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Ascochyta Blight Resistance in Lentils: Genetics and Supporting Techniques for Breeding

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Plant Genetics and Breeding

At

Lincoln University

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Canterbury
New Zealand

2001

ACKNOWLEDGEMENTS

I am indebted to my supervisors, Associate Professors David McNeil and George Hill for their kind support and guidance throughout the period of this study.

A special thanks to Dr. A. J. Conner for his valuable advice, useful discussions and help with the all the *in vitro* studies in this project.

I am also grateful to Dr C. J. A. Shelbourne, Senior Scientist in the New Zealand Forest Research Institute for his friendship, encouragement and endless help. Without his help I would surely not have had a chance to study for a PhD in New Zealand.

I would like to thank Dr Frank Wood, former chief executive of the New Zealand Forest Research Institute, now Vice Chancellor of Lincoln University, for his understanding, advice and support.

I should like most of all to thank my mother, Wang Shufen. Without her love and encouragement I would have not even got a chance to be educated. Thanks also to my brothers and sisters for their continual support throughout the period of my education. Thanks also to my loved daughter Ruby Ye for sacrificing some of the time I should have spent with her.

I wish to thank all friends I have made at Lincoln University for the constructive discussions on different aspects of the work.

I am also thankful to Dr Jian Wang, Scientist in the New Zealand Crop and Food Research Institute, Mr DeZhi Luo and Chongjian Du for their friendship.

Finally, my most heartfelt thanks and love must go to my wife, Tianpei Sun, her love, reassurance, patience, encouragement, never ending support have made the completion of this project possible.

ABSTRACT

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy at Lincoln University

ASCOCHYTA BLIGHT RESISTANCE IN LENTILS: GENETICS AND SUPPORTING TECHNIQUES FOR BREEDING

by

Guoyou Ye

To promote breeding for Ascochyta blight in lentil, the genetics of resistance to lentil Ascochyta blight was investigated. In addition, some techniques needed to support the genetic research and breeding were developed in this study. An efficient procedure for producing genetically true-type plantlets was established based on stimulating elongation of the axilary buds. This technique was then used to multiply F_1 hybrid populations to make the population sizes of different generations large enough for careful genetic analyses.

Two regeneration systems, one based on multiple shoot induction from intact seedlings and the other based on cotyledonary node culture, were developed to facilitate the transfer of useful genes from wild lentils and/or other sources into cultivated lentil using tissue culture and genetic engineering techniques.

Ten major genes were identified for foliar resistance. The inheritance models for these genes were: one dominant gene for high resistance and one dominant gene for moderate resistance (ILL 5588); a single dominant gene (ILL 5684 and W6 3241), two complementary dominant genes (W3 3192 and Titore), two recessive genes with additive effect (Indian head); one recessive gene (Laird); and one partial dominant gene with large effect and one dominant gene with less effect (W3 3261). The gene in ILL 5684 is allelic to the one in ILL 5588 for high resistance.

The contributions of minor genes to Ascochyta blight resistance were established for the first time by creating recombinant inbreds with the same major genotypes but different minor genotypes from two crosses (ILL 5684 × Titore, W6 3241× Titore). A mixed model based analysis carried out for the cross ILL 5588 × Titore indicated that about 30% of the phenotypic variations in segregating populations were due to the minor genes.

The overall genetic effect and the partition of the genetic effect into additive, dominant and epistatic effects were done for four crosses using generation-mean analysis. The underlying genetic mechanisms for seed infection rate were more complicated than that for foliar disease severity. The six basic generations were sufficient to model foliar disease severity, whereas they were not sufficient for seed infection rate in two crosses. Dominance played an important role in all crosses for both seed and foliar resistance. Except for foliar resistance in the cross ILL 5684 x Titore, at least one type of inter-gene effect (epstastic) contributed to the increased/reduced resistance. Therefore, selection for resistance would be more efficient if the dominance and epistasis effects were reduced after a few generations of selfing.

The major gene for foliar resistance in ILL 5684 is linked to the genes for seed yield/plant or it has pleiotropic effect on seed yield/plant. The gene is independent of the genes for plant height and days to flowering. Within each set, there were significant differences among inbreds for all the three traits. The estimates of heritability based on inbred means were high for seed yield/plant and days to flowering, and moderate for plant height. For the set with major resistance gene, 1) disease severity was not correlated with seed yield/plant and plant height, but weakly and negatively correlated to days to flowering when measured under disease pressure. 2) Seed yield/plant was strongly and positively correlated with plant height, moderately and negatively correlated with days to flowering, and plant height was weakly and positively correlated with days to flowering under both testing conditions. 3) Inbred × environment interaction was not important and selection can be done with or without artificial inoculation. Thus, selection within the set of inbreds with the major resistance gene is required and is feasible for the improvements of yield and other traits and for the utilisation of resistance conferred by minor genes.

Based on the results from this study and previous studies, a breeding procedure suitable for the current situation was developed. This procedure is based on crossing resistant and high yielding cultivars and multi-location testing. Gene pyramiding, exploring slow blighting and partial resistance, and using genes contained in wild relatives will be the methods of the future. Identification of more sources of resistance genes, good characterisation of the host-pathogen system, and identification of molecular markers tightly linked to resistance genes are suggested to be the key areas for future study.

PRE-PUBLICATION LIST

G. Ye, D.L. McNeil and G.D. Hill 2000. Two major genes confer Ascochyta blight resistance in *Lens orientalis*. *New Zealand Plant protection* 53: 109-133.

G. Ye, D.L. McNeil and G.D. Hill 2000. Lentil Ascochyta blight and breeding for its resistance. *New Zealand Plant protection* 53: 97-102.

G. Ye, D. L. McNeil, A. J. Conner, and G.D. Hill 2000. Improved protocol for the multiplication of lentil hybrids without genetic change by culturing single node explants. *SABRAO Journal of Breeding & Genetics* 32:13-21

G. Ye, D.L. McNeil and G.D. Hill 2000. Breeding for Ascochyta blight resistance in Lentil. *Plant Breeding* (accepted)

G. Ye, D. L. McNeil, A. J. Conner, and G.D. Hill 2000. Multiple shoot formation from lentil seeds. *New Zealand Journal of Crop and Horticultural Science* (accepted)

G. Ye, D.L. McNeil and G.D. Hill 2000. Genetic variation for minor genes conditioning Ascochyta blight resistance in lentils. *4th European Conference on Grain Legumes* (Accepted)

G. Ye, D.L. McNeil and G.D. Hill 2000. Some methods for analysing multisite plant variety trials. I Estimating genotypic means at each site. *New Zealand Agronomy* (Accepted)

G. Ye, D.L. McNeil and G.D. Hill 2000. Some methods for analysing multisite plant variety trials. II Selection for yield and stability. *New Zealand Agronomy* (Accepted)

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Importance of Lentil (*Lens culinaris* Medikus)

Lentil (*Lens culinaris* Medikus) was one of the earliest domesticated crops. The relatively high content of protein makes lentil an important source of proteins in many countries, particularly in developing countries (Muehlbauer *et al.*, 1995). Crop residues from lentil are valuable as livestock feed in many regions where grazing is limited. Lentils are resistant to high temperature and drought and can be grown in infertile soil, they are major crops in drought and semi-drought regions (Muehlbauer *et al.*, 1995). In 1995, lentils were grown on about 3,391,760ha, and total production was estimated at about 2,788,650 metric tons (FAO, 1995). The main producers of lentil are India, Turkey, Canada, Syria, Ethiopia and Morocco, Chile, Argentina, Australia, the United States, Pakistan and Bangladesh (Muehlbauer *et al.*, 1995).

1.2 Ascochyta blight of lentil

Ascochyta blight caused by *Ascochyta fabae* f. sp. *lentis* is one of the important diseases of lentils. It has been reported to be the major disease in many lentil production areas including Argentina, Canada, Ethiopia, India, New Zealand, Pakistan and the Russian Federation (Erskine *et al.*, 1994). The disease has considerable effect on seed quality and yield (Morrall and Sheppard, 1981). Gossen and Morrall (1983) estimated that foliar infection has caused yield losses of up to 40%, but economic losses from infected seed may reach more than 70% in Canada.

Considerable efforts have been devoted to the study of the dispersal of *Ascochyta* (Pedersen *et al.*, 1993; 1994), the influences of environmental factors on disease severity (Pedersen and Morrall, 1994) and chemical control (Beauchamp *et al.*, 1986a; b; Ahmed and Beniwal, 1991). However, efficient and economic disease management techniques are not available. Breeding for disease resistance has been suggested to be the most promising option to eliminate or reduce the losses caused by this disease (Erskine *et al.*, 1994).

To have success in breeding for resistance, the availability of resistant genetic resources, a good understanding of the genetics underlying resistance, the availability of techniques of transferring resistant genes into elite genotypes (introgression) and knowledge of the host and pathogen interaction are necessary. Many valuable resistant resources have been identified by germ plasm screening in cultivated and wild lentil species (Morrall and Sheppard, 1981; Singh *et al.*, 1982; Iqbal *et al.*, 1990; Kapoor *et al.*, 1990; Erskine and Bayaa, 1991; 1993; Bayaa *et al.*, 1994). Several authors studied the genetics of blight resistance in lentils using segregation analysis (Tay, 1989; Tay and Slinkard, 1989; Andrahennadi, 1994; 1997; Sakr, 1994; Ahmad *et al.*, 1997b; Ford *et al.*, 1999).

Although studies of the mechanism of host-pathogen interactions are very few, it was clear that isolates from different sources have differences in virulence, and the interactions between host and isolate may or may not be significant (Ahmed *et al.*, 1996a; b; Ahmed and Morrall, 1996a; b). Though the presence of pathotypes was recently established (Nasir and Bretag, 1997b), no further reports were published in this aspect. Therefore, highly virulent isolates should be used for testing resistance, until the existence of races is confirmed.

1.3 Justification

Understanding the underlying genetic basis for a target trait is a prerequisite for achieving maximum return from any breeding program, though it may not be necessary for starting a breeding program. The genetic basis underlying Ascochyta blight resistance in lentils is not yet well understood. The allelic relationships among the reported major genes are mainly unknown, although it seems that several major genes are involved in the determination of resistance. All previous studies used segregation analysis to infer the models of inheritance. Although it provides some information about the genetics of major genes, the reliability of the results from such an analysis relies on the assumption that the effects of minor genes and interactions between major and minor genes can be ignored. Otherwise, the conclusions from this method may be misleading. Parh (1998) has already suggested difficulties in the major gene approach. The facts that many varieties show different resistance levels and no complete resistance has been found indicate that polygenes are probably involved in the resistance. It is, therefore, necessary to quantify the effect of minor genes in resistance. If the effects of minor genes are proved important, they should be explored in breeding for resistance. Classical generation-mean analysis can be used to detect the relative importance of additive, dominant and epistatic genetic effects of genes controlling one trait, which are important parameters for designing a breeding program. Recently a modified version of the classical generation- mean analysis has been developed by Gai and Wang (1998). This can quantify the effects of major gene and minor genes together (the so-called "mixed genetic model"). In this thesis, the genetics of Ascochyta blight resistance in lentils was studied from different aspects. The inheritance models of major resistance genes and the allelic relationships between major genes were tested using segregation analysis. The contribution of minor genes to resistance was proved using recombinant inbred lines homozygous for the major resistance genes and a mixed-model based method. The overall genetic effects and gene action model were analysed using generation-mean analysis.

One difficulty associated with genetic analysis and breeding when wild lentil species are involved is that it is not easy to obtain sufficient hybrid seeds. *In vitro* micorpropagation may be a way to overcome this problem, since it has been successfully used in many plant species to multiply rare elite materials (Kyte and Kleyn, 1996).

Several studies have developed *in vitro* systems for lentils with different efficiencies (Bajaj and Dhanju, 1979; Williams and McHughen, 1986; Saxena and King, 1987; Singh and Raghuyanshi, 1989, Malick and Saxena, 1992a; Warkentil and McHughen, 1993; Ahmad *et al.*, 1997a). However, no system has been developed specifically for obtaining genetically true-to-type hybrids, and the efficiencies of the published systems were not high enough to allow biotechnology-based methods to be efficiently applied in lentil breeding. In this thesis, shoot segment culture was used to develop a system for producing genetically true-to-type hybrids. Cotyledonary node and seed cultures were refined to develop systems that have the potential to be used for genetic transformation.

Ascochyta blight resistance is only one of the traits of interest to a lentil breeder. Other traits such as yield, plant height and maturity are important for the success of a new cultivar. Therefore, simultaneous improvement of several traits (multitrait selection) is necessary. The genetic relationships among the traits are required for the design of an efficient multitrait selection strategy (Agrawal, 1998). There are some studies about the genetic correlations among important agronomic traits, but none of these studies have taken Ascochyta blight resistance into account. Moreover, the existence of major genes for resistance suggests that breeding for resistance will incorporate one or more major resistance genes. Thus the effects of a major resistance gene on other traits, particularly yield, need to be investigated. In this thesis these aspects were studied using recombinant inbreds from two crosses.

1.4 Aims

The aims of this study are to provide genetic information for the design of an efficient breeding program for Ascochyta resistance in lentil and to establish some supporting techniques for its efficient implementation.

1.5 Objectives

The major objectives of studies reported in this thesis are:

1. To gain a good understanding of the genetics of Ascochyta blight resistance in lentils.

2. To investigate the genetic correlations among Ascochyta blight resistance and other agronomic traits.

3. To establish *in vitro* propagation protocols for multiplying hybrids and possible application of genetic engineering.

To achieve these objectives the following studies were carried out.

1. To detect inheritance models of major resistance genes and study the allelic relationships among them.

2. To test whether minor genes contribute to Ascochyta blight resistance.

3. To determine the gene actions of resistance using generation-mean analysis

4. To analyse the inheritance using a mixed genetic model.

5. To study the genetic correlations among Ascochyta blight resistance and other agronomic traits.

6. To establish an efficient *in vitro* protocol for producing genetically true-to-type hybrids.

7. To develop efficient *in vitro* regeneration systems.

CHAPTER 2
LITERATURE REVIEW

2.1 Basic Biology

2.1.1 The taxonomy of the genus *Lens*

Genus *Lens* Miller is a member of the tribe Viceae, sub-family Papilionaceae, family Fabaceae. According to crossability and chromosomal diversity, two biological species were suggested in the genus *Lens*: *L. culinaris* with three subspecies *culinaris*, *orientalis* and *odemensis*, and *L. nigricans* with subspecies *ervoides* and *nigricans* (Ladizinsky *et al.*, 1984). Recently, based on results from isozyme and DNA restriction fragment length polymorphisms (RFLPs), Ladizinsky (1993) revised the taxonomy of *Lens*. He elevated the subspecies *odemensis*, *ervoides* and *nigricans* to species status.

2.1.2 Flowering biology of lentils

Papp (1980) described the flowering biology of lentils and reported that lentil flowers open in an acropetal order. When opening, the petals move in a peculiar way; the standard gradually expands and the wings loosely close in on the keel. At the end of flowering the standard curves forward and the wings fit tightly to the keel.

2.1.3 Cytological characteristics

All the karyotypic investigations in lentil have found that *L. culinaris* has a symmetrical karyotype with 2n = 14 chromosomes, all chromosomes were either metacentric or sub-metacentric. However, the average chromosome length, total chromatin length, chromatin volume, arm ratio and position of centromere varied from variety to variety (Raziuddin *et al.*, 1990; Bahraei, 1991; Nandanwar and Narkhede, 1991; Reddy and Annadurai, 1992; Ramesh and Salimuddin, 1992; Ladizinsky, 1993; Salimuddin and Ramesh, 1994a; b; Patil *et al.*, 1995).

2.1.4 Phylogenic relationships of species in the genus *Lens*

According to breeding experiments and cytogenetic analysis of hybrids between the cultivated and wild lentils, Ladizinsky (1979) suggested that *L. culinaris* is much closer to *L. orientalis,* and that *L. nigricans* and the cultivated species may have a monophyletic origin. However, later researchers have shown that *L. odemensis* is closer to *L. culinaris* than to *L. nigricans*. *L. orientalis* has been shown to be the most likely progenitor of the cultivated lentil by many studies using morphological characters (Ladizinsky, 1979; Ladizinsky *et al.*, 1984; Hoffman *et al.*, 1988), seed protein profiles (Ladizinsky, 1979; Ahmad *et al.*, 1995a), isozymes (Ferguson and Robertson, 1996; Hoffman *et al.*, 1986), restriction sites of ctDNA (Oss *et al.*, 1997), restricted fragment length polymorphism (RFLP) (Havey and Muehlbauer, 1989a; Muench *et al.*, 1991; Mayer and Soltis, 1994), randomly amplified polymorphic DNA Markers (RAPDs) (Abo-Elwafa *et al.*, 1995; Sharma *et al.*, 1995; Ahmad *et al.*, 1996; Ford *et al.*, 1997) and the amplified fragement length polymorphism (AFLP) (Sharma *et al.*, 1996).

2.2 Ascochyta blight of lentil

2.2.1 Basic characteristics of Ascochyta blight in lentils

Bondartzeva-Monteverde and Vassilievsky (1940) first identified *Ascochyta lentis* as a pathogen of lentils in 1938. They made detailed investigations of disease symptoms. Mitidieri and De (1974) reported necrotic lesion development on leaflets, stems, pods and seeds of lentils associated with *A. lentis* infection.

Based on comparisons among isolates of *A. fabae* (the causal agent of Ascochyta blight of faba bean) and isolates of *A. lentis*, for cultural and morphological traits, Gossen *et al.* (1986) suggested that *A. lentis* should be synonymised with *A. fabae*. They proposed two special forms: *A. fabae* f. sp. *fabae* for isolates from faba bean and *A. fabae* f. sp. *lentis* for isolates from lentil. This proposal has been widely accepted. However, based on the RAPD banding patterns, morphological characteristics and pathogenicity

tests, Kaiser *et al.* (1997) suggested that the causal pathogen of Ascochyta blight in lentil is a species that is distinct from *A. Fabae*, and renamed back to *A. lentis*.

Kaiser and Hellier (1993) found the sexual state of *A. fabae* f. sp. *lentis* on lentil straw in the USA in 1992. They showed that the fungus is heterothallic with two mating types, and that it is probably a species of *Didymella*. Ahmed *et al.* (1996a) confirmed the presence of two mating types of *A. lentis* by controlled crossing in the laboratory. The roles of the two mating types in the field in promoting variability in the pathogen population and in the disease cycle are not known.

Roundhill *et al.* (1995) described the infection process based on investigations under light and electron microscopes:

a) Spores usually germinated within 6 h of inoculation.

b) Germ tubes grew for varying distances along the leaf surface before forming an appressorium, sometimes within 10 h.

c) A penetration peg then either directly entered the underlying epidermal cell, or grew as a sub-cuticular hypha for a short distance before entering the cell.

d) The first response of epidermal cells to the presence of the fungus was an aggregation of cytoplasm abutting the site of infection.

e) This was followed closely by deposition of a papilla. Some relatively thick papillae were seen at 29 h post-inoculation.

f) The fungus then grew into the papilla and formed an infection vesicle.

g) In susceptible host cells, the protoplasm became necrotic before hyphae grew into the lumen of the cell from the infection vesicle. In more resistant cells, the infection vesicle often became surrounded by electron-dense wall material developed by the host.

h) The fungus remained in susceptible epidermal cells for up to 4 d, amongst remnants of the protoplast, before spreading to the adjacent mesophyll.

i) Hyphae then grew into intercellular spaces of the mesophyll and remained there for 2 - 3 d before penetrating the cells.

j) The mesophyll reacted to infection in a similar way to the epidermis, with only host cells close to the fungus becoming affected.

k) After the degeneration of host, tissue, pycnidia, which contained spores, formed 10-14 d after inoculation of the leaf.

Similar infection processes were observed for both the tolerant and susceptible varieties tested except that in cultivars that are more tolerant the infection vesicle more frequently became surrounded by electron-dense wall material formed by the host. In stem tissue of the cv. Laird, a tolerant cultivar, the middle lamella was also occasionally thickened with electron-dense material deposited on either side of it.

Infection and disease development and spread are favoured by cool, wet weather (Nene *et al.*, 1988). The highest infection frequency occurred with a wet period of 24 or 48 h. The latent period was shortest at 20 °C and longest at 10 °C. Temperature had little effect on lesion size and number of pycnidia per lesion, but infection frequency was higher at 10 °C and 15 °C than at 25 °C (Pederson and Morrall, 1994). Pederson and Morrall (1994) observed that disease severity was tissue-age-related, tissue below the top 4 or 5 nodes on the main stem and secondary branches were almost completely free of disease. This effect was most apparent at podding.

