genotype *aabb* would be susceptible (segregation ratio 15R : 1S). However, results of Chapter 5 indicated the resistance of Acc622, relative to Icarus, is controlled by a co-dominant or partially recessive gene. If it is assumed resistance of Acc 622 is due to a co-dominant gene, A, then A_1A_1 is fully resistant, A_1A_2 is intermediate and A_2A_2 is susceptible (Chapter 5). The resistance of the alternative line is assumed to be due to a second dominant gene B where *BB* and *Bb* are resistant and *bb* is susceptible. Thus, Acc622 is A_1A_1bb while the alternative line is A_2A_2BB. For a cross between Acc622 and the alternative line, all F_2 progeny of the genotypes - -*B*- and A_1A_1- - would be resistant, A_1A_2bb would be intermediate while plants of genotype A_2A_2bb would be fully susceptible. In view of the environmental variation present in the experiments it is unlikely that the intermediate category could be identified therefore the exact segregation ratio observed would be in the range of 15:1 where the A_1A_2bb genotype had the resistant phenotype to 13:3 where Aabb had the

susceptible phenotype. The intermediate heterozygous plants could be identified by progeny testing at the F_3 generation.

A low frequency of susceptible plants in crosses to Acc 680 and 297, together with the occurrence of a single susceptible plant among the Acc 622 controls, raises some question as to whether the apparent segregation in these populations was due to genetic or environmental effects. For all crosses where susceptible progeny were observed, several methods could be employed to identify whether the high level of symptom expression was due to genetic or environmental effects. Diseased F_2 progeny could be transplanted and grown to maturity and progeny tested in the F_3 generation. If the symptoms were due to genetic effects the F_3 progeny should have a high frequency of susceptible plants, the exact proportion depending upon the genetic control of resistance for the two parents. For example, if the susceptible plants were homozygous for the susceptible allele at two loci all progeny would be susceptible. Alternatively, susceptible plants could be crossed to either of the parents. The F_2 of this cross would segregate and include a much higher proportion of susceptible plants than the initial cross. Again, the proportion of susceptible plants would depend upon the genetic control of resistance for the two parents. An additional method of determining whether the susceptible plants were the consequence of genetic or environmental effects would be to identify molecular markers linked to the resistance genes and test the progeny for the markers. Susceptible plants would not carry the markers linked to the resistance gene of either parent. This method would rely on identifying markers tightly linked to the resistance genes and would be subject to error if recombination occurred between the marker and the gene.

Three accessions of northern European origin, Acc 948 and 970, which showed highly resistant reactions to a range of isolates (Chapter 4) and Acc 1046,

appeared to carry different resistant genes from Acc 622. Transgressive segregation was also observed in the F_2 populations between Acc 970 and Acc 303 and 484, the latter two assumed to carry the same resistance gene(s) as Acc 622. The reaction of Acc 970 relative to Acc 622 concurs with Lawsawadsiri (1994) who reported that Acc 970 carries a single dominant gene for resistance to isolate A26 of *A. fabae*, while current experiments indicate Acc 622 carries a single co-dominant, or partially recessive, gene.

The reaction of Acc 680 relative to Acc 622, 303, 484 and 970 was not conclusive. The initial test of Acc 680 x 622 indicated transgressive segregation, although at a low frequency (one plant in 80 rated as 7, Figure 6.2). There was no indication of transgressive segregation in crosses between Acc 680 and either 303 or 484 suggesting they all carried the same resistance gene(s), while the cross of Acc 680 x 970 showed transgressive segregation at a low level.

As Acc 680 and Acc 484 were both originally derived from ILB 37, and the F_2 of the cross between Acc 484 and Acc 680 did not segregate in reaction to *A. fabae*, it is probable they carry the same resistance gene(s). The initial conclusion that the resistance of Acc 680 is conferred by a different gene to the resistance of Acc 622 was based on a single susceptible F_2 plant among the progeny of Acc 622 x Acc 680. In view of results for Acc 303, 484, 680 and 970 it is possible that the apparent segregation in Acc 622 x Acc 680 was due to environmental, rather than genetic effects and the resistance of Acc 622 is the same as Acc 484 and 680. Acc 680 was therefore classified as carrying the same gene as Acc 622 (Table 6.3). As transgressive segregation was observed among the F_2 progeny of Acc 970 crossed to Acc 622, 303, 484 and 680, it is highly likely that the resistance of Acc 970 to *A. fabae* is different to the four other accessions.

There is evidence that lines with different resistance genes might be selected from the one landrace. For example, Acc 299 (BPL 471) and Acc 303 (BPL 472) were both derived from the Lebanese landrace ILB 287. The F_2 populations from crosses of these two accessions to Acc 622 responded differently with no segregation for Acc 303, but segregation in the F_2 of Acc 622 x Acc 299 indicated Acc 299 carries a different resistance gene to Acc 622. Hanounik and Robertson (1989) also reported a difference in the resistance of BPL 471 and BPL 472. BPL 471 was resistant to *A. fabae* in 13 trials conducted in six countries over a number of years, while BPL 472 was rated as susceptible in France in the same trials. Thus, the procedure of single plant selection within a highly heterogeneous landrace, with selfing during the development of the BPLs, followed by selection for resistance to *A. fabae*, resulted in the development of lines carrying different resistance genes. Similarly, Rashid et al (1991b) reported different genes for resistance to *A. fabae* in two selections derived from BPL 2485 (Acc 680), with BPL 2485-1 having the genotype *Af1Af1 Af7Af7* while BPL 2485-2 was *Af1Af1*.

Accessions 290, 342, 508 and 668 produced F_2 populations when crossed with Acc 622 that showed segregation, but as the parental controls were heterogeneous the segregation could not necessarily be attributed to transgressive segregation. Acc 342 (Giza-4) was included as a susceptible control but appeared to be heterogeneous for reaction to Australian isolates of *A. fabae*. All other parents were progeny tested (Chapter 3), however, it appears that this was not effective in identifying homozygous resistant plants for all populations. Rashid et al. (1991a) suggested that as faba bean is a partially cross-pollinated crop, three cycles of testing and mass selection could reduce the level of heterogeneity, although no population was homogeneous for resistance to *A. fabae*.

The experiments described in this chapter have identified a number of lines that differ from Acc 622 for genetic control of resistance to *A. fabae*. However it is not possible to determine whether the lines that differ from Acc 622 all carry the same resistance gene(s) or if there is further variation among them. The only indication that one line might have a different genetic control of resistance was for Acc 1046 which produced a much higher proportion of susceptible plants than other critical crosses (Fig 6.3, Table 6.2). On the other hand, there was a higher proportion of the parent controls that rated 3 or 5 in the cross of Acc622 * Acc1046 than for other crosses which might indicate a higher disease pressure in the test of this cross which might account for the high frequency of susceptible plants among the F_2 progeny.