Seed infection occurred up to 250 m from the primary inoculum source, but gradients generally levelled off within 50 m (Pederson *et al.*, 1993). The larger splash droplets, which followed a ballistic trajectory, were important in short-range disease spread. Longer-range spread was the result of wind dispersal of detached leaflets and conidia in small air-borne droplets (Pederson *et al.*, 1994).

2.2.2 Control methods

Methods for the control of lentil Ascochyta blight involve chemical control and/or improved cultivation practices, plus the use of resistant or tolerant varieties. Beauchamp *et al.* (1986a) found that single applications of chlorothalonil, captafol, folpet or metiram at early bloom to early pod set provided the best protection. Seed yield was increased and seed infection was reduced. Yield losses and seed infection were prevented by early

rather than late application, two or three applications were better than a single application, but these effects were linked to weather conditions (Beauchamp *et al.*, 1986b).

Benomyl [Benlate] (France *et al.*, 1987; Ahmed and Beniwal, 1991), Daconil [chlorothalonil] (Ahmed and Beniwal, 1991; Iqbal *et al.*, 1992), and a mixture of tridemorph and maneb (as Calixin M) provided good disease control and highest yields. Research on fungicides has led to the registration of Bravo (chlorothalonil) as a foliar protectant and Crown (thiabendazole + carbathiin) for the control of seed-to-seedling transmission in Canada (Morrall, 1997). However, effective, chemical control can often not be used by farmers because of economic reasons. This is particularly true in developing countries, which are the main producers of lentil.

Ahmed and Beniwal (1991) found that hot water and dry heat treatments at 55 °C for 25 min and 70 °C for 24 h partially inhibited fungal growth from seed (Ahmed and Beniwal, 1991). Pedersen and Morrall (1994) reported that using a 10 m wide barrier of lentil sprayed with chlorothalonil at early bloom or a 14 m non-host barrier effectively controlled development of the disease. Based on the observation that disease incidence was generally less in mixed than in the sole crops, and that sowing date has an obvious effects on disease development and spread (Mittal, 1997), cultivation practices such as crop rotation and intercropping have been developed (Bedi, 1990). However, the acceptance of altered cultivation practices is limited and they only help to reduce the loss caused by Ascochyta blight.

2.2.3 Genetic resources for Ascochyta blight resistance

Genotypic differences for Ascochyta resistance are present in cultivated lentil. Disease symptoms on different accessions ranged from small flecks (resistant) to extensive lesions on both leaves and stems with death of some plants (highly susceptible) (Nasir and Bretag, 1998). Khatri and Singh (1975) tested 947 lines and found one line without pod infection. Morrall and Sheppard (1981) reported significant differences among breeding lines of lentil with regards to Ascochyta disease reaction. Singh *et al.* (1982) identified 11 moderately resistant or partially resistant varieties/lines from 118

varieties/lines. Among 152 tested genotypes, Iqbal *et al.* (1990) found that 17 were highly resistant, and 34 showed an average reaction while the rest ranged from susceptible to highly susceptible. Kapoor *et al.* (1990) found two highly resistant varieties and 13 highly resistant advanced lines out of the 1,047 lines that they tested. Ahmed and Beniwal (1991) identified 35 resistant accessions out of 139 accessions tested but none were free of disease. Erskine and Bayaa (1991; 1993), reported that two lines, ILL 358 and ILL 5684, developed by ICARDA (International Centre of Agricultural Research for Drought Areas) were resistant or highly resistant over five locations in different countries. Other lines were resistant at one location, but not at others. This suggests the presence of different isolates of the fungus. Among 76 lentil lines tested for resistance to *A. lentis* by Alam *et al.* (1993), none was immune or highly resistant; 3 (88518, 88527 and 88547) were resistant, two were moderately resistant and five, 17 and 49 lines were moderately susceptible, susceptible and highly susceptible, respectively. Nasir and Bretag (1998) tested the reactions of 488 lentil accessions from 25 different countries to three Australian isolates of *A. lentis* in a glasshouse. The 488 accessions showed differential resistance to the three isolates of *A. lentis* and could be divided into five resistance groups. Twenty-six accessions were resistant to all three isolates, while 142 accessions showed variable reactions and 320 accessions were susceptible to all three isolates. Varietal variation for resistance in cultivated lentil was also reported by Singh *et al.* (1994), Andrahennadi *et al.* (1996), Nasir and Bretag (1996) and Russell (1996). The varieties reported to be resistant are summarised in Table 2.1.

Resistance to blight has also been found in wild *Lens* species. Erskine and Bayaa (1991) found 30 accession of wild lentils with a strong resistance reaction and a disease score of one. In 1993, they identified another 12 lines with resistance scores of one to three. Bayaa *et al.* (1991; 1994) tested the response of wild lentils to *A. lentils* from Syria and found that 24 *Lens orientalis* collections, 12 *L. odemensis* collections, three *L. nigricans* and 36 *L. ervoides* collections were resistant.

Table 1.1: Ascochyta blight resistant cultivars/lines in cultivated lentil.

Country	Cultivars/lines
Canada	ILL358, ILL5588, ILL5684, Laird
Chile	ILL358, ILL4605
Ethiopia	ILL358, ILL 857
India	HPL5, L442, L448, LG 169, LG170, LG171, LG172, LG173, LG174, LG176, LG177, ILL179, ILL195, ILL201, LG209, LG217, LG218, LG219, LG221, LG223, LG225, LG231, LG232, LG236, Pant L406,
	Piant 4,
Morocco	ILL5698, ILL5700, ILL5883, ILL6212
New Zealand	ILL5684, ILL5588, ILL5714, Rajah
Pakistan	FLIP84-27L, FLIP84-43L, FLIP84-55L, FLIP84-85L, FLIP86-9L, FLIP86-12L, ILL358, ILL4605, ILL5588, ILL5684, ILL6024, 78 S 26018, 78 S 26052, 88518, 88527, 88547, Masoor-93
Syria	ILL857, ILL2439, ILL4605, ILL5244, ILL5588, ILL 5562, ILL5590, ILL5593, ILL5684, ILL5725

2.2.4 The genetics of Ascochyta blight resistance

The genetics of blight resistance in lentils has been studied using segregation analysis. Several resistance genes have been discovered and are summarised in Table 2.2. The following genetic models were suggested.

1. Single dominant gene (Tay and Slinkard, 1989; Ahmad *et al.*, 1997b; Vakulabharanam *et al.*, 1997; Ford *et al.*, 1999).

2. Single recessive gene (Tay, 1989).

3. Two duplicated recessive genes (Andrahennadi, 1994).

4. One dominant gene and one recessive gene (Sakr, 1994)

5. Two complementary genes (Tay, 1989; Andrahennadi, 1994; Ahmad *et al.*, 1997b).

Table 2.2. Ascochyta blight resistance mechanisms in lentils.

Resistant Parent	Susceptible Parent	organ	Inheritance model	Reference
Indian head	PI 345635	seed	Two duplicated recessive genes	Andrahennadi, 1994
W6 3241 (*L. orientalis*)	Invincible	foliar	one dominant gene	Ahmad *et al.*, 1997b
W6 3261 (*L. orientalis*)	Invincible	foliar	one dominant gene	Ahmad *et al.*, 1997b Parh, 1998
W6 3261	Titore	foliar	one dominant gene	Ahmad *et al.*, 1997b
W6 3261	Titore	foliar	one partial dominant gene with large effect and one dominant gene with less effect	Ye *et al.*, 2000
W6 3261	Olympic	foliar	one dominant gene	Ahmad *et al.*, 1997b
W6 3192 (*L. ervoides*)	Titore	foliar	two dominant complementary genes	Ahmad *et al.*, 1997b
W6 3192	Olympic	foliar	two dominant complementary genes	Ahmad *et al.*, 1997b
W6 3222 (*L. odemensis*)	Titore	foliar	two dominant complementary genes	Ahmad *et al.*, 1997b
Laird	Eston	seed	one recessive gene	Tay and Slinkard, 1989
ILL5588	Eston	foliar	one dominant gene	Ford *et al.*, 1999
ILL5588	Eston	seed	one dominant gene	Andrahennadi, 1997 Vakulabharanam *et al.*, 1997
ILL5588	Eston	seed	two dominant genes one recessive gene	Tay, 1989
ILL5588	Eston	seed	one dominant genes one recessive gene	Sakr, 1994
ILL5684	Eston	seed	one dominant gene	Tay and Slinkard, 1989
ILL5684	Eston	seed	two dominant genes	Tay, 1989
ILL5684	Eston	seed	two dominant genes	Tay, 1989

2.3 Methods for detecting genetic mechanisms underlying disease resistance

From the above discussion, it is clear that the genetics of Ascochyta blight resistance in lentil is not yet fully understood. For instance, the extensive presence of inter-varietal differences seems to suggest segregation analysis, which is the only genetic analytical method that has been used, is not suitable. The genetic analytical methods suited to a trait depend on the phenotypic distribution of the trait in a segregating population such as the F_2. If the distribution is continuous, the methods for analysing a quantitatively inherited characteristic should be used. If the distribution is discontinuous and the grouping of phenotypes leads to a ratio that can be fitted to a model for a qualitatively inherited characteristic, the result from Mendelian segregation analysis would be reliable for major genes. In this case, if the variation within each phenotypic group is not large, the contribution of minor genes would be minimal and would not be important in practical plant breeding and genetic analysis for minor genes would not be necessary. Otherwise, selection aimed at accumulating the effects of minor genes might be an efficient breeding method. Because of the feasibility of correctly classifying major genotypes, it is possible to create materials that are the same for major gene(s) but different for minor gene(s) and then to use them to quantify the contribution of the minor genes. However, if the distribution is not continuous and the grouping of phenotypes do not fit any inheritance models of a qualitative trait, both of the effects of major and minor genes should be utilised in a breeding program. In this case, analytical methods based on a mixed inheritance model would have to be used.

2.3.1 Mendelian segregation analysis

Mendelian segregation analysis is the classical method to detect an inheritance model underlying a qualitative trait. The principle of fitting inheritance models using segregating generations is to test whether the observed segregation ratio fits the expected ratio under an assumed genetic model. Two methods can be used for this purpose. The most commonly used is the χ^2 test. This method is appropriate when each expected value

is not too small (roughly speaking, at least 1.5, Smith, 1986). The other is the so-called G- Test (Garcia-Dorado and Gallego, 1992). It is a likelihood ratio test. It compares the log-likelihood of a log-linear model that fixes the genotypic frequencies at their expected frequencies from the assumed model to that of a log-linear model that sets the genotype frequencies at their observed values.

To use segregation analysis, it is important to compute the expected phenotype segregation ratio of an assumed inheritance model. Mendelian segregation predicts that in a given sample of gametes from diploid individuals, on average, one-half will carry one allele and one-half will carry the alternative allele for a locus. Random union of gametes produced by female and male parents form the genotypes. Therefore, the expected genotype segregation ratio can be computed using the expected frequencies of gametes from both parents. The phenotype segregation ratio is the same as the genotype segregation ratio if there are no complete dominant or inter-loci interactions. If some loci are completely dominant and/or interact, the phenotype segregation ratio is computed by summing up the proportions of the different genotypes with the same phenotype. If all loci segregate independently and do not interact, the multiloci phenotype segregation ratio is the product of the segregation ratios of each locus. Because the phenotype segregation ratio for a single locus can easily be worked out and remembered, in practice this simple rule is very useful.

If the genes are linked, recombination rates between genes have to be taken into account when gamete frequencies are worked out (see later sections). In the case of disease resistance, only a few phenotypic groups can be easily distinguished since the effects of different loci may not result in sufficiently large phenotypic differences that can be detected by a scaling system. This usually prevents researchers from estimating the linkage of two genes. Therefore, independent segregation of genes is normally assumed when an inheritance model is fitted. Table 2.3 gives some useful phenotype segregation ratios.

Table 2.3. Expected segregation ratios of inheritance models commonly found in disease resistance studies.

Model	F_2	BC_1 AABB	BC_2 aabb
One dominant gene	3:1	all resistant	1:1
One recessive gene	1:3	1:1	all susceptible
Two dominant genes	15:1	all resistant	3:1
Two complementary dominant genes	9:7	all resistant	1:3
One dominant and one recessive gene	13:3	all resistant	3:1

2.3.2 Conventional quantitative genetic methods

When many genes each with a small effect control a trait, i.e., the trait is polygenic, the phenotype of a genotype is jointly determined by the genetic effects of genes and the environmental effect. The genetic effects are of three kinds: additive (homogeneous), dominant (intra-locus interaction) and epistatic (inter-loci interaction). Because the genotypes of individual plants are not known, the genetic control of a trait is detected by investigating the similarity and difference among related individuals and families using statistical methods. This forms the core of conventional quantitative genetics. There are many good texts available in this field (e.g. Mather and Jink, 1982; Facloner, 1989; Kearsey and Pooni, 1996).

For self-pollinated species, a simple method is the so-called generation-mean analysis, first proposed by Mather (1949) and elaborated by several other workers (Griffing, 1950; Anderson and Kempthorne, 1954; Jinks, 1956; Hayman, 1954; 1957; 1958; Gardner and Eberhart, 1966; Mather and Jinks, 1982). The method uses the information from different generations to detect the genetic control and relative importance of additive, dominant and epistatic effects. Expressed in a simple way, this method fits the observed means of each generation to the their expected means under a

assumed genetic model. If the goodness of fit is high judged by the χ^2 test, the assumed model is accepted. Otherwise, it is rejected and a new model is fitted. Similarly, the observed variances can be fitted to the expected variances. In practice, the simplest model is fitted first, further parameters (a more complicated model) are added only if a simple model cannot fit the observed means (variations). Sometimes, two different models fit the data, in which case the one with the fewest and biologically most plausible parameters is accepted (Kearsey and Pooni, 1996). The advantages of this method are

1. The generations used are the ones produced in a practical breeding program; thus, it can be easily integrated with a breeding program.
2. Very simple field design can be used, and
3. All the computations can be done by standard statistical software.

The disadvantage is that the results can not be related to any ancestral population, as the estimates obtained from each cross may be, to varying degrees, unique.

Other analytical methods use special mating designs to develop particular sets of progenies and use the variations between and within these progenies to estimate additive, dominant and genetic variance components. The general principle of all the methods is based on phenotypic records from progenies tested in designed trial(s), variance components of different sources are estimated using methods such as analysis of variance (ANOVA), the maximum likelihood method and the restricted maximum likelihood method. The relationships between genetic variance components and the estimated variance components of the different sources are used to obtain the estimates of genetic variance components. Commonly used designs are North Carolina (NC) designs I, II and III, triple test cross and diallel cross (Kearsey and Pooni, 1996). The NCI design is also known as the hierarchical mating design. In this design, each of 'm' random male parents is crossed with 'f' random female parents to produce 'mf' full-sib families. It is the least restrictive design. However, it provides less genetic information and does not allow an independent test for the dominant variance component. The NCII design is also known as a factorial mating design. In this design, every male parent is crossed with every female parent to form full-sib and half-sib families. It allows independent tests and estimates of additive, dominance and maternal effects. However, it requires that every

male and female be crossed many times, it cannot be used in species that are less reproductive and/or are difficult to cross. The NCIII design involves taking a number of F_2 plants from a cross between two inbred lines and crossing each back to the two parental lines. In the triple cross design, like the NCIII, all the F_2 plants are mated to both parental lines, in addition, they are crossed to the F_1 as well. These two designs provide the most genetic information (all major sources of variation and their relative importance). However, the extensive crosses needed limits their application to highly reproductive and easily crossable species. In addition, like generation mean analysis, the results obtained are cross specific.

In a diallel cross, a set of random individuals are used as both male and female parents. It is the mating design discussed most in plant genetics literature, and several different analytical methods have been proposed (see Christie and Shattuck, 1992 for a review). Because of the high number of crosses for a fixed number of parents, the number of parents used is rarely sufficient to provide accurate estimates of the genetic variance components. Therefore, it may not be a suitable design for estimating genetic variance components.

2.3.3 The mixed-inheritance model

All of the above methods assume that many genes with minor effects control a trait. However, recent studies have shown that effects of genes controlling a trait may differ from each other. One or few genes may have large effects (major genes) and many other genes may have minor effects (minor genes). Genetic models with both major gene(s) and minor genes are called mixed-inheritance models.

Several methods have been developed for analysing mixed-inheritance models and are collectively called complex segregation analysis by human and animal geneticists (Edwards, 1960; Elston and Rao, 1978; Bonney, 1984; Hoeschele, 1988; Demenasis and Bonney, 1989; Mitchell-Olds and Bergelson, 1990; Elston, 1993). Most of these methods are for human and animal populations and may be hard to apply to plant traits. However, see Tourjee *et al.* (1994; 1995) and Mitchell-Olds and Bergelson (1990) for applications in plant genetics. The two methods most suited for analysing plant traits are Elkind and

Cahaner 's method (Elkind and Cahaner, 1986) and Wang and Gai's method (Gai and Wang, 1998). In Elkind and Cahaner's method, the F_3 families produced from heterozygous F_2 plants heterozygous for a major gene are used. Wang and Gai's method is an extension of the classical generation-mean analysis. The distribution of each generation is described as a mixture of normal distributions. In addition, the component parameters (genetic parameters) are estimated via the maximum likelihood method. The best inheritance model is selected based on Akaike's information criterion. Both methods assume that there is only one major gene. Wang and Cai's method also assumes that there is no interaction between the major gene and minor genes, and that major genotype groups in each segregating population have the same variance. The first assumption, though unrealistic, does not restrict the application of this method because it is only one of the models that researchers can fit to the data. The second assumption is not required for the method and could be removed if computing resources are not limited. Elkind and Cahaner's method requires that the major genotypes be known. This limits its application in practice. However, if a neutral marker tightly linked to a major gene was available, major genotypes could be worked out and regarded as known. A big advantage of Elkind and Cahaner's method is that it estimates and characterises the interaction between the major and minor genes.

When major genotypes can be identified, the contribution of minor genes can be quantified by comparing recombinant inbred lines with same major genotype. This method was used by Rebetzke *et al.* (1998) to study the genetics of reduced saturated fatty acid content in soybean.

2.3.4 Quantitative trait loci mapping

Quantitative trait loci (QTL) mapping was developed as a result of the rapid development of modern molecular techniques (Tanksley, 1993; Lee, 1995; Jansen, 1996). It provides more information than conventional quantitative genetic methods. For instance, the positions of the QTLs on chromosomes can be determined. The number of QTLs controlling a trait can be more precisely estimated (Lee, 1995; Carbonell and Asins, 1996; Kearsey and Pooni, 1996). In recent years, the genetic basis underlying

disease resistance in many species for various diseases has been studied using QTL mapping (Bent and Yu, 1999).

The principle underlying QTL mapping is to follow the segregation of genes controlling quantitative traits by the use of linked observable genetic markers (Sax, 1923). Therefore, it is an extension of linkage analysis. The difference is that in classical segregation analysis the genotypes of both loci can be easily determined, while in QTL mapping only the genotypes of the marker loci are known. The existence of traits recorded in quantitative (QTL) and qualitative (marker) scales implies that methodologies for analysing continuous and discrete traits need to be used simultaneously in QTL mapping. All individuals can be classified into groups according to the marker genotypes and then the means for marker genotypic groups are compared statistically using analysis of variance. If the means are not significantly different, the grouping using marker genotypes is equivalent to grouping individuals randomly, that is, the QTL and marker are independent. If the differences between marker genotypic groups are significant, the QTL must link with the marker (Soller and Beckmann, 1983; 1990; Weller, 1986; Edwards *et al.*, 1987; Stuber *et al.*, 1987; Asins and Carbonell, 1988; Cowen, 1988; Simpson, 1989; Dudley, 1993). Since normally there are more than one type of QTL genotypes, within each marker genotype, the expected values for the marker genotypes are weighted means of the expected values of these QTL genotypes. The expected genotypic values of QTL genotypes can be determined in terms of the genetic model underlying the trait. The weights are the frequencies of the QTL genotypes within the marker genotypes. i.e. the conditional probability of the QTL genotype given the observed marker genotype is M_j.

To estimate QTL effects and recombination rate between QTL and marker(s) (hence the position of the QTL), methods based on regression analysis (Knapp *et al.*, 1990; Knapp, 1991; Haley and Knott, 1992; Jansen, 1992; Martinez and Curnow, 1992) and the maximum likelihood method (Weller, 1986; Lander and Botsein, 1989; Luo and Kearsey, 1989; 1991; Carbonell *et al.*, 1992; Jansen, 1992; Knott and Haley, 1992; Hackett, 1994) are used. For the maximum likelihood based methods, the joint likelihood function of the trait values in a mapping population is established based on an assumed distribution of trait values in each marker genotype class. This likelihood function is the

function of observed trait values, expected QTL genotype trait values and the conditional QTL genotype probabilities. The estimates of the unknown parameters (recombination rate and QTL effects) are then obtained by maximising this likelihood function with respect to all the parameters.

For the regression method, a regression model is established to relate observed trait values and expected QTL genotype trait values and the conditional QTL genotype probabilities. Then the least-squares method is used to estimate recombination rate and QTL effects. Estimates from maximum likelihood are sound statistically with several desirable characteristics. However, it is not an easy job to estimate the parameters, particularly if the number of unknown parameters is large and specialised software is required. Regression based methods are computationally simple and can be easily implemented using standard statistical software, but the estimates may not be statistically sound.

Based on the number of markers used for mapping a QTL, QTL mapping methods can be grouped into three kinds,

1. single marker analysis,
2. flanking marker analysis and
3. multiple marker analysis.

The simplest way to detect the existence of a QTL is to investigate its association with each single marker available separately. As mentioned above, ANOVA can be used to test the differences among marker genotype groups and significant differences indicate the association between QTL and marker locus. The advantages of this method are that the computation involved is simple, and the gene order and a complete linkage map are not required. However, because the putative QTL genotypic means and the QTL positions are confounded, the estimate of QTL effects are biased, statistical power is lower and the QTL position cannot be precisely determined (Lui, 1998). In addition, it does not give information on the number of QTLs associated with a marker. Therefore, ANOVA based method for single marker analysis should be regarded as a preliminary analysis only in a QTL mapping study to simply detect the presence of a QTL. The

maximum likelihood method has been developed to estimate the genetic effects of a QTL linked to a marker and the recombination rate between them (Luo and Kearsey, 1989; 1991)

If a known, accurate marker linkage map is available, (i.e. marker positions and order are assumed known without error), a natural extension of QTL mapping is to use a pair of markers flanking the potential QTL loci. This method is called interval mapping (Lander and Botstein, 1989; Knapp *et al.*, 1990; Knapp, 1991; Carbonell *et al.*, 1992; Haley and Knott, 1992; Jansen, 1992; Martinez and Curnow, 1992; Jansen, 1993; Jansen and Stam, 1994). Because a pair of markers provides more information than a single marker, the resultant estimates of both genetic effects and recombination rates are more accurate. Interval mapping also is less sensitive to the violation of normality.

The number of QTLs underlying a quantitative trait must be more than two, and all the QTLs for the trait are not necessarily independent of each other. Thus, the estimates of the effects and position of a QTL may be biased by other linked QTLs. So-called composite interval mapping was developed to overcome this problem by using other markers as cofactors (Jansen, 1993; Zeng, 1993; 1994; Jansen and Stam, 1994).

The main factor limiting QTL analysis is the higher cost involved in genotyping segregating populations. In most of the studies, only one or two mapping populations are used and this raises concerns over generalisation of the results. To overcome this problem, a lower cost methodology has been proposed and used in several studies (Michelmore *et al.*, 1991). It is based on local mapping of a target quantitative trait. The tightly linked molecular markers in two DNA pools consisting of opposite phenotypes respectively were sought first. These markers were then mapped using a segregating population and a local linkage map was obtained. Because only two samples were screened in the first step, many probes and/or primers can be examined and the possibility of finding tightly linked markers is increased. Moreover, only markers that showed tight linkage with a target quantitative trait were tested for segregation in the mapping population. Thus a large sample can be examined and the mapping efficiency can be increased. Furthermore, this method makes it possible to use several mapping populations and compare the results obtained from them. From a genetic mapping point

of view, the main disadvantage of the method is that only one quantitative trait can be analysed per effort. However, from an applied genetics and breeding point of view, this is more of an advantage than a disadvantage. This is because at present usually only well defined, important traits are worth using molecular markers to undertake deep analysis.

2.4 Marker-aided selection

One of the objectives of gene mapping is to identify markers linked to agronomic traits and to use these markers to assist selection for the improvement of the trait. When marker information is used to assist selection, it is referred as marker-aided selection (MAS). For a monogenic trait, selecting for the presence/absence of a tightly linked marker is essentially the same as selecting on the genotype itself. For a polygenic trait, selection for a single QTL is less useful except where the major QTL is identified; marker information is combined with phenotypic value to increase selection response.

2.4.1 Using markers in selection for monogenic resistance

A single gene conferring resistance is conventionally introgressed into an elite background by repeated backcrossing. If, instead of the gene, a marker tightly linked to the gene is used to trace its segregation in the selection process, the method is referred to as maker-assisted introgression (MAI). The use of MAI has several obvious advantages.

1. The tedious and troublesome test for resistance is not required, and selection can be done in normal environments;
2. Selection can be done at an earlier stage and hence generation intervals may be reduced;
3. The transfer of both dominant and recessive genes is feasible.
4. The results from both theoretical study and practical application have shown that MAI reduces the necessary population size and the number of

generations required for developing commercial varieties (Hyland and Quaas, 1991; Hillel *et al.*, 1990; Melchinger, 1990; Tansley, 1993; Edwards and Page, 1994; Visscher *et al.*, 1996).

Flanking markers can be used to identify the backcross lines that are heterozygous for target genome regions and by only advancing these selected lines linkage drag will be reduced. It also allows for the selection of genotypes with the maximum percentage of the recurrent parent genome.

Because mongenic resistance is a qualitatively inherited trait, classical Mendelian linkage analysis can be used to identify linked markers. Marker genotypes and genotypes for resistant genes are determined for plants in a segregating population and the linkage between the marker and the trait is confirmed by estimating the recombination rate. However, generating DNA markers is both costly and time-consuming. Better strategies are required to facilitate the identification of markers linked to a target trait. Ideally, few DNA samples are used to screen markers and most of the DNA markers used for genotyping individual plants are linked to the target trait. Near-isogenic lines (NILs) produced from backcrossing programs to introgress resistance genes are ideal material to identify linked markers (Paran *et al.*, 1991; Penner *et al.*, 1993). As NILs are genetically identical except for the locus conferring resistance, markers represented by bands, which appear only on DNA from resistant NILs, must link to the gene for resistance. The NILs for many important diseases are now available in major crops. Bulk segregation analysis proposed by Michelmore *et al.* (1991) does not require special materials and has become a standard practice in this field. Two DNA pools formed by mixing DNA from highly resistant individuals and highly susceptible individuals are used to detect polymorphism. Markers represented by bands, which appear only on one of the DNA pools are likely to be linked to the gene for resistance or susceptibility. Then the linkage and recombination rates between these markers and the resistant gene are confirmed and estimated using Mendelian linkage analysis.

2.4.2 Using markers in selection for polygenic resistance

Like any quantitative trait, accumulating the effects of QTLs by selection is required to increase resistance controlled by polygenes. In this context, MAS can be used to increase selection efficiency. Increased genetic gain from MAS can be achieved via increasing selection accuracy, selection intensity and reducing generation turnover. The increased selection accuracy is achieved by incorporating marker information in selection criterion. The increased selection intensity is achieved by reducing the number of plants required for each line to obtain accurate estimates of genetic value. The reduced generation turnover is achieved by selecting at earlier stages (Lande and Thompson, 1990; Lande, 1994; Hosptial *et al.*, 1997; Moreau *et al.*, 1997). Clearly, MAS will be used mostly in early generations when it is not possible to increase the selection efficiency by increasing the number of plants tested per line. For traits, which are difficult or expensive to measure, additional benefit can be obtained from MAS, since less or even no phenotypic evaluation may be needed.

2.5 Gene mapping and marker-aided selection in lentil

2.5.1 Gene mapping

The first linkage group of lentil was described by Zamir and Ladizinsky (1984). They showed that Gs (cotyledon colour), Got2 (glutamic oxaloacetic transaminase) and Me1 (malic enzyme) are in one linkage group and that Got3 and Adh1 (alcohol dehydrogenase) are in another. The remaining loci were independent of each other.

Muehlbauer *et al.* (1989) studied the inheritance and linkage relationships of morphological and isozyme loci in lentil using seven crosses between nine *L. culinaris, L. orientalis* and *L. odemensis* genotypes carrying four morphological marker genes and alloenzyme polymorphism at 18 loci. Six linkage groups were identified containing 14 of the loci analysed. A comparison of the isoenzyme phenotypes and linkage groups

observed with those found in *Pisum sativum* revealed that several linkage groups were conserved between these two crops.

Havey and Muehlbauer (1989b) constructed a genetic linkage map of lentil spanning 333 cM from 20 RFLP, eight isozyme and six morphological markers in an interspecific cross. Weeden *et al.* (1992) developed a 560 cM linkage map, which consisted of 64 morphological, ioszyme and DNA markers, from an interspecific cross of *L. ervoides* × *L.culinaris*. In addition, nine markers were scored that assorted independently of any of the multiple linkage groups. Vaillancourt and Slinkard (1993) analysed the F_2 progeny from 18 crosses involving five, two and one parents of *L. culinaris*, *L. orientalis* and *L. odemensis* respectively, using 28 genetic markers (11 morphological traits and 17 isozyme markers). These 28 markers formed four linkage groups (I to IV), which represent linkage relationships in the cultivated lentil and in its wild progenitor *L. orientalis*.

Tahir *et al.* (1993) produced a compiled linkage map based on combined linkage data and regions of homology shared with pea including seven morphological, 25 isozyme, 38 RFLP and six other loci. Tahir and Muehlbauer (1994) developed a genetic map using eight sets of recombinant inbred lines developed from interspecific hybrids between *L. culinaris* and *L. orientalis* based on isozyme and morphological markers. Six linkage groups included 17 isozyme loci and four morphological trait loci were identified.

Recently, RAPD markers have been tested for genetic mapping. Eujayl *et al.* (1995) generated 63 RAPD markers using primers. However, only 28 RAPD markers were allocated into nine linkage groups. The map constructed by the use of these 28 markers and three oligonucleotides and one morphological marker covered 173 cM of the genome. In a further study, Eujayl *et al.* (1997) generated 78 RAPD markers using 390 primers. A genetic map of nine linkage groups including 28 RAPD, three oligonucleotide markers and one morphological marker were constructed which covered 206cM. These studies clearly showed that RAPD markers are valuable for genetic mapping, although many marker loci segregated independently of the established linkage groups presumably caused by the small number of F_2 plants used.

Eujayl *et al.* (1998a) constructed a genetic linkage map using 89 RAPD, 79 AFLP, six RFLP, and three morphological markers. This map covered 1073 cM of the lentil genome with an average distance of 6.0 cM between adjacent markers. It is the most extensive genetic map of lentil to date.

Similar to many other self-pollinated crops, interspecific hybrid populations have been used to maximise polymorphism for map construction. However, reduced recombination or chromosomal rearrangements in interspecific crosses may lead to segregation distortion and non-representative genetic distances and linkage relationship (Tadmor *et al.*, 1987; Paterson *et al.*, 1990). In lentil, Vaillancourt and Slinkard (1993) found that in crosses between *L. odemensis* and the other *Lens* species, markers of linkage groups III and IV showed disturbed segregation and pseudo-linkage. Eujayl *et al.* (1997) showed that in one of the two crosses they used, most of the RAPD, isozyme and morphological markers showed segregation distortion, while another cross showed little distortion. This indicated that it is both necessary and possible to develop a mapping population that has little segregation distortion.

2.5.2 Markers linked to agronomic traits

Abbo *et al.* (1992) studied the association between seed weight and morphological and DNA markers. They found that factors affecting seed weight were linked to morphological and DNA markers distributed over several linkage groups. A high seed weight in segregating generations was usually associated with alleles of marker loci originating from the cultivated parent.

Using recombinant inbreds derived from interspecific (*L. culinaris* × *L. orientalis*) hybrids, Tahir *et al.* (1994) studied the association of isozyme markers with seven quantitative trait loci (days to flowering, days to maturity, plant height, biomass, seed yield, harvest index and seed weight). They found that 14 tested isozyme markers could detect the loci affecting variation in all seven quantitative traits. Detected QTL were located in six of the seven linkage groups on the lentil genetic map. Regions of the genome represented by linkage groups 1, 5 and 7 appeared to affect a greater number of traits than other genomic regions represented by linkage groups 2, 3 and 4.

Eujayl *et al.* (1998b) identified four RAPD markers linked to the gene conferring resistance to lentil vascular wilt (*Fusarium oxysporum*). The closest marker is OPK-15_{900}, which is 10.8 cM away from the resistance gene. Eujayl *et al.* (1999) also found that a single major gene, which is linked to RAPD marker OPS-16_{750} at 9.1cM, controls frost tolerance. Ford *et al.* (1999) identified seven RAPD markers linked to the resistance locus conferring Ascochyta blight resistance in ILL 5588. Five of the seven RAPD markers were within 30 cM of the resistance locus and the closest flanking markers were approximately 6 (PV) and 14cM (PB18) away from the resistance locus.

Sarker *et al.* (1999) reported that seed coat pattern and peduncle pubescence were linked to flowering.

2.6 *In vitro* multiplication

In vitro multiplication is the primary requisite for biotechnology-based breeding such as *in vitro* selection and genetic transformation and micropropagation of rare genetic material. Genetic transformation is a more flexible method to improve agronomic traits of simple inheritance such as disease and insect resistance than conventional backcross based methods. The most feasible application of *in vitro* multiplication in lentil at present is for rescuing and multiplying hybrids. This may help to overcome the difficulty of obtaining sufficient F_1 hybrid plants for genetic analysis.

Bajaj and Dhanju (1979) first reported *in vitro* lentil regeneration from meristem tips. Williams and McHughen (1985) described a protocol for regenerating lentil from callus cell. Hypocotyls and epicotyls cultured on Murashige and Skoog (MS) medium containing 3 % sucrose, 10 mg/L kinetin, 1 or 0.1 mg/L gibberellic acid (GA_3) produced callus which regenerated shoots spontaneously. These shoots were transferred to basal medium to promote development. Shoots about 5 cm in length were transferred directly into a mist chamber to develop roots. In 1986, using shoot meristem and epicotyl as explants, they also (Williams and McHughen, 1986) obtained whole fertile plants from

calli induced on MS media containing 10 mg/L of kinetin with either 1 or 0.1 mg/L GA_3. The induced callus was able to regenerate shoots in relatively large numbers, even after several subcultures.

Saxena and King (1987) obtained whole plants from callus induced from embryonal axes cultured on MS medium or on a modified B5 medium supplemented with 2,4-D (1 - 10 mg/L). On transfer to B5A medium without hormones or with benzyladenine (BA) and (IAA), certain peripheral areas of the callus turned green. Such green patches later differentiated into several embryoid-like structures. Further subculture of the embryoid-like structures (or embryoids) on a glutathione-supplemented medium produced well organised embryos having cotyledons, shoots and roots which were able to develop into whole plants.

Polanco *et al.* (1988) studied the factors affecting callus and shoot formation using three cultivars. They found that medium with 2,4-D induced the formation of calluses in all explants, but no organ regeneration occurred. Singh and Raghuvansi (1989) found that the best callus formation was on medium containing 1.0 mg/L kinetin and 10.0 mg /L 2,4-D. Whole plants were formed by transferring callus to media containing only kinetin to induce buds and culturing the buds on MS basal medium.

Malick and Rashid (1989) observed that root, leaf and hypocotyl of young seedling underwent callusing on a medium rich in cytokinin or auxin, and the tissue formed was only able to regenerate roots. Ghanem (1995) found that when leaf, stem and root explants were cultured on supplemented MS medium, no callus was induced in the absence of 2,4-D. The best callus induction was achieved with 0.5 mg /L 2,4-D + 2 mg/L kinetin.

Polanco *et al.* (1988) reported multiple shoot formation was obtained with 33 – 92 % of explants in a medium supplemented with 2.25 mg/L BA and 0.186 mg/L NAA + 2.25 mg/L BA. Root formation from explants occurred only in media with NAA or IAA.

Singh and Raghuvansi (1989) reported that plants could be regenerated directly from nodal segments and shoot tip explants as well as from callus. Nodal segments and shoot tip explants each produced a single shoot and roots in 4 weeks on hormone-free MS medium. Only shoots regenerated on a medium containing kinetin and multiple shoots

formed without intervention by callus or root formation. Ahmad *et al.* (1997a) developed a protocol to regenerate shoots *in vitro* from nodal segments without a callogenic phase. It used an MS medium containing 2.89 μM GA_3 and 1.11 μM BA, and no sucrose. Best root induction from these shoots was obtained on MS medium supplemented with 5.37 μM NAA. However, Parh *et al.* (1998) found there was serious shoot tip necrosis on *in vitro* seedlings when the Ahmad *et al.* (1997a)' s protocol was used. They overcame the problem by doubling the calcium content of the basic MS salts.

Warkentin and McHughen (1993) obtained multiple shoots from cotyledonary nodes. Halbach *et al.* (1998) reported that best multiple shoot formation was from bisected embryos, and that keeping the cotyledons attached to the embryo-axis was beneficial. Oktem (1999) showed that cotyledonary node culture was a good system for genetic transformation in lentil.

Using intact seedlings as explants, Malick and Rashid (1989) showed that multiple shoots generated from cotyledonary nodes on a medium with 10^{-5} M BA. The best morphogenetic responses were from nodes and the poorest from leaves. Malik and Saxena (1992a) reported that multiple shoots developed after the culture of seeds on MS media supplemented with 1-50 μM thidiazuron (TDZ). Differentiation of shoot buds occurred in cultures exposed to TDZ for 4 - 6 weeks but only from nodal and subjacent areas. There was frequent secondary shoot formation. Developing shoots were able to form roots and eventually whole plants on a modified MS medium containing 2.5 μM NAA. No genotypic difference for morphogenesis was observed. Shoot formation from intact seedlings was also reported by Halbach *et al.* (1998).

Cohen *et al.* (1984) established a two stage *in vitro* technique for the development of interspecific hybrid embryos. They used 14 day old fertilised ovules which were cultured on MS medium supplemented with zeatin. This was followed by the release of the embryos from the ovular integuments. These embryos later developed into viable and vigorous plants. Ladizinsky *et al.* (1985) also obtained vegetatively normal *L. culinaris* × *L. ervoides* hybrids using embryo culture techniques. No *in vitro* protocol has been developed for rescuing hybrids between *L. culinaris* × *L. nigricans*.

2.7 Conclusion

The presence of germplasm with resistance to lentil Ascochyta blight provides the materials for breeding and makes breeding for resistance possible. However, the genetic basis of resistance is yet not well understood. Minor genes may be involved in the determination of resistance, thought major genes appear to be the main controllers. If both major genes and minor gens were proved to be involved, Mendelian genetic analysis would provide oversimplified description of genetic mechanism. Some quantitative genetic methods such as generation mean analysis are more informative if the effect of individual genes is not of interest. However, an important assumption underlying all the classical quantitative genetic methods is that the trait is under control of many genes, which have small and equal effects. They may not be suitable if major genes are present. In this case, methods for analysing so-called major gene and polygenes mixed model are more suitable, though they are more computation intensive. Quantitative trait loci mapping with the aid of molecular markers has the potential to quantify the effects of individual genes. However, at present the resolution powers of this kind of method are still not high enough to be useful for this purpose.

One of the difficulties of carrying out genetic analyses in lentil is to obtain sufficient hybrid seeds. This is particularly true when wild relatives are used as parent(s). *In vitro* propagation of limited hybrid seeds has the potential of overcoming this problem.

For successful breeding for Ascochyta blight resistance, relationships among Ascochyta blight resistance and other agronomic traits need to be investigated, because multitrait selection is necessary. Moreover, useful techniques for the utilisation of more efficient breeding methods such as marker-aided selection and genetic engineering need to be developed. Identification of markers linked to resistance genes is likely to be greatly promoted by the development of modern molecular techniques. Genetic transformation has been less successful in lentil presumably due to the lack of an efficient regeneration system.

Therefore, an effort in this study was made to study the genetics of Ascochyta blight resistance in lentil using various analytical methods, to investigate the relationships

among Ascochyta blight resistance and other agronomic traits, and to develop some useful techniques for the efficient implementation of a breeding program. The results of these studies were used to develop breeding strategies for current and future breeding.

CHAPTER 3

AN EFFICIENT *IN VITRO* SYSTEM FOR PROPAGATION OF LENTIL HYBRIDS

Summary

A sufficiently large population of hybrid plants from a cross is required to perform genetic analysis using Mendelian and quantitative genetic methods. The limited success of interspecific crossing in lentil necessitates an efficient method to multiply the limited number of hybrid seeds produced. In this report, single nodal segment culture was explored as a mechanism to serve this purpose. Basal medium, sucrose content and plant growth regulators all influenced the elongation of axillary shoots (from axillary buds). An MS medium with 3 % sucrose and 1.0 mg/L gibberellic acid (GA_3) or 0.17 - 0.18 mg/L indole-3-butyric acid (IBA) was recommended to obtain strong, healthy shoots with the highest total number of nodes per segment. A medium of 0.67 strength MS supplemented with 2.0 mg/L α-naphthaleneacetic acid (NAA) and 5 % sucrose was the best medium for root formation. The application of this multiplication approach produced about 20 plants from a single seed in 3 months or about 100 plants in 4 months.