The genotypes of a number of *A. fabae* resistant lines were proposed by Rashid et al. (1991a), and selections from several of the populations were included in the present study. However, the limited number of lines in common, possible differences in the actual selections between the two studies, together with different isolates of *A. fabae* prevent inferences being made on the genotypes of the alternative resistant lines identified in the present study. Several methods could be used to distinguish whether the various alternative sources of resistance carry the same or different genes to each other. One method would be to test them with a differential set of *A. fabae* pathotypes. However, as such a set of pathotypes is not available an alternative method would be to intercross these accessions and test the F_2 progeny for reaction to *A. fabae*, with segregation in the F_2 indicating a genetic difference. Susceptible F_2 plants could be progeny tested to confirm their genotype.

The identification of alternative sources of resistance to *A. fabae* to that of the Australian faba bean var Ascot should enable the Australian faba bean breeding program to diversify the resistance genes in future varieties. The elucidation of the relationship among the alternative sources of resistance would ensure even greater diversification and therefore minimise the likelihood of changes in the pathogen *A. fabae* overcoming the resistance of all varieties. In addition, knowledge of the alternative genes should facilitate the various genes being combined in the one variety and further increase the durability of resistance of Australian faba bean varieties to *A. fabae.*

Chapter 7. AFLP analysis of genetic differentiation among Ascochyta blight resistant accessions of faba bean

7.1 Introduction

To optimise crossing and selection programs, information about genetic variability is important. The optimum level of genetic distance between parents in a breeding program will depend on the breeding strategy. For example, the minimum genetic variation between donor and recurrent parent might be sought for backcrossing where a major gene controlling a single trait is introduced into a well adapted background. On the other hand, a moderate to high level of genetic diversity would be more appropriate where the expression of heterosis is sought. A high level of genetic variation between parents also improves the efficiency and accuracy when constructing genetic maps. Further, it might be postulated that genetically distant genepools are more likely to include different genes, such as for disease resistance, than closely related genepools.

Molecular marker technology has proven very useful in contributing to studies of genetic distance of several crops, including faba beans. Link et al. (1995) utilised RAPD markers to classify germplasm and identify genetic distance among inbred faba bean lines, including European small-seeded lines, European large-seeded lines and Mediterranean lines. AFLP markers have been used to measure genetic distance of several crops, such as rice (Zhu et al., 1998), maize (Marsan et al., 1998), lentils (Sharma et al., 1996) and soybean (Maughan, et al., 1996), but, there has been no report of application of AFLP methodology to studying genetic distance in faba bean.

Ascochyta blight resistant faba bean accessions, and susceptible controls, were analysed by the AFLP technique with the aims of (1) identifying the genetic similarities among the Ascochyta blight resistant faba bean accessions, (2) identifying

whether there was a relationship between genetic similarity and the genetic control of resistance to *A. fabae*, and (3) determining whether there was a relationship between genetic similarity and the origin of the accessions.

7.2 Materials and Methods

7.2.1 Plant materials and DNA samples

The twenty-two accessions of faba bean used for the present study are listed in Table 7.1. Leaf samples of each accession were harvested from six plants grown in a glasshouse. These plants were grown from seed obtained from plants that had been selfed and progeny tested to confirm they were homozygous resistant to *A. fabae* (Chapter 3). DNA extraction was carried out on leaf samples from individual plants and the extracts were combined to serve as the DNA sample of each accession.

7.2.2 DNA extraction

DNA was extracted by the method of Weining and Langridge (1991), as follows. The leaves were placed in a 2 ml Eppendorf® centrifuge tube, dipped in liquid nitrogen and ground to a fine powder using a knitting needle. 750 ml of extraction buffer (Appendix 7.1) was added to the samples which were then homogenised by a quick vortex. 750 ml of phenol/chloroform/iso-amyl-alcohol (25:24:1) was added and mixed on a rotor for 30 min. The phases were separated by centrifuging at 5000 rpm for 5 min, and the supernatant was transferred to a new 1.5 ml Eppendorf® centrifuge tube. The phenol chloroform step was repeated (equal volumes of transferred supernatant and phenol chloroform). Following centrifuging, 650 µl of the upper phase was transferred to a new 1.5 ml tube

Table 7.1 Faba bean accessions used for the AFLP study of genetic similarity of *A. fabae* resistant accessions

Waite Acc.	ICARDA Acc[a] Name	Original population/ pedigree	Origin	Reaction to *A. fabae*	References
290	BPL 230 A884	ILB 142	Morocco	Resistant	1, 5
295	BPL 460 A888	ILB 284	Lebanon	Resistant	1, 3, 5
297	BPL 465 A889	ILB 285	Lebanon	Resistant	1, 5
299	BPL 471 A8710	ILB 287	Lebanon	Resistant	1, 2, 3, 5
303	BPL 472 A8713	ILB 287	Lebanon	Resistant	1, 3, 5
342	Giza-4	-	Egypt	Susceptible	3, 5
484	BPL 74 A883	ILB 37	Iraq	Resistant	1, 3, 5
496	BPL 365 A886	ILB 227	Morocco	Resistant	1, 5
508	BPL 818 A8815	ILB 549	Ethiopia	Resistant	1, 2, 3, 5
622	Ascot component		Naxos	Resistant	4
668	L 83120 A882	BPL 460* ILB 37	ICARDA	Resistant	1
674	L 83124 A8824	ILB 37* BPL 2485	ICARDA	Resistant	1
680	BPL 2485 A88302	ILB 37	Iraq	Resistant	1, 2, 3, 5
683	L 83114 B8826	ILB 938* BPL1390	ICARDA	Resistant	7
712	L 82003 B8834	BPL 472*BPL 261	ICARDA	Resistant	7
722	L 82009 B8837	A2* ILB 938	ICARDA	Resistant	7
948	Quasar		UK	Resistant	6
970	ILB 752		Sweden	Resistant	1, 2, 5
975	L 83125	BPL 460*BPL2485	ICARDA	Resistant	1
976	L 83136	ILB 37*BPL 460	ICARDA	Resistant	7
1046	BPL 646	ILB 382	UK, USSR	Resistant	1, 2, 3, 5
Icarus	BPL 710	L81-248571	Equador	Susceptible	4

[a]) The ICARDA accession number includes the selection number of the line provided to the Waite faba bean breeding program

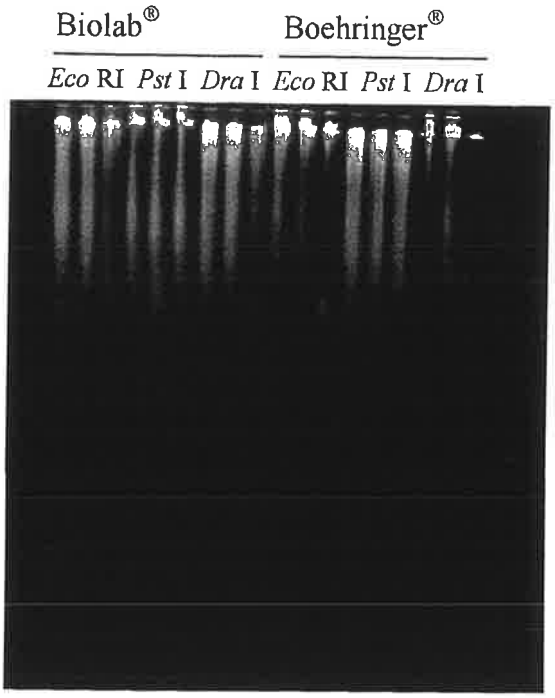
1 Bond et al., 1994.
2 Rashid et al., 1991a.
3 Hanounik and Robertson, 1989.
4 Ramsey et al., 1995.
5 Hanounik and Maliha, 1984.
6 Lockwood et al., 1985.
7 Robertson, L. pers comm.

to which 650 µl of chloroform was added and after mixing the sample was centrifuged. 600 µl of supernatant was transferred to a new 1.5 ml tube. The DNA was precipitated by adding 60 µl 3 M Na-acetate pH 4.8 and 600 µl of isopropanol. The mixtures were centrifuged at 15,000 rpm for 15 min. The supernatant was discarded and the white DNA pellet was washed with 1 ml 70% ethanol. The ethanol was drained and the pellets were vacuum-dried. The DNA was resuspended in 50µl of TE buffer and stored at 4°C.

7.2.3 Digestion of DNA

Three DNA samples (Acc 622, Acc 970 and Icarus) were digested separately using *Pst* 1, *Eco* R1 and *Dra* 1 (either Biolab® or Boehringer®) in order to identify the most appropriate of these digestion enzymes for further steps of the experiment. As all enzymes digested the DNA samples (Fig. 7.1), *Eco* R1 (Biolab®) was initially used. However, in AFLP analysis, primer combinations based on the enzyme *Eco* R1 did not produce either any bands, clear bands or polymorphisms. Therefore, *Pst* 1(Boehringer®) was used as the alternative enzyme. DNA was digested at 37°C for 4 hours using 2 µl *Pst* I, 1 µl BSA, 1 µl Spermidine, 1.2 µl 10x reaction buffer (supplied with the enzymes) and made up to a final reaction volume of 12 µl with sterile water.

Figure 7.1 Three DNA samples (Acc 622, 970 and Icarus) digested by *Pst* I, *Eco* R I and *Dra* I


Biolab®
Boehringer®
Eco RI *Pst* I *Dra* I *Eco* RI *Pst* I *Dra* I

7.2.4 Gel electrophoresis

Digested DNA was loaded onto a 1.5% agarose gel which was electrophoresed in 1 X TAE buffer for 2 hours at a current of 40 mA. Gels were stained in 10μg ml^{-1} ethidium bromide for 30 min and checked for complete digestion under UV light.

7.2.5 AFLP Analysis

The AFLP analysis was undertaken in the laboratory of Dr. Peter Langridge, Waite Institute, Adelaide University, using a method derived from Vos et al, (1995) with modifications suggested by Greg Penner (unpublished).

7.2.5.1 Preparation of template DNA

(1) *Restriction Digest*

1μg of DNA was digested at 37°C for 3 hours using 5 μl R-L buffer (Appendix 7.2), 5 U *Mse* I, 5 U *Pst* I and made up to a final volume of 50 μl with sterile water.

(2) *Annealing of Adapter*

Stock solutions containing both *Mse* I adapters (Table 7.2) at 50 μM each, and of both *Pst* I adapters (Table 7.2) at 5 μM each were prepared. The stocks were heated at 90°C for 3 min then the adapters were allowed to anneal at room temperature for more than 30 min.

(3) *Ligation of Adapters*

Ligation of adapters occurred by adding 1 μl *Mse* I adapters, 1 μl *Pst* I adapters, 1 μl 10 x R-L buffer, 1.2 μl 10 mM ATP, T4 DNA ligase (1U/μl) and 4.8 μl

H_2O to the digested DNA sample (60 µl total volume), and incubated at 37°C for 3 hours and then placed at 4°C overnight.

The next day the DNA was precipitated by adding 120µl ethanol and 6 µl 4.8 mM sodium acetate (pH 4.8) and placed in liquid nitrogen for 5 min. The mixtures were centrifuged at 15,000 rpm for 15 min, the pellets were washed with 70% ethanol and then dried in a speed vac concentrator (Savant®) for 10 min. The DNA was resuspended in 60 µl 0.1 M TE (Appendix 7.3).

(4) *Pre-amplification of DNA*

Pre-amplification of prepared template was performed with complementary primers, using a single *Pst* I (*Pst* ACA) and three *Mse* I primers (*Mse* I CAG, *Mse* I CCA, *Mse* I CGA) (Table 2). This pre-amplification of DNA was prepared with 4 µl of the digested and ligased DNA mixed with 1µl 75ng/µl *Pst* I + 1 primer (Table 7.2), 1µl 75ng/µl of *Mse* I + 1 primer (Table 7.2), 4 µl 1.25 mM dNTPs, 2.5 µl 10 x Taq buffer, 1.5 µl 25 mM $MgCl_2$, 0.2 µl *Taq* polymerase (5U/µl) with sterile water added to a total volume of 25 µl. The PCR reaction conditions were: 20 cycles of 30 sec at 94°C, 1 min at 56°C, and 1 min at 72°C. Following PCR, 100 µl H_2O was added to each sample. This sample served as the template DNA.

7.2.5.2 Selective Amplification of Template DNA

(1) *End labelling*

Only *Pst* I compatible primers were labelled. The primer was prepared by mixing 1µl 50 ng/µl primer 1, 1.5 µl ^{32}PγATP (10 Ci/ µl), 1 µl 10 x PNK buffer (Appendix 7.4), 0.2 µl 10 U/µl T4 PNK, in a total volume of 10 µl. The mixtures were incubated at 37°C for 30 - 60 min.

(2) *Selective PCR*

Selective PCR was performed with 1µl of labelled primer *Pst* I, 0.5 µl (50 ng/µl) of unlabelled primer *Pst* I, 0.6 µl (50 ng/µl) *Mse* I, 2 µl 10 x *Taq* buffer, 1.2 µl 25 mM $MgCl_2$, 3.3 µl 1.25 mM d NTPs, 0.2 µl (5U/µl) *Taq* polymerase and 9.2 µl H_2O (total volume 18 µl). 2 µl template DNA was added to the reaction mixture. PCR conditions for the first cycle were: 30 sec at 94°C, 30 sec at 65°C, and 1 min at 72°C. This was followed by 9 cycles over which the annealing temperature was decreased by 1°C per cycle followed by 30 sec at 94°C, 30 sec at 65°C, and 1 min at 72°C for 25 cycles.