3.1 Introduction

Lentil (*Lens culinaris* Medik) was one of the first crops and was cultivated by Neolithic man. The relatively high protein content of lentil grains makes this crop an important source of protein in many countries, particularly in developing countries (Muehlbauer *et al.*, 1995). The genetic base of cultivated lentil is narrow, but many agronomically useful traits have been found in wild lentils (Erskine *et al.*, 1994). Therefore, it has been suggested that transferring these traits from wild species into

cultivated species will be an important strategy to improve the quality, yield, adaptation and stress-resistance of cultivated lentil (Erskine *et al.*, 1994). At present interspecific hybridisation, by hand-pollination, is the only practical way to implement this strategy. Several biological characteristics of wild lentils such as very small flower size, low number of seeds/pod, and post-fertilisation abortion has made artificial crossing very difficult. Therefore, a highly successful crossing technique is not available and is unlikely to become available in the near future. However, because Lens spp are a strictly self-pollinated species, the F_1 hybrid plants of a particular cross are all genetically identical. Thus, micropropagation of limited F_1 materials may be a way to enlarge the F_1 population and eliminate the requirement for large-scale pollination to obtain enough hybrid material for further genetic study and breeding.

When hybrids are multiplied by *in vitro* culture, somaclonal variation must be minimal for the plantlets produced to be used for genetic analysis. Alternatively, somaclonal variation itself could be explored if the resultant plants are to be used as breeding material for selection and further mating. To service the first goal, an *in vitro* propagation method aimed at producing genetically true-to-type plants is useful. Among methods of *in vitro* propagation, nodal segment culture has been shown to produce no or fewer genetically off-type plants (Ahuja, 1993; Kyte and Kleyn, 1996). In lentils, Singh and Raghuvansi (1989) reported plant regeneration directly from nodal segment and shoot tip explants. Ahmad *et al.* (1997a) developed a protocol to regenerate shoots *in vitro* from nodal segments without a callogenic phase. However, Ahmad *et al.* (1997a) used the number of shoots per segment as one of the criteria to determine the optimal protocol. The presence of more than one shoot per segment in their experiments made the results less relevant to true-to-type hybrid production as there was the possibility of somaclonal variation in the adventitious buds that arose. In this Chapter I describe single-node segment culture, that will enable more safe and efficient production of genetically true-to-type lentil hybrids.

3.2 Materials and methods

3.2.1 Plant materials and explant preparation

The cultivar Titore of cultivated lentil (*Lens culinaris*) was used to develop the system, and eight intra- and inter-specific hybrids (see Table 3.4) were used to verify the applicability of the system. Seeds were surface sterilised by rinsing in 95 % ethanol for 30 seconds, then soaking them in 1.25 % sodium hypochlorite containing a few drops of the surfactant Tween-20 with continuous stirring for 20 min followed by five - six washes in sterile deionised water. The seeds were germinated in an incubator at 24 °C in the dark on MS salts and vitamins (Murashige and Skoog, 1962) with a doubled concentration of calcium chlorate (Parh *et al.*, 1998), 3 % sucrose and 8 % agar in plastic tissue culture containers. There were 10 seeds per container. After 3 - 4 d, they were transferred to a growth room with a light intensity of 60 $\mu molm^{-2}s^{-1}$ at 24 °C with 16 h daylength. Nodal segments about 1.0 cm were prepared from 3 to 4 weeks old seedlings.

3.2.2 Shoot elongation experiments

1) Basal medium and strength: full- or half-strength salts, full-strength vitamins of the following media: MS, B5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972) and LM (Verma *et al.*, 1983).

2) Sucrose concentrations: the MS medium was supplemented with different concentrations of sucrose (0 %, 1 %, 3 %, 5 % and 7 %).

3) Plant growth reguator concentrations: the MS medium supplemented with different concentrations of IBA or BA (0.025, 0.05, 0.1 and 0.2 mg/L) or NAA (0.025, 0.05, 0.1 and 0.2 mg/L) or GA_3 (0.1, 0.2, 0.4, 0.8 and 1.0 mg/L).

4) Second round culture: nodals of first round shoots obtained from several different media (MS basal medium supplemented with 0.6 mg/L GA_3, MS basal medium supplemented with 3% sucrose and 0.6 mg/L GA_3, and MS basal medium supplemented with 3% sucrose) were cultured on MS medium supplemented with 3 % sucrose and 1.0 mg/L GA_3.

A randomised complete block design was used with three replications of single containers. Five explants were cultured per container.

3.2.3 Root induction experiments

1) Basal medium and salt strength: MS at 1, 1/2, 1/4 or 1/8 strength salts or B5 basal medium.

3) Sucrose concentrations: MS supplemented with different concentrations of sucrose (1 %, 2 %, 3 %, 4 % or 5 %).

3) Plant growth regulator concentrations: MS supplemented NAA (0.2, 0.5, 1.0 or 1.5 mg/L) or IBA (0.2, 0.5, 1.0 or 1.5 mg/L).

A randomised complete block design with three replications of two containers was used. Five shoots were cultured per container.

3.2.4 Multiplication of hybrids

The following intra- and inter-specific hybrids were multipled using the optimal protocol as determined from above shoot elongation and rooting experiments (Shoot production medium was basal MS medium supplemented with 1.0 mg/L GA_3 and 3 % sucrose. Root initiation medium was 2/3 strength MS salts and MS vitamins supplemented with 2.0 mg/L NAA and 5 % sucrose).

Lens culinaris cv Titore x *Lens orientalis* W 6 3261, *Lens culinaris* cv Titore x *Lens erovides* W 6 3192, *Lens culinaris* cv ILL 5588 x *Lens orientalis* W 6 3261, *Lens culinaris* cv ILL 5588 x *Lens erovides* W 6 3192, *Lens culinaris* cv Laird x *Lens orientalis* W 6 3261, *Lens culinaris* cv Laird x *Lens erovides* W 6 3192, *Lens culinaris* cv Titore x *Lens culinaris* cv ILL 5588, *Lens culinaris* cv Laird x *Lens culinaris* cv ILL 5588.

Unless specified, all media were supplemented with 3 % sucrose and all rooting media were supplemented with 1.0 mg/L NAA. Media were solidified with 8 % agar and adjusted to pH 5.8 prior to sterilisation by autoclaving (122 kPa, 20 min). The cultural conditions were the same as for seedling culture.

3.2.5 Data collection and analysis

Data were collected on the number of shoots/segment, average shoot length and number of nodes/shoot for the shoot elongation experiments after 4 weeks incubation. The number of rooted shoots for the root induction experiments was recorded after three weeks culture. The resulting data sets were analysed using the general linear model (GLM) and regression (REG) functions of the SAS package (SAS®, 1990).

3.3 Results

3.3.1 First round shoot production

For shoot elongation, the ranking of the four basal media tested was MS > SH > B5 > LM. On average, shoot production on the MS medium were 6.5 cm high with 6.5 nodes, while on LM medium shoots were 3.2 cm with 2.8 nodes. For all media, full salt strength gave better shoot elongation than half strength.

Sucrose was not necessary for the elongation of axillary buds into shoots. Indeed, the addition of sucrose inhibited elongation. For mean shoot length and the mean number of nodes per shoot, the effects of sucrose were described using linear models (Figure 3.1, 3.2).

However, in spite of the reduction in the mean number of nodes and shoot length, shoots were stronger and healthier when a high sucrose concentration was used. Because strong growing shoots are required for good production of shoots in second round culture, which is necessary for more plants to be produced from a single seed, the commonly used level of 3 % sucrose is recommended to maximise production of healthy plant.

The plant growth regulators NAA and IBA promoted shoot elongation, with IBA being more effective than NAA (Table 3.1). Within the range tested (0.025 - 0.2 mg/L), the relationship between the mean number of nodes per explant (y) and the IBA concentrations (x) was best described as:

$$y = 4.48 + 29.5x - 80.2\,x^2 \; (R^2 = 0.96).$$

The relationship between shoot length (y) in cm and the IBA concentration (x) was best described by:

$$y = 3.95 + 56.7x - 162\,x^2 \; (R^2 = 0.91).$$

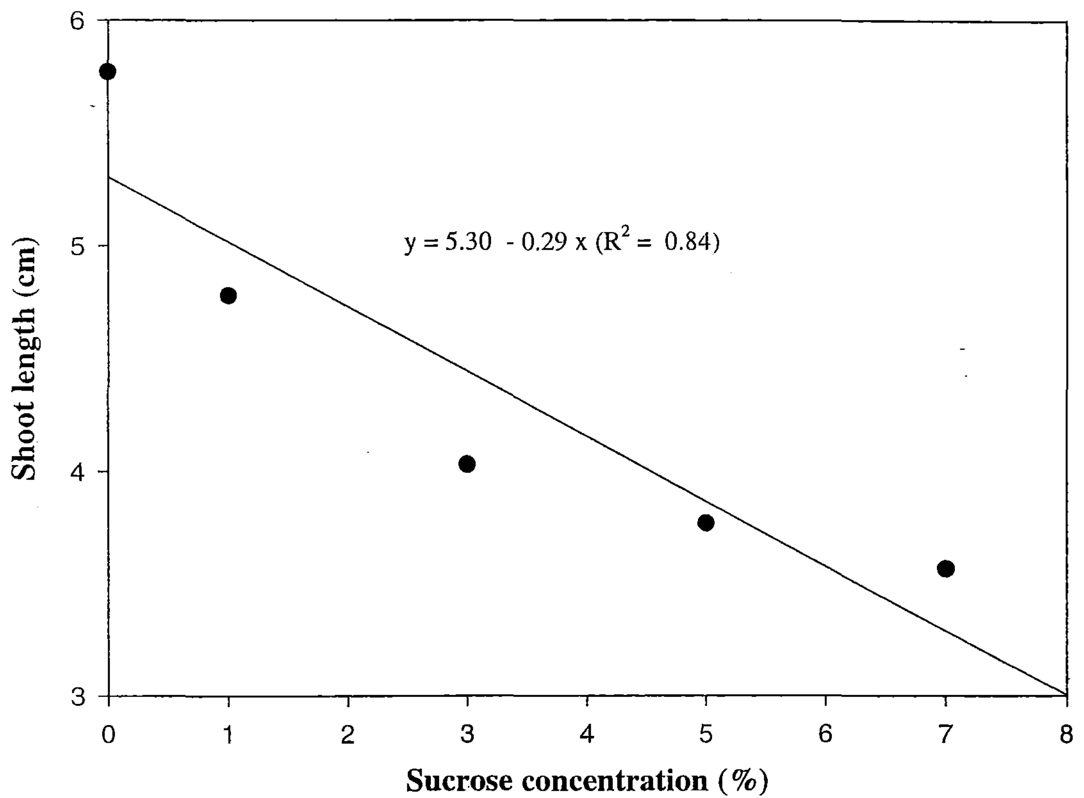

Figure 3.1. Relationship between axillary shoot growth and sucrose content in lentil single node culture. Basal medium: MS salts and vitamins + 0.5 mg/l GA_3.

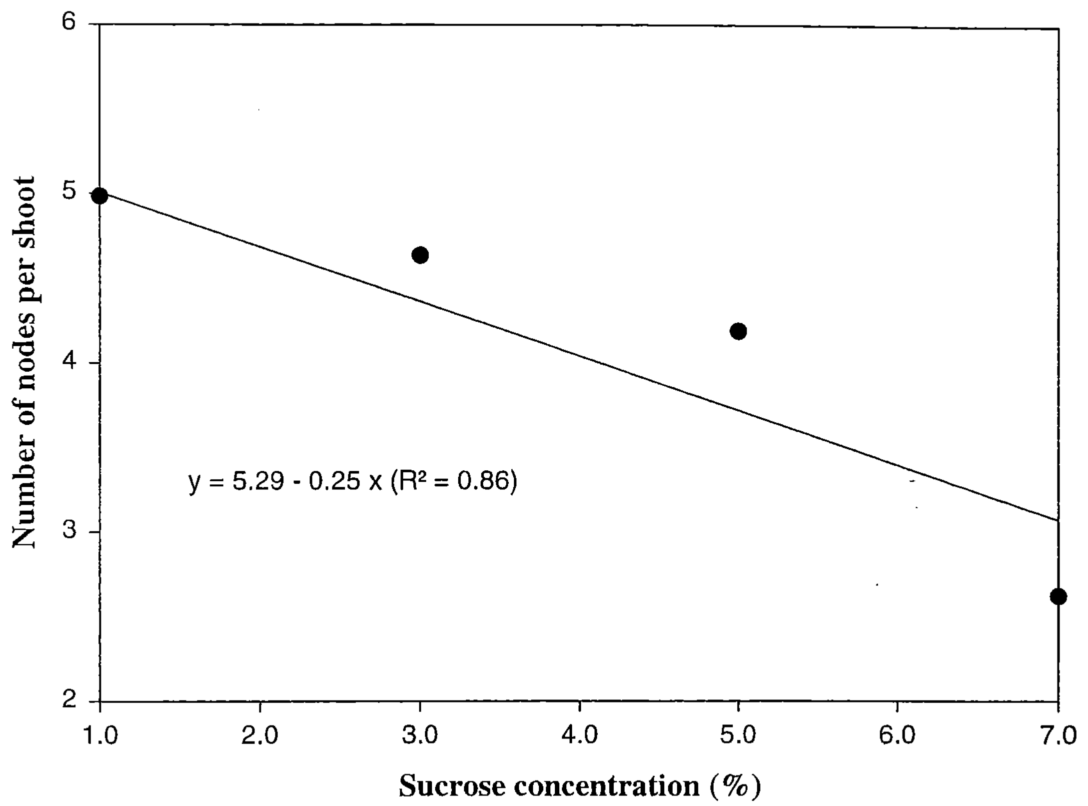

Figure 3.2. Relationship between sucrose concentration and the number of nodes per shoot in lentil single node culture.
Basal medium: MS salts and vitamins + 0.5 mg/l GA_3.

Based on these two equations, the optimum IBA concentration range is 0.17 - 0.18 mg/L. Callus was found on the basal cut surface of all of the explants when NAA was used.

The plant growth regulator GA_3, also promoted axillary shoot elongation (Table 3.1). The shoot length and the number of nodes increased with GA_3 concentrations. Within the tested range (0.1-1.0 mg/L), the relationship between the number of nodes per explant (y) and GA_3 concentration (x) was best described by

$$y = 5.62 + 2.25\,x \ (R^2 = 0.94).$$

Similarly, the relationship between the shoot length (y) and GA_3 concentration (x) was best described by

$$y = 6.80 + 3.33\,x \ (R^2 = 0.79)$$

Although these results suggested that better shoot growth may be obtained by further increasing GA_3 concentration, it is likely that 1.0 mg/L should be efficient enough.

At lower concentrations BA promoted shoot development, while at more than 0.1 mg/L it induced new shoot formation but inhibited shoot development. A concentration of 0.1 mg/L was the best for producing nodes for further culture (Table 3.1).

3.3.2 Second round culture

The shoots produced were as good as in the first round if strong first round shoots were used. The health and vigor of shoots from the first round explants had a great effect on the development of the second round shoots. Segments obtained from shoots produced on medium without sucrose elongated far more slowly and one third as many shoots were produced (Table 3.2). More than four adventitious shoots were produced when 0.1 mg/L BA was used in the first round culture.

Table 3.1. Mean shoot production parameters for single node culture of lentil[1].

PGR	Concentration (mg/L)	Number of shoots/explant	Number of nodes/shoot	Shoot length (cm)
Nil		1	4.1	5.0
NAA	0.025	1	4.4	5.6
	0.05	1	5.1	5.7
	0.1	1	5.8	6.6 [3]
	0.2	1	5.9	6.4[3]
IBA	0.025	1	5.3	5.7
	0.05	1	5.5	5.7
	0.1	1	6.8	8.3
	0.2	1	7.2	8.7[3]
MSE[2]	(Df) = 54	-	0.9	0.9
GA_3	0.1	1	5.8	6.3
	0.2	1	6.0	7.7
	0.4	1	6.8	9.1
	0.8	1	7.1	9.4
	1.0	1	8.0	9.9
BA	0.025	1	4.5	5.5
	0.05	1	4.3	5.5
	0.1	2.22	4.6	5.5
	0.2	3.78	2.3	3.1

[1] MS basal medium supplemented with 3 % sucrose; means were taken from 15 explants.

[2] MSE: Error mean square. Df: degrees of freedom.

[3] Callus formation observed at base of shoots.

Table 3.2. Second round culture of single node segments of lentil[1].

Node source	Number of shoots[4]	Shoot length[4]	Number of nodes per shoot[4]
No sucrose[2]	1.0 b	2.1b	1.8 b
3% sucrose[2]	1.0 b	6.7 a	5.6 a
0.1 mg/L BA[3]	4.5 a	1.2 c	1.4 b

[1] Medium was MS basal medium supplemented with 3% sucrose and 1.0 mg/L GA_3.

[2] First round medium was MS basal medium supplemented with 0.6 mg/L GA_3.

[3] First round medium was MS basal medium supplemented with 3% sucrose.

[4] Treatments followed by the same letter were not significantly different.

3.3.3 Root development

MS was better than B5 for root initiation. On MS medium, 52 % of shoots rooted, compared with only 38 % on B5 medium. Moreover, the shoots on B5 medium became chlorotic. Half-strength MS salts were more effective (64 %) than at full strength (52 %) and the latter was better than 1/4 strength (43 %). At 1/8 strength MS salts, no shoots rooted and all of them became chlorotic. In the range of 1/8 to full strength, the effect of MS salt strength (x) on rooting percentage (y) can be described using a quadratic equation (Figure 3.3). From this equation, the optimum MS salt strength was predicted to be about 2/3.

Sucrose in the rooting medium (more than 1 %) was necessary for shoot viability. A high sucrose content was found to be beneficial to root formation. The highest concentration used in this study gave the best rooting frequency. The effect of sucrose (x) (from 1 % to 5 %) on the rooting percentage (y) can be described using a linear model (Figure 3.4).

Both NAA and IBA promoted root formation and the rooting percentage increased with their concentrations (Table 3.3). The relationship between the rooting percentage (y) and the concentration of NAA (x) was best described by the quadratic equation:

$$y = 11.54 + 61.00x - 15.11x^2 \; (R^2 = 0.99)$$

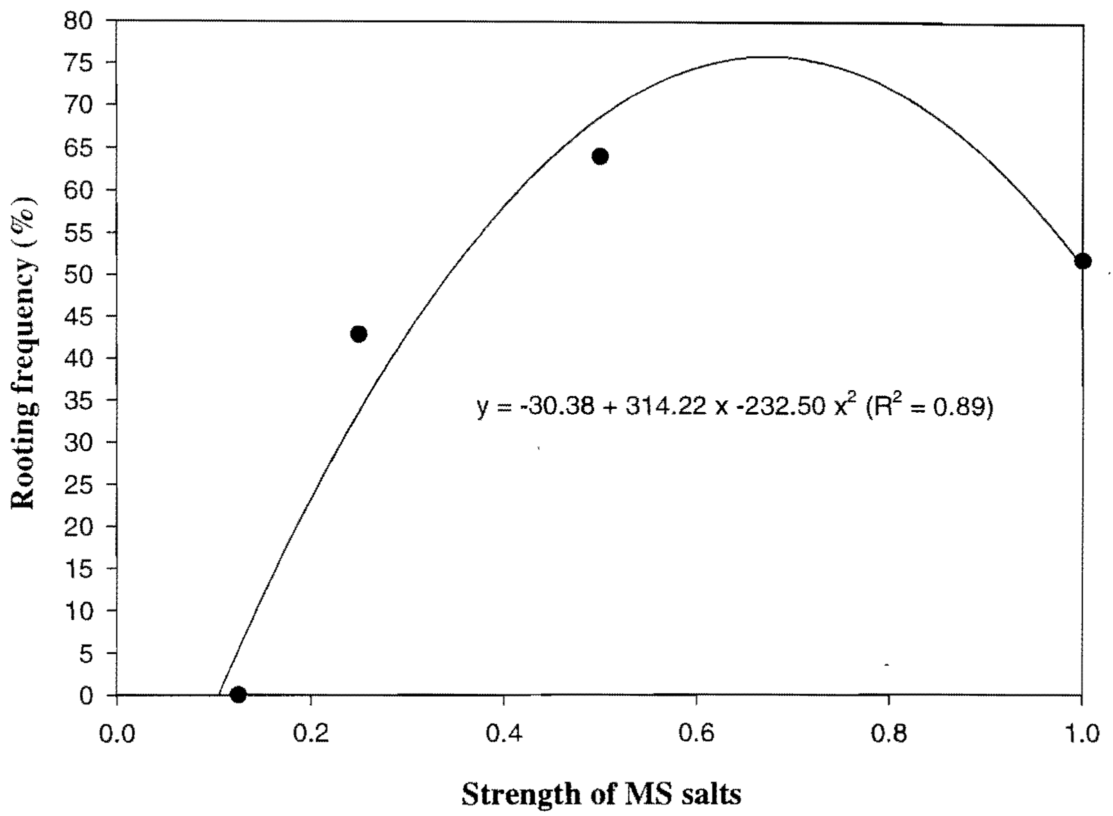

Figure 3.3. Relationship between MS salt strength and rooting frequency of lentil shoots grown from single node explants.
Other medium components: MS vitamins, 3% sucrose, 1.0 mg/L NAA.