7.2.5.3 Gel electrophoresis

The PCR product was mixed with 20 µl of gel loading buffer (Appendix 7.5) and denatured by incubation for 3 min at 90°C. Gels were prepared by mixing 40 ml of sequencing gel (Sequagel-6®), 10 ml of complete buffer reagent (Sequagel®) and 450 µl 1% ammonium persulphate. Samples of 2 µl were loaded in 5x TBE (Appendix 7.6) per track and gels were run for 2 hours at 40 W, transferred to 3 MM chromatography paper and dried on a gel drier (Biopad®) for 45 min at 90°C. Gels were exposed to X-ray film for about 2 days at room temperature.

Table 7.2 Sequences of adapters and primers used for AFLP analysis

Name	Sequence
Pst I adapter 1	5' - CTC GTA GAC TGC GTA CAT GCA - 3'
Pst I adapter 2	5' - TGT ACG CAG TCT AC - 3'
Mse I adapter 1	5' - GAC GAT GAG TCC TGA G - 3'
Mse I adapter 2	5' - TAC TCA GGA CTC AT - 3'
Pst I- 1 primer (A)	5' - GAC TGC GTA CAT GCA GA - 3'
Mse I- 1 primer (C)	5' - GAT GAG TCC TGA GTA AC - 3'
Pst I ACA	5' - GAC TGC GTA CAT GCA GAC A - 3'
Mse I CAG	5'- GAT GAG TCC TGA GTA ACA G - 3'
Mse I CCA	5' - GAT GAG TCC TGA GTA ACC A - 3'
Mse I CGA	5' - GAT GAG TCC TGA GTA ACG A - 3'

7.2.5.4 Data analysis

Bands were scored as present (score 1) or absent (score 0) on autoradiographs. Monomorphic fragments were not included in the analysis, as suggested by Link et al. (1995). Estimates of divergence among genotypes were based on the probability that an amplified fragment from one accession was also present in another. These comparisons were calculated by means of Jaccard's formula (1908), according to the following equation :

$$\text{Genetic distance} = 1 - n_{xy} / (n_x + n_y - n_{xy}),$$

where n_{xy} is the number of bands common to line x and line y,

n_x is the total number of bands in line x,

n_y is the total number of bands in line y.

Genetic distances were used to construct a dendrogram, displaying the hierarchical association among all accessions by the use of the average linkage as determined by the cluster procedure of Phylogenetic Analysis Using Parsimony (PAUP) version 3.11, ©Smithsonian Institution, 1993. Acc 1046 was selected as the outgroup to which other accessions were compared, since this accession had the largest average genetic distance (0.44).

7.3 Results

The preliminary results showed that 63 pairwise combinations of three *Eco* RI primers and twenty one *Mse* I primers tested against four DNA samples (Acc 290, 622, 970 and Icarus) either did not produce bands or bands were unclear or not polymorphic (Table 7.3). Alternative combinations of *Pst* I and *Mse* I primers were then used. Combinations of *Pst* ACA and 21 primers of *Mse* I were tested against four accessions (Acc 290, 622, 970 and Icarus). Three combinations revealed clear polymorphisms, and they were selected to analyse the full set of twenty two accessions.

A total of 176 bands were generated by the three AFLP primer combinations and 104 bands (62%) were polymorphic. The number of polymorphisms detected by individual primer pairs was 42, 23 and 39 for primers *Pst* ACA - *Mse* CAG, *Pst* ACA - *Mse* CCA and *Pst* ACA - *Mse* CGA, respectively. Figure 7.2 shows the DNA finger prints of 22 accessions after PCR with the primer combination *Pst* ACA - *Mse* CAG.

The average genetic distance over all accessions was 0.34. The greatest distance of 0.53 was between Acc 297 and Acc 299 while the most similar (different at 9% of bands) were Acc 299 and Acc 668. The average genetic distance for

individual accessions ranged from 0.30 for Acc 712 and Icarus to 0.44 for Acc 1046 (derived from Table 7.4).

The dendrogram constructed on the basis of shared fragments divided the 22 accessions into two major groups, one of which comprised eight accessions (Acc 290, 295, 712, 508, 948, 674, 970 and 342) and the other group included five accessions (Acc 299, 668, 496, 722 and Icarus). The remaining accessions were either unpaired or as groups of two or three (Fig 7.3). Acc 622 did not occur in either of the major groups but grouped with Acc 297 and Acc 484. When considered on a pairwise-basis (Table 7.4) most accessions had a genetic distance from Acc 622 of less than 0.40, while Acc 1046 was the most divergent with a value of 0.44.

The accessions included in the study originated from a wide range of countries, including the Middle East, North Africa, Northern Europe and Equador. There was no consistent trend between grouping on the dendogram (Figure 7.3) and region of origin. For example, four accessions (Acc 295, 297, 299 and 303) originated from Lebanon but they were evenly distributed across the dendogram and the genetic distance between each pair in this group of four accessions (Table 7.4) was greater that the average genetic distance over all accessions. Similarly, the two accessions of Moroccan origin (Acc 290 and 496) and the two Iraqi accessions (Acc 484 and 680) did not show a high degree of genetic similarity.

Table 7.2 Results of AFLP analysis of Acc 290, 622, 970 and Icarus using *Pst* I and *Eco* RI primers in combination with *Mse* I. Primer combinations that produced clear polymorphic band patterns and were selected for further testing are identified in bold print.

	Pst I	*Eco* RI		
Mse I	ACA	AG	CAG	ACT
A	no band	no band	no polymorp	no polymorp
AC	no band	no band	no polymorp	no polymorp
ACA	no band	no band	no polymorp	no polymorp
ACT	not clear band	no band	no polymorp	no polymorp
C	no band	no band	no polymorp	no polymorp
CAA	not clear band	no polymorp	no polymorp	no polymorp
CAG	**polymorphic**	no polymorp	no polymorp	no polymorp
CAT	not clear band	no polymorp	no polymorp	no polymorp
CCA	**polymorphic**	no polymorp	no polymorp	no polymorp
CCT	no polymorp	no polymorp	no polymorp	no polymorp
CGA	**polymorphic**	no polymorp	no polymorp	no polymorp
CTA	no polymorp	no polymorp	no polymorp	no polymorp
CTG	no polymorp	no polymorp	no polymorp	no polymorp
G	no polymorp	no band	no polymorp	no polymorp
GAA	not clear band	no band	no polymorp	no polymorp
GAT	no band	no polymorp	no polymorp	no polymorp
GCC	low polymorphic	no polymorp	no polymorp	no polymorp
GTG	not clear band	no polymorp	no polymorp	no polymorp
TAA	no band	no polymorp	no polymorp	no polymorp
TG	no band	no polymorp	no polymorp	no polymorp
TGC	low polymorphic	no polymorp	no polymorp	no polymorp

Table 7.4 Similarity matrix of 22 accessions of faba bean as determined by AFLP analysis

Acc	295	297	299	303	342	484	496	508	622	668	674	680	683	712	722	948	970	975	976	1046	Icarus
290	0.33	0.35	0.45	0.43	0.34	0.36	0.39	0.35	**0.42**	0.39	0.46	0.35	0.30	0.28	0.38	0.36	0.42	0.40	0.33	0.40	0.37
295		0.46	0.38	0.44	0.39	0.42	0.28	0.34	**0.39**	0.35	0.39	0.40	0.31	0.32	0.35	0.35	0.39	0.32	0.44	0.47	0.29
297			0.53	0.36	0.38	0.35	0.53	0.45	**0.38**	0.49	0.46	0.34	0.39	0.44	0.43	0.44	0.46	0.44	0.38	0.40	0.45
299				0.48	0.26	0.47	0.15	0.33	**0.32**	0.09	0.24	0.36	0.32	0.33	0.20	0.28	0.33	0.39	0.35	0.49	0.29
303					0.33	0.46	0.48	0.43	**0.42**	0.41	0.47	0.36	0.39	0.44	0.39	0.39	0.35	0.44	0.32	0.40	0.39
342						0.38	0.29	0.30	**0.28**	0.20	0.28	0.29	0.18	0.25	0.23	0.29	0.30	0.36	0.21	0.41	0.25
484							0.46	0.42	**0.29**	0.41	0.43	0.41	0.34	0.37	0.43	0.45	0.40	0.37	0.38	0.51	0.41
496								0.35	**0.33**	0.12	0.25	0.37	0.28	0.31	0.20	0.32	0.32	0.34	0.36	0.46	0.24
508									**0.33**	0.30	0.35	0.37	0.37	0.32	0.34	0.29	0.35	0.37	0.36	0.40	0.30
622										**0.28**	**0.32**	**0.33**	**0.24**	**0.33**	**0.24**	**0.38**	**0.30**	**0.34**	**0.31**	**0.48**	**0.26**
668											0.22	0.29	0.25	0.25	0.14	0.30	0.27	0.33	0.28	0.43	0.22
674												0.38	0.26	0.33	0.26	0.30	0.28	0.40	0.35	0.47	0.31
680													0.31	0.35	0.34	0.33	0.36	0.32	0.27	0.38	0.31
683														0.27	0.23	0.31	0.30	0.31	0.23	0.50	0.23
712															0.27	0.31	0.31	0.42	0.30	0.39	0.22
722																0.32	0.26	0.35	0.32	0.46	0.17
948																	0.35	0.33	0.30	0.45	0.33
970																		0.41	0.33	0.39	0.32
975																			0.32	0.45	0.34
976																				0.44	0.35
1046																					0.50

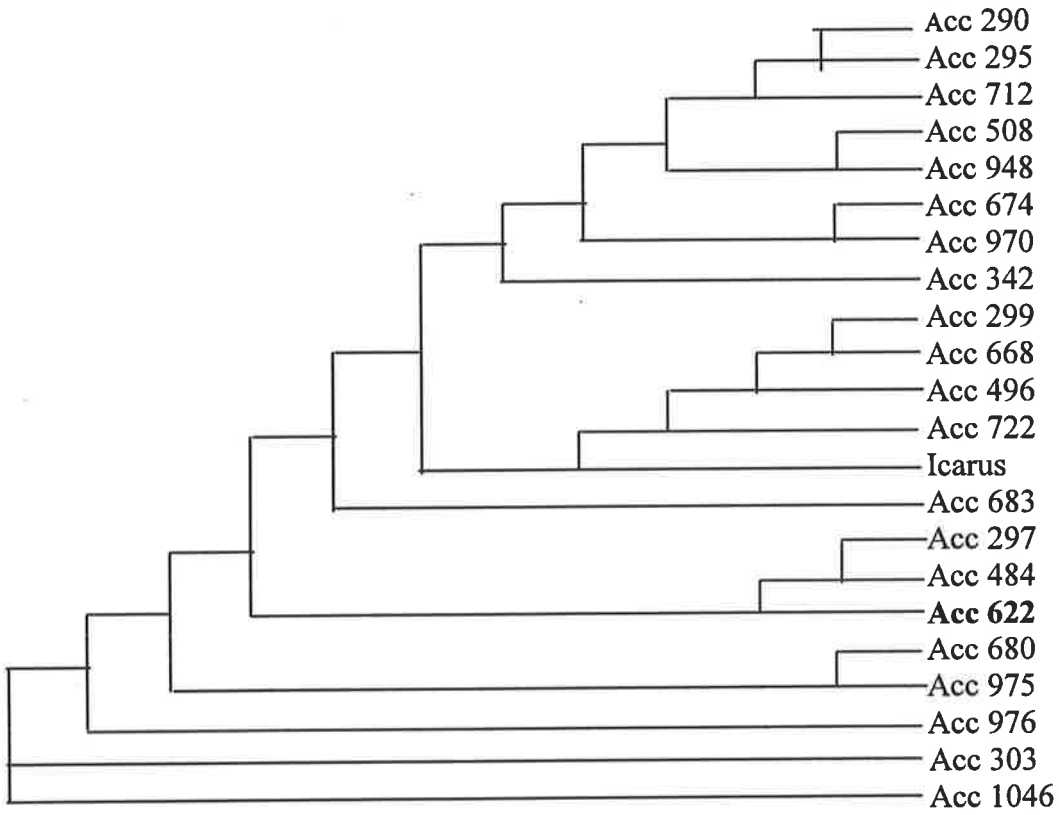
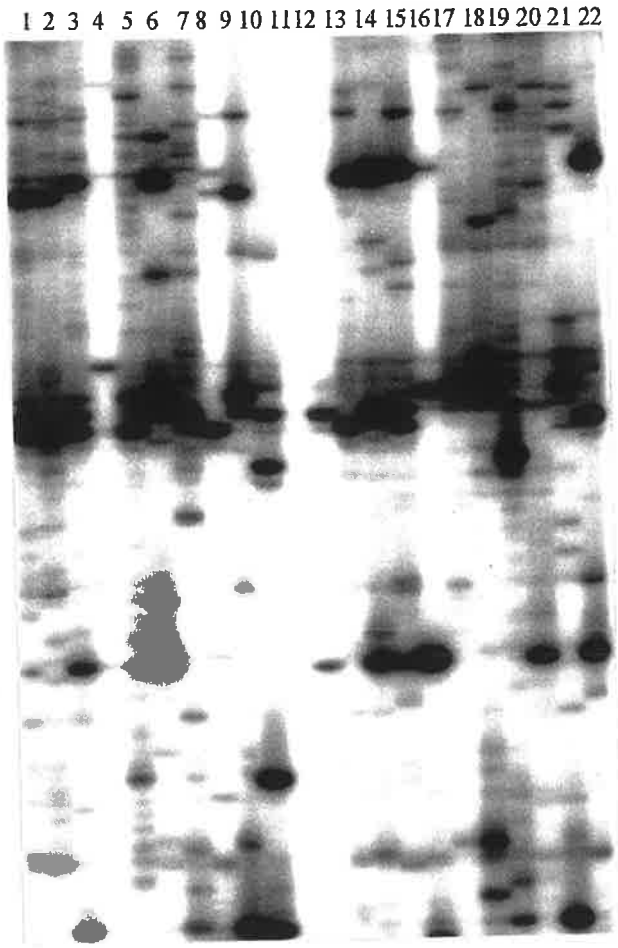


Figure 7.3 Dendrogram of 22 faba bean accessions following AFLP analysis, using Acc 1046 as the outgroup

Figure 7.2 Finger prints of 22 accessions after PCR with primer combination *Pst* ACA - *Mse* CAG.