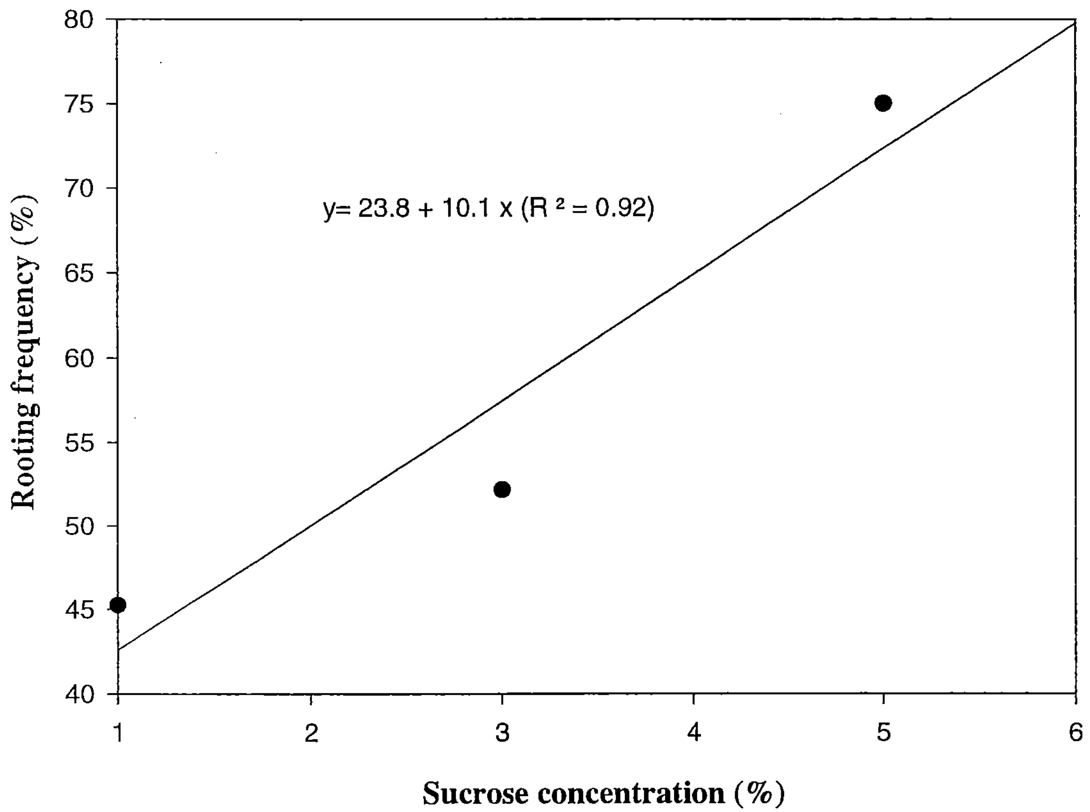

Figure 3.4. Relationship between sucrose concentration and rooting frequency of lentil shoots grown from single node explants on a basal medium of MS salts and vitamins, and 1.0 mg/L NAA.

Table 3.3. Rooting of lentil shoots from single node explants on media containing different concentrations of NAA and IBA[1].

	Concentration (mg/L)					
PGR	0	0.2	0.5	1.0	1.5	2.0
NAA[2]	7.5	25.0[4]	35.0[4]	58.5[4]	70.0[4]	72.5[4]
IBA[3]	7.5	10.0	18.7	34.8	46.3[4]	55.5[4]

[1]Basal medium is MS with 3 % sucrose; means were taken over three replications.

[2]Error mean square of arcsine-transformed data was 0.026 with 68 degrees of freedom.

[3]Error mean square of arcsine-transformed data was 0.038 with 68 degrees of freedom.

[4]Callus formation observed at base of shoots.

Based on this equation the highest concentration used was very close to the optimal concentration (2.02 mg/L). This suggests that further improvement might not be achieved by further increasing the NAA concentration. Similarly, the relationship between the rooting percentage (y) and the concentration of IBA (x) can be described by the quadratic equation:

$$y = 2.18 + 37.42x - 5.35x^2 \, (R^2 = 0.99).$$

Based on this equation, the highest concentration (2.0 mg/L) used is still well below the optimal concentration (3.48 mg/L), indicating that further improvement is possible by increasing IBA concentration. However, the predicted highest rooting percentage using IBA (68%) was lower than that of using NAA (73%). In addition, NAA-induced roots were thicker and shorter than IBA-induced roots. Therefore, NAA was more effective than IBA for root induction, with 73% rooting at 0.2 mg/L NAA.

3.3.4 Micropropagation of hybrids

Based on above results, the protocol used for propagating inter- and intra-specific hybrids was as follows. Shoot production medium was basal MS medium supplemented with 1.0 mg/L GA_3 and 3 % sucrose. Root initiation medium was 2/3 strength MS salts and MS vitamins supplemented with 2.0 mg/L NAA and 5 % sucrose. There was only minor difference among hybrids in terms of the number of nodes per *in vitro* plant, the number of nodes per axillary shoot, and the rooting frequency. However, the number of plants obtained from each hybrid seed differed due to the multiplicative effect of the number of nodes from each *in vitro* plant and axillary shoot, and the rooting frequency (Table 3.4).

3.4 Discussion

For in vitro shoot growth, an MS basal medium was the best of the four tested in this study. The superiority of MS over other media has been demonstrated with other legume species, such as *Prosopsis cineraia* (Shekhawat *et al.*, 1993), *Swartzia madagascariensis* (Berger and Schaffner, 1995) and *Bauhinia vahlii* (Upreti and Dhar, 1996). However, MS was ineffective in other legume species, such as the soybean (*Glycine max*) (Kaneda *et al.*, 1997; Shetty *et al.*, 1992).

A suitable strength of MS salts is important for good rooting and about 2/3 strength was determined to be the best in this study. However, using shoots induced from intact seedlings by use of thidiazuron, Malik and Saxena (1992a), found that rooting percentage was no different in full or half strength MS salts. The effects of salts on rooting may function through two paths. One is through providing necessary nutrients and the other is through influencing the absorption and/or function of plant growth regulators. When nutrition was limited, no roots were formed. This was the case for the 1/8 MS as indicated by yellowing and poor shoot performance. When nutrition was not a limiting factor, the effect of salt strength may be through influencing the absorption and/or functioning of plant growth regulators.

Table 3.4. In vitro multiplication of lentil intra- and inter-specific hybrids using single node culture.

F_1 hybrid	Number of nodes/plant[1]	Number of Nodes/shoot[2]	Rooting (%)[3]	Number of plantlets
Titore x W 6 3261[4]	6	8	71	34
Titore x W 6 3192[4]	5	8	70	28
ILL 5588 x w 6 3261	5	7	74	26
ILL 5588 x W 6 3192	6	7	69	29
Laird x W 6 3261	5	7	71	25
Laird x W 6 3192	6	8	71	34
Titore x ILL 5588	6	8	71	34
Laird x ILL 5588	6	8	71	34

[1]Three-weeks-old *in vitro* seedling on a modified MS medium (MS salts with doubled content of calcium chlorate and MS vitamins) supplemented with 3 % sucrose; means were taken from 5 - 10 seeds.

[2]First round shoots on MS medium supplemented with 1.0 mg/L GA_3 and 3 % sucrose for four weeks; means were taken from 25 - 60 shoots.

[3]Rooted in 2/3-strength MS medium supplemented with 2.0 mg/L NAA and 5 % sucrose for four weeks. Transfer the plantlets to a soil mix in glasshouse, the average survival rate of plantlets was 85 % with a range of 80 - 95 %.

[4]W 6 3192 is an accession of *L. ervoides*, W6 3261 is an accession of *L. orientalis*, and the others are cultivars of *L. culinaris*.

Sucrose in the rooting medium is necessary to obtain healthy plants. The effect of sucrose on root formation has not been previously investigated in lentil tissue culture. However, similar results have been reported by other researchers for other species (Ahuja, 1993; Kyte and Kleyn, 1996). However, in many other plant species,

a high carbohydrate content has been shown to inhibit root formation and development (Ahuja, 1993; Kyte and Kleyn, 1996).

Root formation was induced using NAA in all reported lentil tissue culture studies. However, the optimum concentration varied from one study to another. For example, 0.47 mg/L was the best concentration in that of Malik and Saxena (1992a), but 1.0 mg/L was found to be best by Ahmad *et al.* (1997a).

The initial studies, aimed at optimising shoot elongation and root development, were used cultivated inbred lentil seed. The resulting best medium was then used to multiply interspecific hybrids. Because culture response parameters are under genetic control (Hernandez-Fernandez *et al.*, 1984; Lazar *et al.*, 1984), a protocol suited to the parents should also work for hybrids if the genes affecting response in cultivated lentil are not recessive to the genes in wild lentils. However, the efficiency may differ. This is the case in this study. The differences among hybrids in terms of shoot length and the number of nodes/shoot were marginal. This result is surprising because genotypic variation for *in vitro* response are well documented in plant tissue culture literature (Ou *et al.*, 1989; Ghaemi and Sarrafi, 1993). However, the protocol was based on the elongation of axillary buds and the elongation of axillary buds into branches is relatively easy to achieve in lentil because they are naturally multi-branched species. Therefore, the genetic variation for this particular trait may not be large. Moreover, the inherently different elongation rates of the species and their hybrids may have been reduced by the limited height of the culture container. These data therefore suggested that single nodal segment culture is a genotype-independent system for the micropropagation of lentil and its hybrids.

When an *in vitro* protocol is developed for the true-to-type type plant production for genetic study, the following three criteria must be satisfied.

1. the protocol induces no or minimal somaclonal variation;
2. the protocol plants with consistent developmental stage; and
3. the protocol is highly efficient.

Since somaclonal variation may result from adventitious shoots, the selection of a type and concentration of cytokinin must ensure that only axillary shoot growth is stimulated. Direct shoot formation without a callogenic phase will, to large extent, reduce the possibility of inducing adventitious shoots. Because it is difficult to

distinguish axillary shoots from adventitious shoots, and the protocol developed will be used for enlarging hybrid populations, the selection of the best cytokinin concentration was very conservative. That is no more than one shoot was induced from each segment. The possibility of using multiple shoot induction ability of BA to develop an efficient micropropagation method in lentil is under investigation and will be reported in another report.

To obtain a population of plants at a similar developmental stage, a two-step procedure should be followed. Sufficient explants are produced first, and then are subcultured under the identical conditions to produce plants. Micropropagation using single node segments as explants seems to be the most compatible with this two-step procedure. With regard to the efficiency, the method produced at least 25 plants from a single seed in 3 months or about 100 plants in 4 months. It is therefore much more efficient than performing repeated hybridisations to obtain a similar number of hybrid plants.

CHAPTER 4

IN VITRO SYSTEMS FOR EFFICIENT REGENERATION OF LENTIL

Summary

Two *in vitro* systems were developed to regenerate lentil plants based on cotyledonary node and seed culture. For cotyledonary node culture, explants from 3 to 12 d seedlings on MS basal medium supplemented with BA at 0.6 mg/L or Thidiazuron (TDZ) at 0.01 mg/L gave best useable shoot production from one round of culture. Continual culture on a medium with 0.2 mg/L BA gave the best long-term result. More, and longer, shoots were produced when cotyledon and epicotyl parts were embedded into the medium so the nodal region was directly in contact with the medium. More than 70 % of shoots produced roots in a 2/3 strength MS medium supplemented with 1.5 mg/L NAA and 5 % sucrose. Cytokinins BA, TDZ and Kinetin all induced multiple shoot formation from intact seedlings (seed culture). Kinetin produced less, but longer shoots than BA and TDZ. In terms of the number of useable shoots (> 1 cm) per seed, BA and TDZ at optimum concentration had similar efficiency. However, TDZ was more inductive and toxic. For shoot elongation, stumps (tissue remaining after excising useable shoots) from media containing high levels of cytokinin should be transferred to a medium without cytokinin but with 0.5 mg/L GA_3 or 0.05 mg/L NAA. Stumps from media with lower concentrations of cytokinin can be transferred to a fresh medium with the same composition. Up to 20 useable shoots were produced in 6 weeks using 0.2 mg/L BA.

Multiple shoots were induced from 10 inbreds of four species using MS medium supplemented with 0.2 mg/L BA. However, there were evident genotypic differences. Transfer of shoots longer than 2.0 cm to a 2/3 strength MS medium supplemented with 1.5 mg/L NAA gave the best root formation.

4.1 Introduction

Lentil (*Lens culinaris* Medikus) was one of the first crops cultivated by Neolithic man. The relatively high protein content of lentil grains makes this crop an important source of protein in many developing countries. However, the genetic base of the cultivated lentil is narrow. This makes it susceptible to many diseases such as Ascochyta blight. Progress towards improving disease resistance has proved to be slow (Muehbauer *et al.*, 1995).

The development of modern biotechnology offers plant breeders several valuable new tools to utilise useful genes from wild species and/or heterogeneous sources and to create novel genetic variations. For instance, *in vitro* culture of interspecific hybrids has been suggested to promote the formation of novel recombinations (Larkin and Scoweroft, 1981). Somatic hybridisation can be used to produce distant hybrids, which are not possible using sexual hybridisation (Bajaj, 1995). *In vitro* mutation and selection offer much higher selection pressure and selection intensity than any conventional method and consequently increase the range of variation and the possibility of obtaining novel mutants (Larkin, 1987). Genetic transformation is a more flexible method for improving simply inherited traits such as disease and pest resistance than classical backcross-based introgression (Lee, 1995). However, a prerequisite to applying these methods to practical plant breeding is the availability of an efficient regeneration system. As with other large-seeded legume species the *in vitro* culture of lentil has proved difficult. However, successful regeneration has been achieved using different types of explants using both organogenesis and embryogenesis (A detailed summary of *in vitro* culture of lentil is given in Chapter 2).

Cotyledonary nodes are a good source of explants for regeneration in many legume species including pea (*Pisum sativum*) (Malick and Rashid, 1989; Jordan and Hobbs, 1993), dry beans (*Phaseolus vulgaris*) (Franklin *et al.*, 1991), pigeonpea (*Cajanus cajan*) (Prakash *et al.*, 1994), soybean (*Glycine max*) (Kothari *et al.*, 1991), and white clover (*Trifolium repens*) (Voisey *et al.*, 1994).

Combined with *Agrobacterium*-mediated gene transfer, cotyledonary node culture has been used to successfully obtain transgenic plants in pea (Jordan and

Hobbs, 1993) and white clover (Voisey *et al.*, 1994). In lentil, cotyledonary node culture combined with *Agrobacterium*-mediated gene transfer has also been tried (Warkentin and McHughen, 1993). The induction of multiple shoot formation was successful, while transformation was not. Recently, Oktem (1999) obtained transgenic plants from particle bombarded cotyledonary nodes.

Seed culture has been successfully used in the regeneration of several species including legumes such as pea and chickpea (*Cicer arietinum*). Compared to other types of explants, seeds offer several advantages. For example, no specific explant preparation is needed and surface sterilisation of seeds is relatively simple. This means the establishment of cultures from seeds is much easier and is more reliable than from other sources.

This chapter describes how cotyledonary node and seed culture procedures were optimised to develop efficient regeneration systems for lentil.

4.2 Materials and methods

4.2.1 Plant material

Seeds of *Lens culinaris* cv. 'Titore' were obtained from the New Zealand Institute of Crop and Food Research Ltd. All the other lines/cultivars (see Fig 4.1) were from Turkey.

4.2.2 Explant and medium preparation

Seeds were surface sterilised by rinsing in 95 % ethanol for 30 seconds, soaking in 1.25 % sodium hypochlorite containing a few drops of the surfactant Tween-20 with continuous stirring for 20 min, followed by five - six washes in sterile deionised water. The seeds were germinated in an incubator at 24 °C in the dark on MS salts and vitamin (Murashige and Skoog, 1962) with doubled calcium chlorate concentration, 3 % sucrose and 8 % agar in plastic tissue culture containers. There were 10 seeds per container. After 3 - 4 d, containers were transferred into a tissue culture room with a light intensity of 60 $\mu molm^{-2}s^{-1}$ at 24 °C with 16h days. Nodal segments (about 1.0 cm) were prepared from 3 to 4 week old seedlings. Cotyledonary

nodes were prepared by excising hypocotyl, epicotyl and cotyledons at 3 mm from the cotyledonary node.

All media were supplemented with 3 % sucrose (except in the sucrose content experiment), solidified by 8 % agar and adjusted to pH 5.8 prior to sterilisation by autoclaving (122 kPa, 20min). All experiments were done in a tissue culture room with a light intensity of 60μmolm^{-2}s^{-1} at 24 °C with 16h days.

4.2.3 Experiments on cotyledonary node culture

Shoot elongation

1. Basal medium and strengths: explants from 3 d old seedlings were cultured on MS, B5, SH media with full- and half- strength salts, all supplemented with 0.6 mg/L BA.
2. Seedling age: explants from seedlings of 3, 5, 7, 9, 11, 13, 15 or 21 d old were cultured on MS medium supplemented with 0.6 mg/L BA.
3. Plant growth regulator concentrations: MS medium supplemented with BA (0.1, 0.2, 0.4, 0.6, 0.8 and 1.5 mg/L) or TDZ (0.01, 0.02, 0.03, 0.04 and 0.1 mg/L), (explants were from 3 d old seedlings).
4. Culture duration: MS medium supplemented with BA at 0.2 or 1.5 mg/L for 1, 2, 3 or 4 weeks.

Root induction

Shoots (three to four cm) were transferred onto MS medium with 2/3 strength of salts supplemented with1.5 mg/L NAA and 5 % sucrose.

4.2.4 Experiments on seed culture

Shoot induction

1. Basal medium and BA concentrations: MS medium, MS medium with a doubled content of $CaCl_2$, B5 medium and B5 medium +750 mg/L $CaCl_2$ were tested in combination with four concentrations of BA (0.2, 0.4, 0.6 or 0.8 mg/L).

2. Plant growth regulator concentrations: MS supplemented with different concentrations of kinetin (0.2, 0.4, 0.6, 0.8, 1.0 or 1.5 mg/L) or TDZ (0.1, 0.2, 0.5, 0.6, 0.8, 1.0, or 1.2 mg/L).

3. Species and inbreds: Shoot formation capabilities of the following ten inbred/lines of four lentil species were tested using MS medium supplemented with 0.4 mg/L BA.

Lens culinaris: Titore, Olympic; *Lens nigricans*: W6 3210, W6 3218, W6 3221, and W6 3208; *Lens ervoides*: W6 3173, W6 3176 and W6 3192, and *Lens odemensis*: W6 3244.

Elongation of induced shoots and further induction of multiple shoots

1. Shoots of 0.5-1.5 cm length were isolated with or without a small piece of seed segment and transferred to MS medium supplemented with different concentrations of NAA, IAA, IBA, GA_3 and BA.
2. Stumps (the part remaining after excising useable shoots) were transferred to fresh medium of same composition or MS basal medium supplemented with 0.05 mg/L BA and 0.05 mg/L NAA.

Root induction

Shoots longer than 2.0 cm induced from the seeds on a medium consisted of MS salts with doubled $CaCl_2$ content and 0.2 mg/L BA were transferred to MS medium supplemented with different concentrations of NAA or IBA for rooting.

4.2.5 Experimental design and analysis

For shoot induction, a randomised complete block design was used with three replications of five containers. Five explants were cultured per container. For root induction, a completely randomised design with three replications of two containers was used. Five shoots were cultured per container. After 3 or 4 weeks

incubation, data were collected on number of shoots/explant, shoot length in the shoot induction experiments and the number of rooted shoots in the root induction experiments. The data sets were analysed using the general linear model (GLM) and regression (REG) functions of the SAS statistical package (SAS, 1990). Percentage data was arcsine transformed for statistical analysis.