Lane 1 Acc 290
Lane 2 Acc 295
Lane 3 Acc 297
Lane 4 Acc 299
Lane 5 Acc 303
Lane 6 Acc 342
Lane 7 Acc 484
Lane 8 Acc 496
Lane 9 Acc 508
Lane 10 Acc622
Lane 11 Acc 668
Lane 12 Acc 674
Lane 13 Acc 680
Lane 14 Acc 683
Lane 15 Acc 712
Lane 16 Acc 722
Lane 17 Acc 948
Lane 18 Acc 970
Lane 19 Acc 975
Lane 20 Acc 976
Lane 21 Acc 1046
Lane 22 Icarus


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

7.4 Discussion

AFLP analysis using three primer combinations was used to construct a phylogenetic tree of 22 faba bean accessions resulting in two major groups of 8 and 5 accessions, and several groups of two or three accessions. The average genetic distance over all lines tested was 0.34, with the greatest distance among accessions being 0.53 (53%) and the closest distance 0.09 (9%). These values are on average lower than those reported by Link et al. (1995) for genetic distances determined by RAPD analysis, although there is considerable overlap in values between the two studies and the range in the present study is greater. Link et al. (1995) reported a range in genetic distance over 28 inbred lines of faba bean, each represented by a single plant, of 0.306 - 0.646. One possible reason for the difference in range in genetic distance between the present study and Link et al. (1995) could be selection of germplasm tested. The objective of Link et al.'s (1995) study was to identify genetic distance between and within germplasm pools, thus entries were selected to represent a wide geographic range. In the present study the major criterion for selection of entries was resistance to *A. fabae*, while two susceptible entries were included for comparison. This narrow focus could reasonably be expected to include a lower level of genetic diversity. In addition, the majority of accessions in the present study fall in the "Mediterranean" category that Link et al. (1995) described as having a lower level of genetic diversity than European germplasm.

The number of AFLP primer pairs used to classify cultivars for purposes of determining genetic diversity differs between authors. Paul et al. (1997) analysed 32 genotypes of tea with 5 primer combinations which revealed a total number of 73 polymorphic amplified DNA fragments, and these classified the genotypes to three

groups. Sharma et al. (1996) used a single primer combination to analyse 54 genotypes of *Lens*, and revealed 23 AFLPs from which a dendogram was constructed. Similarly, Zhu et al. (1998) concluded that one primer combination which can generate more than 30 polymorphic markers could be sufficient to classify 57 cultivated rice accessions, although as more markers were included in the analysis, there were fewer ambiguities.

There was little relationship between genetic similarity determined by AFLP analysis and region of origin. Although two accessions from northern Europe, Acc 948 and 970 fell in the same group, each Lebanese accession, Acc 295, 297, 299 and 303 occurred in a different group, as did the accessions from Morocco (Acc 290 and 496). This could indicate there is as much diversity on a regional scale as across the Mediterranean basin from where most accessions were derived. In addition, it could be a consequence of the criterion of selecting the entries that resulted in a restricted gene pool tested. If a greater proportion of lines from non-Mediterranean regions, such as northern Europe and China, had been included, the entries in the present study might form one major group among several. It is interesting to note that although the susceptible cultivar, Icarus, is derived from an Ecuadorean landrace, it groups with accessions of Mediterranean basin origin. This probably reflects the fact that the Spaniards introduced faba beans to South America in the post-Columbian era.

Accessions derived from the same source (ILB) did not necessarily fall within the same group. Acc 299 and 303 were both derived from ILB 287 but were genetically distant with a pair-wise genetic difference of 0.48, while Acc 484 and 680 derived from ILB 37 had a genetic distance of 0.41, higher than the average genetic distance overall accessions of 0.34. This indicated that there is a large degree of genetic variation within the landrace populations. On the other hand, Acc 722 and

Icarus were closely related with a genetic distance of 0.17. One parent of Acc 722 is ILB 938, the same population BPL 710 (source of Icarus) was derived from.

Acc 622, which was the standard accession for comparison of resistance genes in Chapter 6, did not fall within either the two major groups. Most accessions with different resistance genes to Acc 622 were in the one group (Acc 712, 948, 674 and 970) while Acc 299 was in the other major group (Figure 7.3). Despite the accessions with alternative resistance genes occurring in different groups to Acc 622, when examined on a pair-wise basis there did not appear to be a relationship between *A. fabae* resistance genes and genetic distance from Acc 622 (Table 7.4).

Acc 484 grouped with Acc 622 and the value of genetic distance was less than the average over all accessions (0.29). This is consistent with the result of Chapter 6 where it was concluded that both accessions have the same resistance gene. Acc 297, which was considered to also carry the same resistance gene as Acc 622, was also in the same group as Acc 622.

In summary, AFLP analysis offers a potential means of assessing genetic variations among accessions in faba bean. The analysis did not identify clear-cut separation of accessions on the basis of either origin or gene controlling resistance to *A. fabae*. This was possibly due to the large amount of genetic variation among accessions representing resistance to *A. fabae*, including accessions derived from the same origin. The high level of polymorphism displayed should enable the AFLP technique to be utilised for genetic mapping of faba beans. The results of genetic distance between pairs of accessions (Table 7.4) should enable selection of appropriate parents for mapping populations to maximise the level of genetic variation while including the specific traits to be mapped.

Chapter 8. General Discussion

Several aspects related to resistance of faba bean to *A. fabae* have been studied in this thesis. The results should contribute to both the efficiency and long-term effectiveness of breeding *A. fabae* resistant cultivars.

All accessions included in this study, other than the susceptible controls, were selected on the basis of published resistance to *A. fabae*. Prior to undertaking the genetic studies, single plants of all accessions were self-pollinated and progeny tested to identify homozygous resistant plants. This process demonstrated that several of the accessions that were derived from ICARDA inbred lines were heterogeneous for reaction to *A. fabae*. These accessions included Acc 290, 299, 508 and 1046. Further selection and progeny testing of self-pollinated plants of these selections is required to develop homogeneous homozygous resistant lines. There was evidence of a reduction in heterogeneity of Acc 299 when the progeny of selfed plants and bulk samples were tested with a number of Australian isolates of *A. fabae* (Chapter 4). A similar procedure could be adopted to produce *A. fabae* resistant selections from heterogeneous populations that are well adapted to southern Australia. This method was used to select the Ascochyta blight resistant cultivar Ascot from well-adapted heterogeneous cultivar Fiord (Knight, unpublished).