4.3 Results

4.3.1 Cotyledonary node culture

***Shoot induction*:** Basal medium and salt strength influenced shoot induction from cotyledonary nodes (Table 4.1). Full strength salts were better than half strength in all the four tested basal media. The best medium was MS regardless of salt strength. Therefore, full strength MS basal medium is recommended as the best for shoot induction from cotyledonary nodes (Table 4.1).

Table 4.1. Shoot number and useable shoot percentage induced from lentil cotyledonary nodes cultured on different basal media[1].

Basal medium	Number of shoots[2]	% useable shoots[3]
MS	35	70
1/2 MS	20	50
B5	14	30
1/2 B5	20	30
SH	25	50
1/2 SH	20	10

[1]All media supplemented with 3 % sucrose and 0.6 mg/L BA; means were taken from 35 - 45 explants.

[2]Error mean square was 32.86 with 68 degrees of freedom.

[32]Error mean square of arcsine-transformed data was 0.94 with 68 degrees of freedom.

Seedling age was important in shoot organogenesis. More shoots were initiated from cotyledonary nodes of young seedlings. Explants from seedlings 3 to 7 d after germination produced over 16 shoots/node, while those from 13 to 21 d old

seedlings produced only about seven shoots/node. Within the range tested, the effect of explant age (x) on shoot number (y) can be described as:

$$y = 21.5 - 1.14\,x + 0.02x^2 \quad (R^2 = 0.78)$$

Although, shoots from nodes of seedlings older than 13 d were stronger and longer, the best source of nodes was from seedlings of 3 to 12 days old.

Cytokinins BA and TDZ all induced shoot development, with BA being more effective than TDZ (Table 4.2). When TDZ was used, shoots were more stunted, with more than 0.1 mg/L of TDZ all explants died within a week. Shoot elongation was inhibited by BA at more than 0.4 mg/L. The best concentration range of BA was from 0.2 to 0.6 mg/L. At 0.6 mg/L it produced the highest number of shoots over 1 cm high. Lower BA concentrations could be beneficial if the explants were to be cultured on medium with the same composition over a prolonged period. Otherwise, 0.6 mg/L of BA is recommended.

***Shoot elongation*:** Throughout this study, short shoots gave poor root initiation. Thus, elongation of the induced shoots was necessary to develop a suitable *in vitro* multiplication system. Three options were investigated to elongate the short shoots induced on the medium containing 0.6 mg/L BA. First, shoot clusters (after cutting shoots longer than 2.5 cm) were subcultured onto fresh medium containing 0.6 mg/L BA. In this case, more than 60 shoots from each stamp were formed within 4 weeks.

However, less than five shoots were longer than 2.5 cm. Most of the new shoots were initiated at the base or at the lower nodes of existing shoots. Moreover, some shoots died or became chlorotic. Second, shoot clusters were subcultured onto a MS basal medium without cytokinin. Total shoot number was about 40, and more than ten shoots were longer than 2.5 cm. Again, most of the new shoots were initiated at the base or at the lower nodes of existing shoots. Third, all induced shoots which were longer than 1.0 cm but less than 2.5 cm were transferred to an elongation medium and the remaining clumps of shorter shoots were transferred to fresh medium containing 0.6 mg/L BA. The elongation media used were the basal MS medium supplemented with different plant growth regulators. When IBA was used at 0.2 or 0.4 mg/L it gave the best shoot elongation and most healthy shoots. Shoots on NAA-containing media

elongated, but could not be used because they were chlorotic. Callus formed at the cut surface of shoots in NAA-containing media. Shoot elongation occurred at 0.2 mg/L BA, but no shoot elongation was observed at 0.4 mg/L BA. Furthermore, on fresh medium, about five well-developed shoots were obtained from the basal clumps of the shorter shoots with many other small shoots. Out of these three approaches, the third option using an elongation medium containing 0.4 mg/L IBA is the preferred method, even though this approach requires more space.

Table 4.2. Shoot formation and development from lentil cotyledonary nodes cultured on MS medium supplemented with different concentrations of BA or TDZ [1].

Kind	Concentration (mg/L)	Number of shoot	%shoot ≥ 1.0 cm
BA	0.2	11.8	100
	0.4	12.6	100
	0.6	17.8	90
	0.8	17.2	70
	1.5	17.2	30
EMS[2]	-	25.25	0.14[3]
TDZ	0.01	10	30
	0.02	15	5
	0.03	13	5
	0.04	9	5
	0.1	5	0
EMS[2]	-	29.36	0.11[3]

[1]All media supplemented with 3 % sucrose; mean were taken from 45 explants.

[2]Error mean square with 68 degrees of freedom.

[3] Based on arcsine transformed data.

***Root initiation*:** When shoots (>3.0 cm) were transferred to a rooting medium consisted of 2/3 strength MS salts, 2.0 mg/L NAA and 5 % sucrose, on average, 70 % of shoots from shoot induction medium initiated roots after three weeks. After a cycle on the elongation medium with 0.4 mg/L IBA, 78 % of the shoots initiated roots within 3 weeks.

4.3.2. Seed culture

***Shoot induction*:** Basal medium, BA concentration, and their interaction were significant for both of the total number of shoots/seed and the number of useable shoots/seed. However, the contribution of the interaction to the overall variance was much less than that of the basal medium and BA concentration (Table 4.3). Therefore, the best BA concentration can be chosen without considering the interaction. The MS medium with doubled $CaCl_2$ content was better than MS at lower BA concentrations, but the reverse was true at higher BA concentrations.

The two media containing MS salts were better that the two with B5 salts, regardless of the BA concentration (Table 4.3). There were no significant differences for shoot number between B5 and B5 with 750 mg/$CaCl_2$, and between MS and MS with doubled $CaCl_2$ content (Table 4.3).

Adding BA to the four media at all four concentrations induced multiple shoot formation (Table 4.3). More useable shoots (> 1cm) were produced at lower BA concentrations, while more shoots were induced in higher concentrations (Table 4.3).

Shoot-tip necrosis (STN) of main shoots was observed. The most serious STN happened to B5 supplemented with 0.2 and 0.4 mg/L BA (Table 4.3). Compared to the results of B5 and B5 with 750 mg/L $CaCl_2$ and those of MS and MS with doubled $CaCl_2$ content, it was obvious that the addition of calcium (Ca) largely overcame STN.

Kinetin also induced multiple shoots from seed explants as well (Table 4.4). Meaningful differences among the concentrations tested were found for the number of shoots and useable shoots, but not for the average length of useable shoots. The concentration of 0.8 mg/L and 1.0 mg/L were better for the total and the useable

number of shoots per seed. Comparing the number of shoots induced using the best concentration of kinetin (Table 4.4) with that using BA (Table 4.3), it was clear that fewer shoots were produced by using of kinetin. However, the shoots induced by using kinetin were longer, although no designed comparison was made.

Table 4.3. Multiple shoot formation from lentil seeds cultured on different media and BA concentrations.

Basal medium	BA (mg/L)	Number of shoots	Number of long shoots	Shoot-tip necrosis (%)
MS	0.2	7.4	4.1	85.0
MS+440 mg/L $CaCl_2$	0.2	9.7	5.5	2.5
B5	0.2	6.0	3.9	90.5
B5 +750 mg/L $CaCl_2$	0.2	7.5	4.4	18.3
MS	0.4	13.7	4.9	70.0
MS+440 mg/L $CaCl_2$	0.4	12.5	4.4	2.5
B5	0.4	7.4	2.8	89.3
B5 +750 mg/L $CaCl_2$	0.4	9.0	4.4	15.5
MS	0.6	11.5	3.8	55.0
MS+440 mg/L $CaCl_2$	0.6	13.6	3.6	0.0
B5	0.6	8.3	3.4	75.0
B5 +750 mg/L $CaCl_2$	0.6	9.7	4.1	4.8
MS	0.8	13.2	13.2	50.0
MS+440 mg/L $CaCl_2$	0.8	12.1	12.1	0.0
B5	0.8	8.7	8.7	74.5
B5 +750 mg/L $CaCl_2$	0.8	11.5	11.5	5.2

Source	DF	MS[1]	F[1]
Medium	3	322.18/11.70	$78.35^{***}/12.32^{***}$
BA	3	225.36/13.29	$54.80^{***}/13.99^{***}$
Medium*BA	9	25.50/6.15	$6.20^{***}/6.48^{***}$
Error	224	4.11/0.95	

[1] The first and second numbers of each cell are for the number of shoots and long shoots per seed, respectively.

*** $P < 0.001$.

Table 4.4. Multiple shoot formation and development of lentil seeds cultured on MS basal medium supplemented with different concentrations of kinetin[1].

Kinetin (mg/L)	0.2	0.4	0.6	0.8	1.0	1.5	EMS[2]
Number of shoots	1.1	1.1	1.8	4.5	4.6	2.9	1.14
Number of useable shoots	1.0	1.1	1.5	2.8	2.5	1.9	0.86
Useable shoot length (cm)	5.9	6.4	6.7	5.2	5.2	5.4	0.03

[1]The means were taken from 120-150 seeds.

[2] Error mean square with 82 degrees of freedom.

Thidiazuron induced the formation of multiple shoots at all the concentrations tested. With increasing TDZ concentration, the number of useable shoots per seed was decreased. Seven shoots per seed at 0.1 mg/L were produced compared with one at 1.0 mg/L or more. Within the tested range (0.1 - 1.2 mg/L), the effect of TDZ (x) on the number of useable shoots (y) was best described by

$$y = 1.42 - 0.19\,x \quad (R^2 = 0.94)$$

Compared with BA at the same concentration, more useable shoots were induced by TDZ. This indicated that TDZ was more effective.

Overall, it is recommended that MS salts with doubled $CaCl_2$ content supplemented with 0.2 mg/L BA for inducing multiple shoots from lentil seeds. TDZ at 0.1 mg/L was also efficient, but further experimentation is required to fully justify the use of this growth regulator.

***Shoot regeneration of species and inbred*:** Multiple shoots were induced from all inbred lines tested, and a minimum of about four shoots was produced per seed (Fig 4.1). Therefore, BA-based seed culture can be used for regenerating all lentil species. The shoot formation capabilities varied between species and inbred within species. For example, the most responsive inbred line produced twice as many shoots per seed

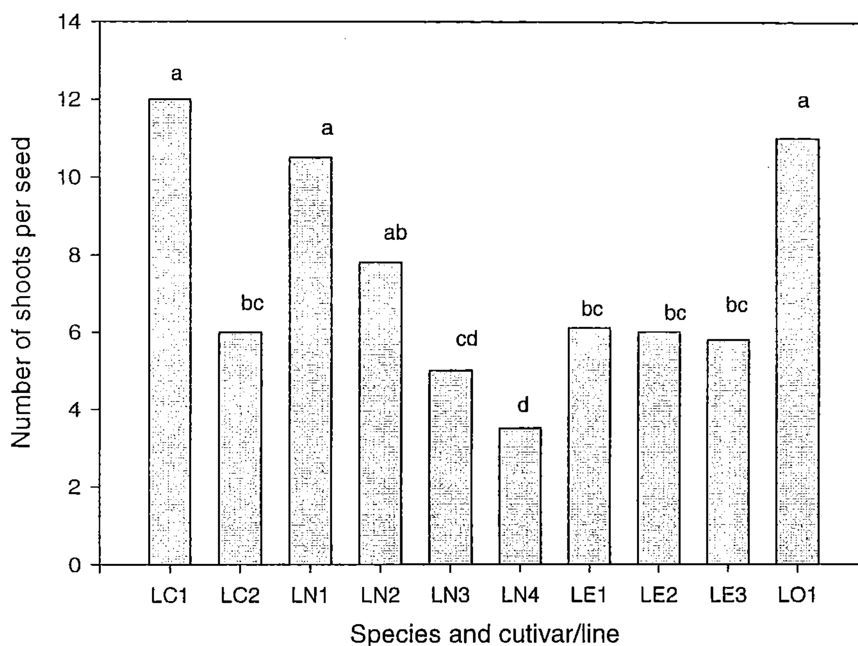

Figure 4.1. Shoot formation from seeds of ten cultivars/lines of four lentil species. The differences among cultivars/lines labelled by the same letter(s) were not significant at $\alpha = 0.05$ level. LC1: 'Titore' of *Lens culinaris*, LC2: 'Olympic' of Lens culinaris; LN1-LN4: accession W6 3210, W6 3218, W6 3221and W6 3208 of *Lens nigricans*; LE1-LE3: accession W6 3173, W6 3176 and W6 3192 of *Lens ervoides* and LO1: W63244 of *Lens odemensis*.

as the least responsive one. The cultivated lentil cultivar Titore produced the greatest number of shoots per seed.

Elongation of induced shoots and further regeneration of shoots: To promote the development of the short shoots, individual small shoots (less than 1.5 cm) with or without seed segment to the basal medium supplemented with different concentrations of NAA, or IBA or IAA or GA_3. It was found that short shoots without seed segments could survive but hardly elongate after 4 weeks. Short shoots with seed segments elongated into useable shoots. However, the multiplication rate was lower than transferring the whole stumps. Therefore, shoots were excised from mother seedlings

when they were longer than 2.5 cm and the stumps were used to produce more shoots. After 2 weeks on fresh medium, on average 9.7 and 11.2 useable shoots were produced from the stumps from 0.2 mg/L BA and 0.1 mg/L TDZ, respectively after removal of longer shoots. However, on the stumps from 0.8 mg/L BA, 0.5 mg/L TDZ, only five and three useable shoots were obtained. When stumps from BA 0.8 mg/L, or TDZ 0.5 mg/L were transferred to medium supplemented with 0.5 mg/L GA and 0.05 mg/L NAA, more useable shoots (5.5 and 5.0, respectively) were obtained. Therefore, stumps from medium containing lower BA (TDZ) can be transferred to a fresh medium of the same compositions, while stumps from medium containing higher BA (TDZ) should be transferred to the medium without BA but with GA_3 or NAA. In addition to the elongation of small shoots, many new shoots/ shoot meristem were induced around the connecting site of cotyledons and hypocotyl.

When stumps became too large to be easily transferred, stumps were subdivided into several (3 - 4) small clusters for further subculture. On the medium supplemented with GA_3 0.5 mg/L or NAA 0.05 mg/L, one or two well-elongated shoots can be obtained from these clusters within 2 or 3 days. Removal of the well-developed shoots is necessary to promote the elongation of other shoots. Up to 20 shoots could be harvested in 3 weeks.

Rooting: From 14 % (on basal MS medium) to 75 % (on MS medium supplemented with 1.5 mg/L NAA) of the shoots rooted within 4 weeks (Table 4.5). NAA was more effective than IBA (Table 4.5). Usually only one well developed root with several short secondary roots were produced per shoot within 4 weeks, but three and four roots with secondary roots can be obtained after a further 2 weeks culture in a fresh medium with the same composition. Callus formation was observed on the cut surface of shoots when NAA was applied. The higher the NAA concentration, the greater the callus development. However, the roots were not initiated from callus. Primary shoots gave a higher rooting percentage than adventitious shoots (Table 4.5).

Table 4.5. Rooting frequencies of lentil shoots induced from seeds on media containing different concentrations of NAA and IBA[1].

	NAA 0.2	NAA 1.0	NAA 1.5	IBA 1.5	IBA 0.2
Adventitious	13.3	43.5	58.5	10	6.7
Main	32.5	66.7	75.0	60.0	25.0

[1]The basal medium was MS salts and vitamin with 3 % sucrose; means were taken from 75 shoots.

[2]Error mean square of arcsine-transformed data was 0.044 with 68 degrees of freedom.

4.4 Discussion

The effect of seedling age on shoot formation from cotyledonary nodes has been observed in many species including legumes such as soybean (Kim *et al.*, 1991), mung bean (*Vigna radiata*) (Gulati and Jaiwal, 1994) and eastern redbud (*Cercis canadensis*) (Distabanjong and Geneve, 1997). The common age of seedlings used in legume cotyledonary node culture is 2 - 12 d (Distabanjong and Geneve, 1997). From the results of the present experiment, it is recommended that explants are prepared from 3 - 12 d old seedlings.

When cotyledonary nodes from 3 d old seedlings were cultured on a basal MS medium, two shoots were produced from the connecting site between the two cotyledons and the axis. They developed well and were about 8 cm long in 4 weeks. They could be used to produce more than 15 single nodal segments in nodal segment culture. The multiplication efficiency of single nodal segment culture can be doubled if instead of using *in vitro* seedlings the two shoots from each cotyledonary node are used to obtain nodal segments. In addition, the remaining cotyledonary nodes can be further cultured on a cytokinin containing medium to form multiple shoots (about seven shoots can be obtained using the cotyledonary node from 21 day old seedlings). Therefore, the combination of single node culture and cotyledonary node culture may

be a good option for obtaining genetically true-to-type plants for genetic studies and large number of plants for breeding from limited hybrid seed.

In this study MS medium was better than the other salt formulations for inducing shoots. The superiority of MS has been shown in other legume species, such as *Prosopsis cineraia* (Shekhawat *et al.*, 1993), *Swartzia madagascariensis* (Berger and Schaffner, 1995) and *Bauhinia vahlii* (Upreti and Dhar, 1996).

Direct shoots were induced by BA from intact seedlings. This has been reported in *Phaseolus* spp (Malik and Saxena, 1992b), *Arachis hypogaea* (Saxena *et al.*, 1992), *Alnus glutinosa* (Perinet and Lalonde, 1983), and *Murraya koenigii* (Bhuyan *et al.*, 1997).

Abnormal shoots were induced from intact seedlings when a BA concentration of more than 0.4 mg/L was used. It is well documented in the plant tissue culture literature that high concentration of cytokinin increases the frequency of abnormal shoots. In other studies using different explant types, much higher BA levels were used to induce shoot formation (Polanco *et al.*, 1988; Polanco and Ruiz, 1997) and abnormal induced shoots were not reported. Therefore, germinating seeds may contain much higher level of cytokinin than other types. This was supported by the observation that some seeds produced two shoots in a basal MS medium without cytokinin.

For shoot induction from intact seedlings, TDZ was the most effective cytokinin, kinetin was the least inductive and BA was in between. Similar results have been reported in lentil with other explant types (Polanco and Ruiz, 1997). The stronger effect of TDZ was consistent with the general trend reported in other studies (Lu, 1993). More stunted shoots were produced when TDZ was used. The inhibiting effect of TDZ on shoot development has been widely documented (Upreti and Dhar, 1996; Distabanjong and Geneve, 1997). However, using TDZ at low concentrations and for a short duration may improve multiplication efficiency. It is possible that BA may be a better cytokinin for lentil *in vitro* culture due to its lower toxicity.

For good shoot induction, it is important to define the right dose of cytokinin (BA or TDZ in this study). Both concentration and duration are important for the best regeneration response. Therefore, two ways can be followed. One is to expose explants to a high concentration for a limited period to initiate shoot differentiation,

and then transfer the explants onto another medium to promote shoot development. Although this can be done without difficulty once a protocol is developed, it is less convenient when the protocol is used in other studies such as genetic transformation. Another method is to continually culture explants in a medium containing low levels of cytokinins. This option would be preferable if the regeneration was satisfactory. This is because it reduces the number of sub-cultures and the experimental cost. In this study, low concentrations of BA or TDZ were highly inductive. Therefore the second option is preferable.

Multiple shoots were induced from 10 inbreds of four lentil species using a BA-containing medium. However there were large genotypic differences. Thus, a universal protocol for lentil *in vitro* culture can be developed based on culturing seeds on a BA-containing media and the screening of inbreds for good multiplication performance should improve the success rate of *in vitro* culture.

Parh *et al.* (1998) first reported that serious STN occurred when basal MS medium was used to produce *in vitro* lentil seedlings. They alleviated the problem by increasing the Ca content of the medium. Studies with other plant species have indicated that Ca deficiency, which can be easily promoted by *in vitro* conditions such as a low transpiration rate and high humidity, may be the main reason for STN (Sha *et al.*, 1985).

In seed culture of this study, on B5 medium with 750 mg/L $CaCl_2$ and on MS medium with doubled the $CaCl_2$ content, the percentages of plants with STN were very different, even though the $CaCI_2$ content was the same. Moreover, only the main shoots showed STN and the STN was less severe when high concentrations of BA were added to the media. Taking the high number of shoots in the medium containing high BA into consideration, the data are consistent with the hypothesis that it is not the absolute amount of $CaCl_2$ but its transport that caused its deficiency at the shoot-tip. The developing shoot tip does not obtain sufficient $CaCI_2$ when it is too far from the source of $CaCI_2$. The inhibiting effect of BA on shoot development delayed the development of STN and consequently reduced the STN percentage.