Rashid et al. (1991a) suggested that three cycles of testing and mass selection could reduce the level of the heterogeneity although this procedure did not produce any population that was homozygous for resistance to *A. fabae*. However, the duration of this project did not allow an extensive period of inbreeding and progeny testing to develop uniform homozygous lines. In addition, seed production from self-pollinated plants was in many cases insufficient to enable rigorous progeny testing with sufficient residual seed for experimentation.

Variability in reaction to *A. fabae* among accessions tested in this study was high. Eight accessions reacted differently when inoculated with isolates that caused infection on susceptible controls (Chapter 4). The variation in reaction was observed between accessions, which were derived from either the same source (e.g. same ILB) or origin. For example, Acc 299 and 303 which were derived from ILB 287, differed in reaction to isolate 331/91. One consequence of the high level of variability within and between accessions was that there was some inconsistency in the conclusions depending upon the method of analysis. Several accessions proposed to carry the same resistance gene as Acc 622, on the basis of F_2 populations (*i.e.* Acc 303, 975 and 680), were quite distant to Acc 622 when investigated using molecular markers (AFLP). This indicates that all lines carrying the one resistance gene are not closely related and the gene is widespread both in terms of genetic backgrounds and geographically.

There was a wide range of pathogenic variability among eight Australian isolates of *A. fabae* tested on eight faba bean accessions. Isolate 331/91 had the highest level of pathogenicity and caused disease on all accessions and the greatest differentiation between resistant and susceptible plants. Based on this result, isolate 331/91 was selected as the inoculum in all experiments investigating the genetic control of resistance to *A. fabae*. Variability among isolates of *A. fabae* in Australia was identified by Lawsawadsiri (1994), but identification of races was uncertain due to the non-uniform reaction within the accessions tested. The sexual stage of *A. fabae* has been identified (Jellis and Punithalingam, 1991) and this will enable the continuous development of new pathotypes through recombination and selection. It is therefore important to both monitor the pathogenicity of Australian isolates of *A. fabae*, particularly to Ascot, to identify any changes in pathogenicity that might

indicate a breakdown in resistance, and also to deploy or combine alternative resistance genes to minimise the impact of a change in pathogenicity of *A. fabae*.

This study indicates that the resistance of Acc 622, one component of cultivar Ascot, to isolate 331/91 of *A. fabae* is conferred by a single, or possibly two, co-dominant or partially recessive gene(s). Ramsey et al. (1995) described the resistance of Ascot to be recessive. The contrasting conclusion between the two studies might be related to the nature of the experiments and populations studies. In the present study the F_1, F_2 and F_3 generations were tested whereas the study of Ramsey et al (1995) was based on the F_2 alone. Lawsawadsiri (1994) identified that Acc 970 carried a single dominant gene for resistance to the Australian *A. fabae* isolate A26. Rashid et al (1991a) reported that resistance in faba bean to *A. fabae* was determined by several genes, some dominant and some recessive, depending on the isolates and the susceptible parent used. As faba beans are partially cross-pollinated, and therefore heterozygous at many loci, the phenotypes of disease resistance and other traits conferred by recessive genes will be expressed at a low frequency in random mating populations. One strategy that could be employed to improve the efficiency and success of a mass selection program for recessive traits with low gene frequency would be to allow one or several generations of self-pollination prior to undertaking selection. This is effectively what occurred at ICARDA in developing the Bean Pure Lines (BPL) where lines with homozygous resistance to *A. fabae* were developed (Robertson and El-Sherbeeny, 1993).

The segregation patterns of F_2 populations enabled several accessions to be classified as having either the same, or different resistance gene relative to Acc 622. Acc 484 is an example of an accession carrying the same resistance gene as Acc 622. The F_2 progeny of the cross between the two accessions did not segregate and in

addition, AFLP analysis indicated a close relationship between the two accessions. Acc 970, which was described by Lawsawadsiri (1994) to carry a dominant *A. fabae* resistance gene, produced transgressive segregation when crossed with Acc 622 and also with Acc 303 and 484. This indicates that the *A. fabae* resistance of Acc 970 is conferred by a different gene to Acc 622 and other similar genotypes. Several other accessions with different resistance genes to Acc 622 were identified, but the relationship among the alternative sources of resistance was not studied. These accessions should be intercrossed and the F_2, F_3 and/or back-cross generations tested for reaction to *A. fabae* with transgressive segregation indicating different genes. This would provide further information regarding the most appropriate accessions to utilize to diversify resistance to *A. fabae* in the Australian faba bean breeding program.

Not all accessions could clearly be allocated to the same, or different, genetic control as Acc 622. This was a result of a low frequency of susceptible plants in both the parental controls and the F_2 progeny. Thus, it was uncertain whether the susceptible parental controls were a consequence of segregation because the initial plant selected was heterozygous, or due to environmental effects which resulted in the critical plants being exposed to conditions that were highly conducive to disease development. To provide a more accurate classification of these accessions, further single plant selections with more stringent progeny testing should be undertaken to develop homozygous resistant lines prior to undertaking genetic studies.

Several procedures could be undertaken to confirm that the presence of F_2 plants with a high level of disease expression was due to transgressive segregation, rather than a consequence of environmental effects. These procedures include (1) progeny testing, (2) crossing the susceptible plant to either parent and observing the

reaction of F_2 progeny and (3) identification of molecular markers linked to the resistance genes.

The availability of resistance genes other than the one in Acc 622 (Ascot) will contribute to the durability of resistance in Australia by allowing either the breeding a range of cultivars, each with a different source of resistance, or the pyramiding of several resistance genes in the one variety. In order to pyramid resistance genes it is necessary to be able to identify the individual genes. This could be done either with a set of differential isolates or markers linked to the individual resistance genes. There was no clear indication of differential reaction to Australian isolates among the more resistant accessions tested, including Acc 948 and 970 which carry different genes to Acc 622. However, the preliminary results of AFLP analysis indicate a high level of polymorphism and this procedure could be adapted to identify markers linked to individual resistance genes.

AFLP analysis was employed to investigate the similarity among 22 accessions of faba bean. The results of this analysis were inconclusive with respect to the relationship between genetic similarity and (a) genetic control of resistance to *A. fabae*, (b) country/region of origin and (c) landrace of origin. This demonstrates a high level of variability both within and between faba bean land races. In addition, the entries included in this study did not represent the full range of faba bean germplasm. If more diverse *A. fabae* resistant lines were included (for example Paull and Ramsey recently identified *A. fabae* resistant plants among germplasm from China and Pakistan) the relationship between genetic similarity and other traits might be more apparent.