A high percentage of rooted plants was obtained when primary shoots were used. This was expected because the main shoots were stronger and healthier, and the accumulation of external cytokinins in the primary shoots may be less since no shoot

initiation stage is involved. The rooting percentage in this study was higher than all the previous studies except that of Ahmad *et al.* (1997a). One reason may be the lower concentration of external BA that was used. However, it was very hard to compare my results with previous studies because of the different types of explants that were used.

During the process of root induction, several shoots were produced simultaneously at the basal parts of some shoots. These shoots were much easier to root. This observation can be exploited to increase the efficiency of multiplication in future study.

CHAPTER 5

MAJOR GENES FOR ASCOCHYTA BLIGHT RESISTANCE IN LENTIL

A. MAJOR RESISTANCE GENES AND THEIR ALLELIC RELATIONSHIPS

Summary

Inheritance of foliar resistance to Ascochyta blight in lentils was studied using four resistant and two susceptible cultivars (lines). Two dominant genes, one for high resistance and one for moderate resistance are present in ILL 5588. One dominant gene, which is allelic to the one for high resistance in ILL 5588 confers resistance in ILL 5684. One recessive gene is responsible for the resistance in cv. Laird. The resistance in cv. Indian head is under control of two recessive genes with additive effects. Two complementary genes, one in accession W 6 3192 and one in cv Titore are responsible for the resistance observed in the segregating generation of the cross between these two susceptible cultivars.

5A.1 Introduction

Ascochyta blight caused by *Ascochyta lentis* is one of the important diseases of lentils. It has been reported to be the major disease in many lentil production areas including Argentina, Canada, Ethiopia, India, New Zealand, Pakistan and the Russian Federation (Erskine *et al.*, 1994). The disease has considerable effect on both seed quality and yield (Morrall and Sheppard, 1981). Gossen and Morrall (1983) estimated that foliar infection has caused yield losses of up to 40 %. However, economic losses from infected seed have reached more than 70 % in Canada. There is no effective and economic established control method. This is despite extensive studies on the control

Ascochyta blight in lentil by agronomic and chemical methods. Breeding for improving host resistance is expected to be the most effective, efficient and environmentally friendly method of control (Erskine *et al.*, 1994).

Resistance sources have bee identified in both cultivated and wild lentil species (Singh *et al.*, 1982; Iqbal *et al.*, 1990; Kapoor *et al.*, 1990; Erskine and Bayaa, 1991; Bayaa *et al.*, 1994; Andrahennadi *et al.*, 1996). The genetics of Ascochyta blight resistance in lentil has been studied using different resistant cultivars (lines) and the following inheritance models were proposed.

1. Single dominant gene (Tay and Slinkard, 1989; Ahmad *et al.*, 1997b; Andrahennadi, 1997; Vakulabharanam *et al.*, 1997; Parh, 1998; Ford *et al.*, 1999),
2. Single recessive gene (Tay, 1989),
3. Two duplicated recessive genes (Andrahennadi, 1994),
4. One dominant gene and one recessive gene (Sakr, 1994), and
5. Two complementary genes (Tay; 1989; Andrahennadi, 1994; 1997; Ahmad *et al.*, 1997b).

Because, in most of the studies, the sample size was small and only one segregating population of each cross was used to infer the inheritance model, these proposed models are inconclusive. Moreover, the allelic relationships between the major genes were unknown and need to be investigated. This information is important for making correct breeding decisions. For instance, if several major genes were not allelic, to pyramid these genes would be a strategy for further improving resistance. Otherwise, the variation among identified cultivars could be an indication of the presence of minor genes. The contribution of minor genes should be quantified and utilised in breeding programmes. Resources containing different resistant genes should be explored.

To improve the efficiency of breeding for resistance for Ascochyta blight an effort was made to gain a better understanding of the genetics of Ascochyta blight resistance in lentils by using several genetic analytical methods. The presence of major genes, their allelic relationships and the genetic models of major genes were detected using segregation analysis. Resistant and susceptible sets of inbred lines produced from two crosses (resistant parents carrying major genes) were used to

investigate the contribution of minor genes. Generation mean analysis and a analysis based on mixed model were done to study the combined gene effects. Results from the segregation analysis are reported in this chapter and other results are reported in later chapters.

5A.2 Materials and methods

5A.2.1 Plant materials

Four resistant cultivars, one susceptible cultivar of *Lens culinaris* and one susceptible line of *L. ervoides* were crossed in all possible combinations excluding the reciprocal to produce F_1 hybrids. To make the F_2 populations large enough, the F_1 populations with few seeds were micropropagated using the protocol reported Chapter 3. The F_1s were selfed to produce the F_2 population and backcrossed to both susceptible and resistant parents to obtain BC1 and BC2, respectively.

Seedlings were prepared by sowing seeds in pots in the glasshouse of Lincoln University. Ten plants were grown in one pot of two litres size. Bark and sand at 60: 40 ratio was used as potting mixture. Pots were arranged randomly on a bench. The maximum and minimum temperatures were maintained at 25 °C and 15 °C.

5A.2.2 Evaluation for blight reaction

A pathogenic isolate of *A. lentis* was obtained from infected seed of cv. Invincible (isolate Rakaia). A random sample of infected seeds were surfaced sterilised in 0.6 % NaOCl for 10 min, soaked on sterile filter paper and plated on V_8-juice (Campbell Soups Ltd.) agar medium with five seeds/plate. After growing for seven days, under near ultraviolet light at 18 °C on a laboratory bench, seeds, which produced a characteristic diurnal colony of *Ascochyta*, were selected. A small plug from these colonies was transferred to fresh medium for further growth and colonisation. Inoculum was prepared from a 15 d culture, grown under similar conditions as for the seed plates. Spores were washed from the culture plates using distilled water and filtered through muslin cloth. Two drops of Tween 20 were added

per 100 ml. The concentration of the spore suspension was measured using a haemocytometer and adjusted to 7.5×10^4 conidia/ml by dilution.

Seedlings with four - five expanded leaves (15 d after sowing) were inoculated with the suspension (1 ml/seedling) using an atomiser. After inoculation, pots were kept in a controlled environment cabinet (18 °C and high humidity) for 24 h and then moved to a green house bench for disease development to occur. Negative control was seedlings of suscetptible cultivar 'Titore' sprayed with distilled water plus Tween 20 only.

5A.2.3 Data collection and analysis

Data on disease development were recorded according to a modified ICARDA (Ahmad *et al.*, 1997b) disease scoring on a 1 - 9 scale. Disease scores were taken at seven, 15 and 21 d after inoculation. Individual plants were classified as resistant (R) with ratings 1 and 3 and susceptible (S) with ratings 5, 7 and 9. Alternatively, plants were categorised into resistant (R) with ratings 1 and 3, moderately resistant (MR) with rating 5, and susceptible (S) with ratings 7 and 9. The data were analysed using the χ^2-statistic to test the goodness of fit of the observed ratio to expected ratios under various genetic models.

5A.3 Results

No plant developed any symptoms in the negative control. This indicated that the casual pathogen of Ascochyta blight did not occur in the surrounding environment of our trial and the disease development on the inoculated plants was due to spraying with the inoculum. The disease symptoms were very similar to those reported by Ahmad *et al.* (1997b). On day 7, disease development was poor and was only sporadically found on a few plants. Thereafter within any group of plants the variability in disease development among the remaining two dates was not significant. Thus, data of any of these two dates could be reliably used for measuring the disease

status of the individual lines. Therefore, the results for 15 after inoculation are used in all later analyses.

5A.3.1 Ascochyta blight reactions of parental cultivars

Table 5A.1 shows the blight reactions of each of the parental lines and cultivars in the order of their observed level of resistance. There was no indication of any lack of trueness to type among the individual plants tested. Thus, the segregation data avoids any potential bias caused by the presence of susceptible plants in resistant parental lines or the presence of resistant plants in the susceptible lines. Indian head, ILL5588 and ILL 5684 were all highly resistant, Laird was moderately resistant and Titore and W6 3192 were susceptible. The reactions among the resistant lines were not exactly the same. Indian head had more plants scored one than ILL 5588 or ILL 5684.

Table 5A.1: Foliar reactions to *Ascochyta lentis* of six lentil cultivars.

Cultivar/lines	Number of plants in different disease rating categories				
	1	3	5	7	9
Indian head	10	10			
ILL5588	5	15			
ILL5684	0	20			
Laird			20		
W6 3192				12	8
Titore				16	4

5A.3.2 Number of major genes

***Inheritance model*:** The first part of the major gene investigation was to examine crosses between resistant and susceptible cultivars (Table 5A.2). The inheritance patterns for the crosses to both susceptible parents (Titore and W6 3192) were entirely

consistent. This confirmed the nature of the pattern. Further discussion will concentrate on the Titore crosses.

In the F_2 progenies of Titore × Indian head, the segregation ratio was close to 1:15 when plants with scores of 1 or 3 were classified as resistant. The backcross with the resistant parent showed a ratio close to 1 resistant: 3 susceptible. In the backcross with the susceptible parents all plants were susceptible. The data suggested that Indian head had two recessive genes for resistance. However, when plants with a score of 5 were separated from other susceptible plants and were regarded as moderately resistant, the segregation ratios in the F_2 progenies were close to 1 highly resistant: 6 moderately resistant: 9 susceptible. Therefore, the data for Indian head were consistent with it containing two recessive genes that conferred moderate resistance. The accumulated effect of these two genes made the plants highly resistant. The segregation in the backcrosses fitted this model well. At this stage to facilitate the later determination of allelic relationships between resistance genes, these two recessive genes were designated Abr1 and Abr2, respectively.

In the cross Titore × ILL 5588, when plants with a scores 1 or 3 were classified as resistant, 73 F_2 individuals were resistant and 27 were susceptible. This could be fitted to a 3 resistant: 1 susceptible ratio (Table 5A.2). In the backcross with the resistant parent all plants were resistant whereas in the backcross with the susceptible parent the segregation ratio was closer to 1:1. Taken together these results suggested that ILL 5588 has one dominant resistance gene.

When plants (20) that scored 5 were classified as moderately resistant and plants with scores of 1 or 3 were regarded as highly resistant, the segregation ratio of the F_2 gave a good fit of a model with one dominant gene for high resistance and one dominant gene for moderate resistance. Therefore, apart from the gene for high resistance, there was another gene conferring moderate resistance.

The backcross data supported this model (Table 5A.2). The frequencies of plants with a score 1 in backcrosses and in the F_2, differed from that in the resistant parent ILL 5588. This indicated that minor genes, with increasing effects, might contribute to the resistance, or that a substantial environmental effect might have occurred between the two groups of plants. The direction of the shift was consistent

with the loss of minor genes for resistance in many of the resistant F_2's. The gene for high resistance was designated AbR3 and that for moderate resistance AbR4.

Table 5A.2: Observed segregation patterns of reactions to *Ascochyta lentis* of five lentil crosses, and χ^2 test for the goodness of fit of various inheritance models.

Cross	G[1]	R	MR	S	Expected ratio	P
Titore × Indian head	F_2	7	40	55	1:6:9	0.887
2 additive recessive for HR						
	BC_1	-	-	59	-	-
	BC_2	16	30	13	1:2:1	0.851
Titore × ILL5588	F_2	73	20	7	12:3:1	0.893
1 dominant HR, 1 dominant MR						
	BC_1	28	17	15	2:1:1	0.819
	BC_2	55	-	-	-	-
Titore × ILL5684	F_2	98		30	3:1	0.683
1 dominant HR						
	F_3	87	-	53	5:3	0.848
	BC_1	35		30	1:1	0.535
	BC_2	60	-	-	-	
Titore × Laird	F_2	-	25	70	1:3	0.767
1 recessive MR						
	BC_1	-	-	60	-	-
	BC_2	-	28	42	1:1	0.146
Titore × W6 3192	F_2	39	-	29	9:7	0.855
2 dominant complementary HR						

[1]G = Generation, HR = highly resistant, MR = moderately resistant, S = susceptible.

In the F_2 generation of the cross Titore × ILL 5684, 98 individuals were resistant (with a score of one or three) and 30 were susceptible. This fitted a 3 resistant: 1 susceptible ratio. In the backcross with the resistant parent all plants were resistant whereas in the backcross with the susceptible parent a segregation ratio close to 1:1 was observed. These results suggested that ILL 5684 has one dominant resistance gene. Because no plants were scored 5, there was no gene conferring moderate resistance. The gene in ILL 5684 was designated AbR5 though its relationship to AbR3 still needs to be determined.

Most plants of Laird were scored 5, thus it was regarded as moderately resistant. In the F_2 progenies of Titore × Laird, 25 plants were scored 5 and 70 were scored 7 or 9. This could be fitted to a 1 resistant: 3 susceptible ratio.

The backcross with the resistant parent showed a 1 resistant: 1 susceptible ratio, and in backcross with the susceptible parent all plants were susceptible. These segregation patterns suggested the presence of one recessive resistance gene in Laird and it was designated Abr6. However, the fit of this model was not as convincing as in some of the others. Whether this gene coincides with Abr1 or Abr2 in Indian head needs to be determined.

The F_1 plants of Titore × W 6 3192 were resistant despite the fact that neither parent was resistant. In the F_2 progenies, 39 plants were resistant and 29 were susceptible, which was close to the 9: 7 ratio. Thus, two dominant complementary genes were responsible for the resistance. The gene in Titore is designated as AbR7 and that in W6 3192 as AbR8.

***Allelic relationships*:** In above analysis, eight resistance genes were detected in the six cultivars (lines). However, Abr6 may be allelic Abr1 and Abr2, AbR5 may be allelic to AbR3, AbR7 or AbR8. To determine the relationships of the loci to each other a series of crosses were used and the data are presented in Table 5A.3.

Because a dominant gene complementary to the dominant gene in W 6 3192 is present in Titore, the susceptible cultivar, the presence or absence of this gene in other resistant cultivars needs to be confirmed before the nature of the genes in these cultivars can be determined. Indian head does not contain AbR7 and AbR8 because crosses of Indian head with both susceptible parents have the same segregation pattern

in the F_2 generation. The same argument holds true for Laird. If the AbR5 gene is allelic to AbR7 (AbR8), both AbR7 and AbR8 must be in pre-set in ILL 5684 (because it is a resistant cultivar), and all the progenies of cross Titore × (ILL

Table 5A.3: Observed segregation patterns of reactions to *Ascochyta lentis* of F_2 populations of lentil crosses for testing allelic relationships.

Cross	HR	MR	S	Expected ratio	P
Indian head × Laird	7	68	50	4:33:27	0.810
ILL 5588 × Laird	130	22	10	48:13:3	0.083
ILL5684 × ILL5588	100	0	-	-	-
Titore × (ILL5684 × W6 3192)	87	-	23	3:1	0.322
Titore × (ILL5588 × W6 3192)	57	9	12	6:1:1	0.735

5684 × W6 3192) should also be resistant. This is not supported by the results. Therefore, AbR5 is not allelic to AbR7. Under the condition that AbR5 is independent, if ILL 5684 contained AbR7 or AbR8 one of the crosses to Titore or W 63192 would give the ratio of 57:7, rather than the 3:1 produced. Therefore, ILL 5684 does not contain AbR7 and AbR8. Consequently, AbR5 is an independent locus of AbR7 and AbR8, and ILL 5684 does not contain either of these two genes. Based on the same arguments, AbR3 is independent of AbR7 and Abr8, and ILL 5588 does not contain these two genes. Therefore, the genes conferring high resistance in ILL 5588 and ILL 5684 do not require a complementary gene to express their resistance, and the dominant gene for moderate resistance in ILL 5588 does not show a complementary effect to the gene in Titore.

In the F_2 progenies of ILL 5588 × ILL 5684, no plants were susceptible (scored 7 or 9), indicating that the dominant gene in ILL 5684 (AbR5) is allelic to the dominant gene conferring high resistant in ILL 5588 (AbR3). The F_2 population of

the cross of Laird × Indian head showed a 4 highly resistant: 33 moderately resistant: 27 susceptible segregation ratio. This indicated that the recessive gene in Laird (Abr6) is not allelic to the recessive genes in Indian head (Abr1 and Abr2). In the F_2 progenies of ILL 5588 × Laird, the observed segregation ratio did not fit the expected ratio under the assumption that the genes from them are independent. Because more plants were resistant and less were moderately resistant, the gene in Laird and ILL 5588 may have an additive effect.

5A.3.3 Genetic categorisation of genotypes

Based on the above results, the genotype of the six cultivars (lines) with regards to their foliar resistance to Ascochyta blight were determined as follows: ILL5588 is AbR_3 AbR_4, ILL 5684 is AbR_3, Laird is Abr_5, Indian head is Abr_1 Abr_2, Titore is AbR_6 and W6 3192 is Abr_7. This is where 'Ab' implies Ascochyta blight, 'R' dominant and 'r' recessive.

5A.4 Discussion

The results show that ILL 5588 had two dominant genes, one for high resistance and one for moderate resistance to Ascochyta blight. Ford *et al.* (1999) proposed a one dominant gene model for foliar resistance and showed that this gene accounted about 90 % of total genetic variation using QTL mapping. Fitting the data of Ford *et al.* (1999) in the same way as this chapter indicates that the gene identified by Ford *et al.* (1999) was probably the one for high resistance (data not shown). This highlighted an important issue in inferring an inheritance model for disease resistance. To make segregation analysis feasible it is necessary to set threshold values to group plants in segregating populations. How to select these values depends on the actual situation. A simple way is to use the resistance levels of the parents as a reference. All plants with scores in the range of the resistant parent are regarded as resistant, and all plants with scores in the range of susceptible parent are regarded as susceptible. Plants with scores that do not fall in both ranges of their parents need to be classified

carefully. In the case of ILL 5588 × Titore, regarding plants with a score of 5 as susceptible (as Ford *et al.* (1999) did) resulted in a one dominant gene model. Regarding them as resistant gave a model with two dominant genes for resistance, and assuming they form a unique group with moderate resistance (as in this chapter) gave a model of one dominant gene for high resistance and one dominant gene for moderate resistance. In practice, a score of 5 may be better regarded as moderately resistant in terms of the scoring system and the third option should be adopted.

Andrahennadi (1997) and Vakulabharan *et al.* (1997) proposed a one dominant gene model for seed resistance. Tay (1989) suggested that two dominant complementary genes for resistance and one recessive gene for moderate resistance were responsible for seed resistance in ILL 5588. No recessive gene for moderate resistance was found in ILL 5588 in the present study, although one dominant gene for moderate resistant was detected.

One dominant gene was responsible for the resistance in ILL 5684 and it was allelic to the dominant gene conferring high resistance in ILL 5588. Seed resistance in ILL 5684 was under the control of two dominant complementary genes (Tay, 1989). The resistance level of ILL 5684 was lower than that of ILL 5588. This indicates either an effect of genetic background on gene expression or the two genes in ILL 5588 have an additive effect. The disease scoring system used does not permit determination of the real reason.

Foliar resistance to Ascochyta blight in Laird was moderate in this study, which is consistent with Andrahennadi *et al.* (1997b). Tay (1989) proposed the same inheritance model for seed resistance.

As in ILL 5588, two models could interpret the inheritance of resistance in Indian head. Two recessive genes with additive effect are responsible for resistance when plants scored 5 are separated from susceptible and resistant classes and regarded as moderately resistant. Two duplicated recessive genes are suitable when they were regarded as susceptible. Because the correspondence between foliar rating and the percentage of seed infection used for grouping plants into resistant and susceptible categories was unknown, these results may, or may not, be consistent with the model of two duplicated recessive genes proposed for seed resistance in this cultivar (Andrahennadi, 1994; 1997).

The segregation pattern of the two susceptible parents W6 3192 and Titore confirmed the model of two dominant complementary genes as proposed by Ahmad *et al.* (1997b). However, their generalisation of this model was not supported, since these two genes did not present in all other cultivars tested.

Most previous studies have used seed resistance to detect the inheritance model. The lack of an established relationship between seed resistance and foliar resistance makes it difficult compare our results with other reported results. For instance, some plants susceptible to foliar infection produced no seeds or insufficient seed for testing for seed infection. Plants classified as susceptible based on seed infection may be classified as moderately resistant to foliar infection. However, Tay (1989) showed that all genotypes with low seed infection also had low foliar infection ratings, indicating that the same genes may be responsible for seed and foliar resistance. If this is true, seed resistance should be used to study the genetics of resistance, because it is more objective (using seed infection rate) than foliar rating (using a scaling system). Consequently, it allows more useful comparisons among the results from different authors. Therefore, the establishment of the correspondence between threshold values used to classify genotypes for seed resistance and for foliar resistance is required. Further, whether the resistance genes for foliar resistance identified in this and other studies also confer seed resistance needs to be tested.