Conclusions

Pathogenic variability of *A. fabae* was high, while variation was also observed within and between several accessions which had previously been identified as resistant. This variability confounded the evaluation of genetics of resistance in some instance, and it was not possible to draw conclusions regarding the genetic similarity, gene(s) controlling the resistance to *A. fabae* and origin among accessions tested. Based on the segregation of F_1 hybrids, F_2 progeny and F_3 families, Acc 622 was identified to carry a single co-dominant or partially recessive gene to isolate 331/92 of *A. fabae* relative to the susceptible cultivar, Icarus. A number of accessions carrying alternative resistance genes to the resistance of Ascot were identified and these should contribute to long-term utilization of resistant cultivars as a means of minimizing the impact of *A. fabae* on faba bean production in Australia.

APPENDICES

Appendix 3.1 Recycled Soil (R.S.)

Three and half tonnes of composted ex-experimental soils are sieved into a sterilising trailer through a 1 cm grid mechanical sieve. This is steamed at 100°C for over 1 hour after which the soil is dumped in the R.S. soil bay to cool.

2/3 cubic metre of this soil is mechanically mixed with 1/2 bale of Peatmoss (which expands to 1/6 cubic metres) and the following fertilisers. R.S. should have a pH of about 6.5.

Fertilisers :

Blood Meal	500 g
Potassium sulphate	200 g
Super Phosphate	100 g
Calcium Carbonate	200 g

Appendix 3.2 Results of preliminary screening of putatively resistant accessions prior to selection of individual plants to be used for further experimentation. Up to nine plants of each accession were self-fertilized and three progeny of each were tested for reaction to *A. fabae*. Boxes with numbers in italics corresponded to selected plants. The number of samples tested varied between accessions due to poor seed set during self-pollination and poor germination in the experiment.

Plant's number	Accession																			
	290	295	297	299	303	342	484	496	508	622	668	674	680	683	712	948	969	970	975	976	1046
1	5 3 5	*1* *1* *1*	3 3 3	*1* *1* *1*	3 3 1	*5* *5* *3*	*1* *1* *1*	5 7	*1* *1* *3*	*3* *1* *1*	*1* *1* *1*	*1* *1*	*1* *1* *1*	7 5 5	*3* *1* *1*	*1* *1*	7 9 9	*1* *1* *1*	3 5 1	*1* *1* *1*	5 3
2	*3* *3*	*1* *1* *1*	7 7 3	9 5 3	*1* *1*	*5* *3* *3*	*1* *1*	3 5	*1* *1* *1*	*3* *1* *1*	*1* *1* *1*	*1* *1*	*1* *1* *1*	*1* *1* *3*	7 9 7	*1* *1*	*9* *9* *7*	*1* *1* *1*	*1* 3 5	*1* *1*	5 5 3
3	*1* *1* *3*	1 1 5	1 3	5 5 5	*1* *1* *1*	9 3 1	*1* *1*	*1* *1* *1*	5 3	*1* *1* *1*	*1* *1*	*1* *1* *1*	3 5 5	*3* *1* *1*	1 3 3	*1* *1*	*9* *9* *9*	*1* *1* *1*	*1* *3* *1*	3 3 1	5 5
4	3 5	*1* *1*	5 1 3	*1* *1* *1*	3 3	3 9 7	*1* *1* *1*	*1* *1* *1*	5 7 7	*1* *1* *1*	*1* *1*	3 3 1	5 5 3	1 3	9 9 3	1 3	7 *9* *9*	*1* *1* *1*	*1* *3* *1*	*1* *1* *3*	*3* *3* *3*
5	*1* *3* *1*	*1* *1*	7 5 3	*3* *1* *1*	*1* *1* *1*	7 9		1 3 5	5 9	1 3	3 1 3	1 3 3	1 5 3	9 9	*1* *1*	*1* *1*	9 9 5	*1* *1* *1*	*1* *1* *1*	*1* *1* *3*	*1* *3* *1*
6	3 5 1	*1* *1*	*1* *1*	3 1 3	1 5	*7* *5* *5*		3 3 3	1 3 3	*1* *1* *1*	*1* *1* *3*	*1* *3* *1*	*1* *1* *1*	5 3	*1* *1*		*9* *7* *7*	*1* *1* *3*	5 7 5	*1* *1* *3*	7 7 5
7		3 1 3	*3* *1* *1*	3 3	1 5 1			1 3	*1* *1* *3*	*3* *1* *1*	*1* *1*		3 5 1	*1* *1*	9 9			*1* *1* *3*	1 9 7	*1* *1*	1 3 5
8		*1* *1*	3 5	3 3 5	*1* *3* *1*				*1* *3* *1*	3 5 1	1 3 3		1 1 5		5 5				*1* *3* *1*	*1* *1* *1*	
9		*1* *1*	*1* *3* *1*							1 3 3	1		*1* *1* *1*		7 7 7					1 1 5	

Appendix 7.1 Extraxtion Buffer

1% Sarkosyl, 100 mM Tris HCl, 100 mM NaCl, 10 mM Na_2EDTA, 0.1M Na_2SO_3

Sarkosyl	1 g
Tris	1.21 g
NaCl	0.58 g
Na_2EDTA	0.48 g
0.1M Na_2SO_3	1.26 g
pH	8.5
H_2O to	100 ml

Appendix 7.2 10 x R-L Buffer

100 mM TrisHAc, 100mM MgAc, 500 mM Kac, 50 M DDT

Tris	1.21 g
MgAc	2.14 g
KAc	4.91 g
DTT	0.77 g
pH	7.5
H_2O to	100 ml

Appendix 7.3 0.1 M TE

10 mM Tris HCl pH 8.0, 0.1 mM Na_2EDTA

1M Tris HCl, pH 8.0	1 ml
0.5 M Na_2EDTA, pH 8.0	10 μl
H_2O to	99 ml

Appendix 7.4 10 x PNK buffer

250 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT, 5 mM spermidine

Tris	3.03 g
$MgCl_2$	2.03 g
DTT	0.77 g
Spermidine	0.13 g
pH 7.5	
H_2O to	100 ml

Appendix 7.5 5x TBE

0.45 M Tris, 0.45 boric acid, 10 mM Na_2EDTA

Tris	108 g
boric acid	55 g
0.5 M Na_2EDTA pH 8.0	40 ml
H_2O to	21 ml

Appendix 7.6 Gel Loading Buffer

98% formamide, 10 mM Na_2EDTA, 0.05% bromophenol blue, 0.05% xylane cyanol

deionised formamide*	98 ml
0.0 mM Na_2EDTA	2 ml
bromophenol blue	0.05 g
xylene cyanol	0.05 g

* the formamide was stirred to deionise for at least 1 hour with mixed bed resin (5g/100 ml formamide) then filtered trough Whatman #541 paper, then stored at - 20°C.

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