CHAPTER 5

MAJOR GENES FOR ASCOCHYTA BLIGHT RESISTANCE IN LENTILS

B. TWO MAJOR GENES CONFER ASCOCHYTA BLIGHT RESISTANCE IN *LENS ORIENTALIS*

Summary

Genetic mechanisms of resistance to Ascochyta blight from wild lentil (*Lens orientalis*) were studied using F_3 families of a cross between the cultivar Titore of the cultivated lentil (*L. culinaris*) and accession W6 3261 of the wild lentil species. The simplest genetic model to adequately fit the data involved two genes, one of which was partially dominant with a large effect (A) and one dominant with less effect (B), which acted additively to confer resistance. One copy of the resistance increasing allele of the A gene is not sufficient for a genotype to be resistant nor are one or two copies of the resistance increasing allele of the B gene alone. For a genotype to be resistant it must have either two copies of the resistance increasing alleles of the A gene or one or two resistance increasing alleles of the B gene plus one resistance increasing allele of the A gene. When fitted to other published data this model was as good as, or better, than alternative models that have been proposed. The importance of sample size was also highlighted. When the sample size is small, the sensitivity of the χ^2 is lower and the confidence ranges of the different genetic models may overlap considerably. In this case, several genetic models may fit the segregation pattern of a generation and model selection based only on the magnitude of the χ^2 was sometimes misleading. Because the sample size required to distinguish two models with similar ratios is very large, it is important to use more segregating populations. Using F_3 has several advantages for inferring inheritance models and is recommended.

5B.1 Introduction

Sources of resistance to Ascochyta blight in wild lentils were identified by Bayaa *et al.* (1994). The genetics of resistance to Ascochyta blight from *Lens orientalis* was first reported by Ahmad *et al.* (1997b). Using F_2 generations, they concluded that the resistance in crosses between resistant *L. orientalis* collections and susceptible *L. culinaris* cultivars was controlled by a single dominant gene. Because the sample size was small and only one type of segregating population was used by Ahmad *et al.* (1997b), the inheritance model they suggested needs to be confirmed. Using the same cross, Parh (1998) found that the one-dominant gene model could be used to interpret his results if F_3 families were classified into non-segregating resistant, non-segregating susceptible and segregating groups. However, this model could not interpret the results from individual plants of segregating F_3 families because the number of susceptible plants was much greater than expected. Parh (1998) did not give a satisfactory explanation for these results.

The objectives of this study were to revisit the genetics of Ascochyta blight resistance from *L. orientalis* using F_3 families and to investigate the effect of sample size on selecting genetic models.

5B.2 Materials and Methods

5B.2.1 Plant material

A segregating F_2 population derived from a single F_1 individual of a cross between a cultivated lentil, *L. culinaris* cv. Titore, and an accession of wild lentil, *L. orientalis*, W6 3261 was used in this study. Eighty F_3 families were obtained by selfing randomly selected F_2 individuals. Each F_3 family plus the parents in the resistance test comprised of 10 plants grown in two, 2 l pots. Bark and sand at a 60: 40 ratio were used as the potting mixture. Pots were arranged randomly on a bench in a greenhouse where maximum and minimum temperatures were 25 °C and 15 °C respectively.

5B.2.2 Isolation of the pathogen, inoculum preparation and pathogenicity test

A pathogenic isolate of *Ascochyta lentis* was obtained from infected seed of cv. Invincible (isolate Rakaia) using the method described in part A of this Chapter. Inoculum was prepared from the 15 d cultures as described in part A of this Chapter. The concentration of the spore suspension was measured using a haemocytometer and adjusted to 7.5×10^4 conidia/ml by dilution. Seedlings with four - five expanded leaves (15 d after sowing) were inoculated with the suspension at 1 ml/seedling using an atomiser. After inoculation, pots were kept in a controlled environment cabinet at 18 °C and high humidity) for 24 h and then moved to a greenhouse bench for disease development to occur.

5B.2.3 Data collection and analysis

Data on disease severity were recorded at four different dates (7, 11, 13 and 15 d after inoculation) using a modified ICARDA disease scoring procedure on a 1 - 9 scale (Table 5B.1).

Table 5B.1: Modified ICARDA disease rating scale (1-9) for lentil Ascochyta blight and equivalent 2-point and 4-point classification.

Classifications		Scale	Disease severity
R[1]	HR	1	No visible lesions
R	HR	2	Very few lesions after careful examination
R	HR	3	Few scattered lesions after careful examination
R	MR	4	Lesions common & easily observed, no defoliation
R	MR	5	Lesions common & easily observed, little defoliation
S	MS	6	Lesions and defoliation common
S	MS	7	Lesions very common, defoliation moderate
S	HS	8	Lesions very common, defoliation high
S	HS	9	Extensive lesions on all plant parts with stem girdling

[1]R = resistant, S = susceptible, H = highly, M = moderately.

Individual plants were classified as resistant with ratings 1 to 5 and susceptible with ratings of 7 to 9. Alternatively, plants were categorised into highly resistant

(HR) with ratings of 1 to 3, moderately resistant (MR) with ratings 4 or 5, moderately susceptible (MS) with ratings 6 or 7, and highly susceptible with ratings 8 or 9.

The data were analysed by using the χ^2-statistic to test the goodness of fit of the observed ratio to the expected ratios under the various genetic models.

5B.3 Results

No plants developed any symptoms in the negative control. This indicates that disease development in the sprayed plants was due to inoculation. Disease symptoms were similar to those reported by Ahmad *et al.* (1997b). The average disease scores of the resistant and susceptible parents at day 15 were 3.33 and 8.00, respectively (Table 5B.2). On day 7, the disease was found sporadically on only a few lines. Thereafter within any group of plants the variability in disease development among the remaining three sample dates was not significant (Table 5B.2). Therefore, the results from day 15 were used in all the later analyses.

Table 5B.2: Ascochyta blight disease ratings for parents and F_3 families from the lentil cross Titore × W6 3261.

	Parental mean		Family mean		
Days	W 63261	Titore	NSR	SEG	NSS[1]
7	2.00 ± 0.73	3.33 ± 2.15	1.71 ± 0.78	2.44 ± 1.41	2.76 ± 1.81
11	2.67 ± 0.76	6.92 ± 1.18	2.49 ± 0.81	4.10 ± 1.66	6.22 ± 1.17
13	3.17 ± 0.94	7.75 ± 1.14	2.73 ± 0.92	4.78 ± 1.71	6.86 ± 1.13
15	3.33 ± 0.90	8.00 ± 1.14	2.93 ± 0.93	4.92 ± 1.67	6.98 ± 1.25

[1] NSR = non-segregating resistant; SEG = segregating; NSS = non-segregating susceptible.

The distribution of disease score of all the tested plants did not fit a normal distribution. There were fewer plants with a score of less than three or more than seven (Figure 5B.1). This indicated that major genes contributed to the resistance in this cross. A similar trend was found when only the plants of the segregating families were used (Figure 5B.2).

Out of the 80 F_3 families, 17 were non-segregating and resistant (all plants were resistant), 38 were segregating (some plants were resistant and some were susceptible) and 25 were non-segregating and susceptible (all plants were susceptible). This ratio can be fitted by 1:2:1 ($P = 0.407$), which would be the expected segregation ratio for one dominant gene. If the analysis was ended here, the "one (dominant) gene model" fitted the data well. However, if instead of using families as the unit of measurement, an analysis was done using individual plant disease scores. The result was far from the expected 5: 3 segregation ratio under the "one gene model" and consequently this model was rejected (Table 5B.3).

To fit the observed segregation pattern properly, a "two gene model" was proposed. This model assumed the existence of one partially dominant gene with a large effect (A) and one dominant gene with less effect (B), which conferred additive resistance. Using this model possession of one increasing allele of gene A would not be sufficient for genotypes to be resistant. The effect of gene B would be so small that the genotypes with one or two increasing alleles of gene B only would be susceptible. However, one increasing allele of gene B would be sufficient to make genotypes with one increasing allele of gene A resistant. Under this "two gene model", the expected segregation ratio of resistant and susceptible in the F_2 plants is 5: 3 (Table 5B.3). The expected ratio of non-segregating resistant, segregating and non-segregating susceptible F_3 families is 1:2:1. The expected ratio of resistant and susceptible F_3 plants is 17:15. The observed segregation pattern fitted these ratios well (Table 5B.2). When plants were further classified into HR, MR, MS and HS, the segregation ratio was very close to the expected ratio of 17:17:21:9 of the "two gene model" (Table 5B.4).

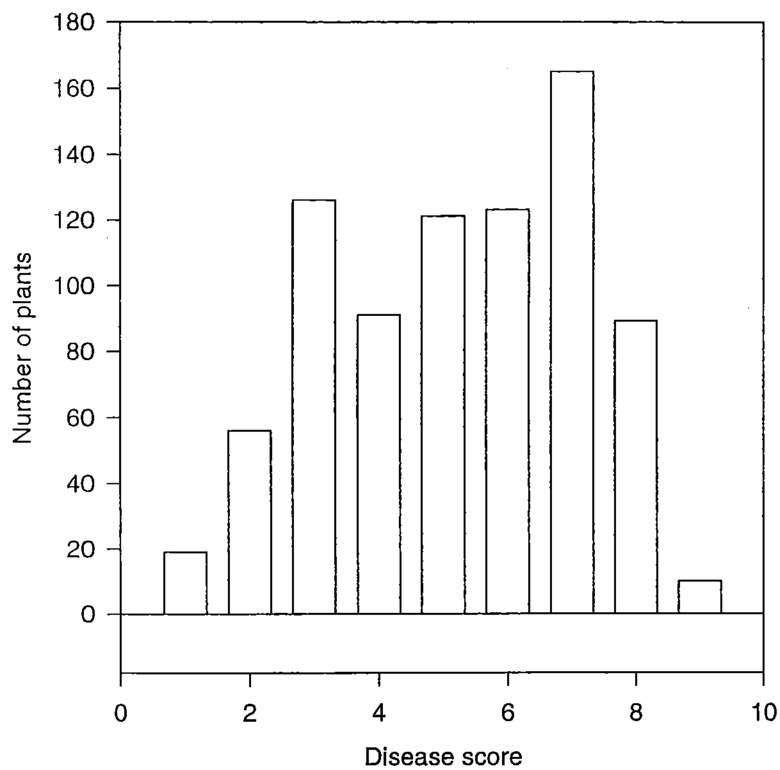

Figure 5B.1. Distribution of disease scores of plants from F_3 families

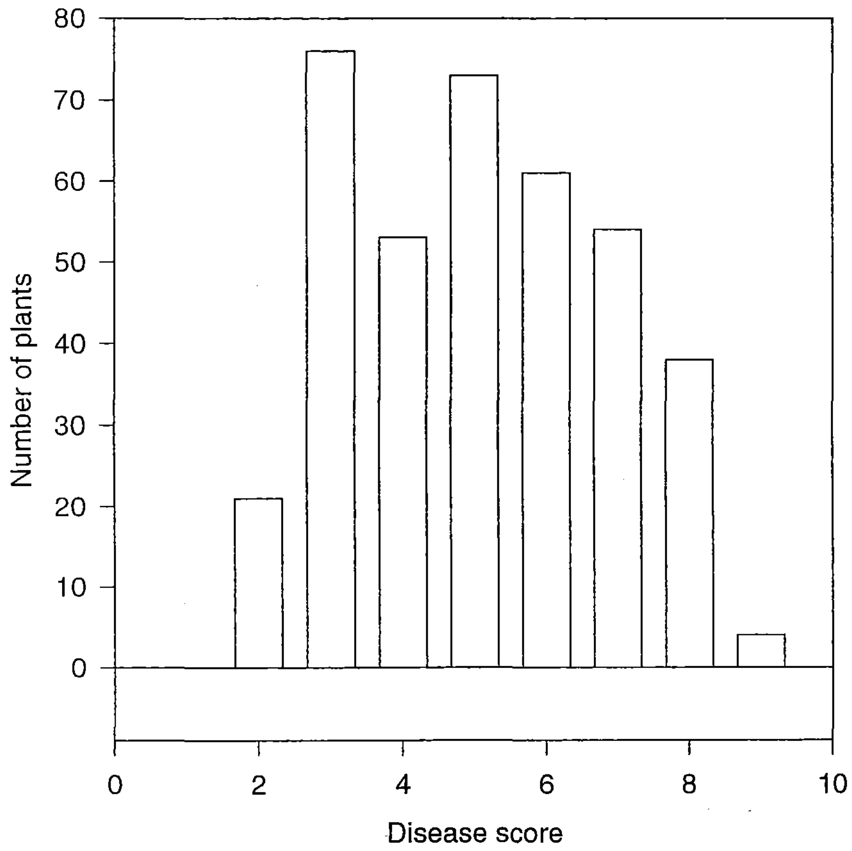

Figure 5B.2. Distribution of disease scores of plants from segregating F_3 Families

Table 5B.3: The segregation of F_2 plants under the proposed "two gene model".

Parents		
W6 3261 (AA/BB)		Titore (aa/bb)
	F_1	
	Aa/Bb(MR)	
	F_2	
1AA/BB (HR)[1]	2Aa/BB (MR)	1aa/BB (MS)
2AA/Bb (HR)	4Aa/Bb (MR)	2aa/Bb (MS)
1AA/bb(MR)	2Aa/bb (MS)	1aa/bb (HS)
Segregation ratio: 5 resistant: 3 susceptible, 3 HR:7 MR:5 MS:1 HS		

[1]See Table 5B.1 for explanation of abbreviations.

Table 5B.4: Observed numbers of F_3 plants in four resistance classes tested against predictions from two genetic models for resistance to Ascochyta blight.

	Resistant		Susceptible		P for χ^2
	HR	MR	MS	HS	(reject if <0.05)
Observed	201	212	288	99	
Expected[1]	212.5 (17)	212.5 (17)	262.5 (21)	112.5 (9)	0.193
Expected[1]	425 (17)		375 (15)		0.604
Expected[2]	500 (5)		300 (3)		<0.000

[1]"two gene model",

[2]"one gene model".

When only plants from the segregating families were used, the observed segregation pattern fitted to the "two gene model" very well. This held whether resistant and susceptible categories were further subdivided. Again the "one gene model" was not supported by the results (Table 5B.5).

Table 5B.5: Observed numbers of F_3 plants from segregating F_3 families in four resistance classes tested against predictions from two genetic models for resistance to Ascochyta blight.

	Resistant		Susceptible		P for χ^2
	HR	MR	MS	HS	(reject if <0.05)
Observed	97	126	115	42	
Expected[1]	83.125 (7)	130.625 (11)	130.625 (11)	35.625 (3)	0.139
Expected[1]	83.125 (7)	130.625 (11)	166.25 (14)		0.224
Expected[1]	213.75 (9)		166.25 (7)		0.339
Expected[2]	285 (3)		95 (1)		0.000

[1] "two gene model"

[2] The model of one dominant gene and one recessive gene.

To cross-validate the two-gene model, data obtained by Parh (1998) using the same cross was analysed. The goodness of fit was excellent as indicated by the P values (Table 5B.6). Therefore, the proposed "two gene model" for Ascochyta blight resistance is appropriate.

5B.4. Discussion

For the F_3 families used in this study both the proposed "two gene model" and the "one gene model" suggested by Ahmad *et al.* (1997b) had the same expected non-segregating resistant, segregating and non-segregating susceptible segregation ratios (1:2:1). If all the segregating F_3 families came from resistant F_2 plants, the one-gene model could be correct. However, if some of the segregating F_3 families came from susceptible F_2 plants the "one gene model" would be rejected. Therefore, taking an F_3 family as the observation unit does not provide enough information to give a firm conclusion.

Table 5B.6: Fitness of the two genetic models to the segregation patterns of the F_3 data of Parh (1998).

Plants from all F_3 families					
	Resistant		Susceptible		P for χ^2
	HR	MR	MS	HS	(reject if <0.05)
Observed	76	88	112	49	
Expected[1]	86.3 (17)	86.3 (17)	106.6 (21)	45.7 (9)	0.620
Expected[1]	172.7 (17)		152.3 (15)		0.336
Expected[2]	203.1 (5)		121.9 (3)		0.000
Plants from segregating F_3 families					
Observed	44	70	74	20	
Expected[1]	45.5 (7)	71.5 (11)	71.5 (11)	19.5 (3)	0.981
Expected[1]	117 (9)		91 (7)		0.675
Expected[3]	156 (3)		52 (1)		0.000

[1] "two gene model"

[2] "one gene model"

[3] one dominant gene and one recessive gene model.

If the "two gene model" is correct, it should also fit the resistance and susceptibility ratios of the F_1 and F_2 generations. The F_2 data reported by Ahmad *et al.* (1997b) was fitted to the "two gene model" using the χ^2 test. This model was rejected at a probability of 0.037 (Table 5B.7). However, only 48 F_2 plants were used by Ahmad *et al.* (1997b) and misclassification of a single susceptible plant as resistant would have lead to non-rejection of the "two gene model" (Table 5B.7). Moreover, there is a data range (grey range) within which neither model can be accepted or rejected. Another two gene model (one dominant and one recessive gene) with a 13: 3 resistance and susceptibility ratio in the F_2 was even more difficult to reject using

Ahmad *et al.*'s (1997b) data. However, this model was rejected using individual plant disease scores (Table 5B.4).

Table 5B.7: Sensitivity analysis of F_2 data reported by Ahmad *et al.* (1997b).

Resistant	Susceptible	P One-gene model (3:1)	P Two-gene model (5:3)	P Other model[1] (13:3)
37[2]	11	0.739	0.037	0.460
36	12	1.000	0.074	0.267
35	13	0.739	0.136	0.139
34	14	0.505	0.233	0.064

[1]"one dominant gene and one recessive gene"

[2]published data.

In order to distinguish among genetic models with close segregation ratios using one segregating generation, a large sample size is required. A simulation indicated that more than 200 F_2 plants were required to unequivocally distinguish the "one gene model" and "two gene model". The difficulty in getting F_1 seeds in lentil inter-specific crosses makes this sample size for the F_2 generation formidable. Micropropagation (e.g. by the method of Chapter 3) of limited F_1 material by *in vitro* culture could be used to enlarge the F_1 population and hence the F_2 population.

Alternatively, other generations with different segregation ratios can be used to reveal the genetic model more accurately. Examples commonly used are backcrosses (difficult in this situation) and F_3 plants. Use of the F_3 generation has the advantages of:

1. being analysable in three different ways,
2. using the average disease scores to represent the performance of the source F_2 plants,
3. having the ability to easily increase sample size.

However, the sample size of the F_3 family must be large enough to avoid misclassifying segregating families as non-segregating ones. Hanson (1959) demonstrated that 11 is the minimum family size to detect segregation for a dominant gene. In this study, the failure to detect segregation would imply that more F_3 families were classified as non-segregating resistant if the one-gene model were correct. This is the opposite of these results. Thus the small F_3 family sample size that was used (n = 10) could have made it harder to reject the "one gene model" if some segregating families were misclassified. Therefore, it does not reduce our confidence in the "two gene model".

The F_1 hybrid would be less resistant than the resistant parent under the "two gene model". Ahmad *et al.* (1997b) tested the resistance of a single F_1 plant and did not find any disease symptom, therefore the F_1 seemed to be highly resistant. This inconsistency may have been caused just by chance escape. More plants are required to accurately measure the resistance of the F_1 generation.

Under the "two gene model", F_3 families from F_2 individuals with genotypes AA/Bb, Aa/BB and aa/Bb are segregating and all nine possible F_2 genotypes can be detected if four categories of resistance level are used instead of two to classify the F_3 families. Theoretically, individuals with genotypes homozygous for each gene (i.e. AA/bb and aa/BB) can be identified, provided the number of plants per family is large enough. These two genotypes can be crossed to the original homozygous recessive parent (aa/bb) to verify the assumptions about their effects. They can also be intercrossed to test the assumption about their relationship.

CHAPTER 6

GENETIC VARIATION FOR MINOR GENES CONDITIONING ASCOCHYTA BLIGHT RESISTANCE IN LENTILS

Summary

In addition to a major gene, minor genes were found to contribute to the genetic regulation of foliar resistance to Ascochyta blight in two crosses between two resistant and one susceptible lentil cultivars (lines). This was established by comparing inbred lines homozygous for the major resistance gene generated from the two crosses. Although the effects of minor genes were not large enough to change phenotypic performance determined by its major genotype qualitatively (from resistant to susceptible, or from susceptible to resistant) based on the measurement scale used, they substantially and significantly modified the level of resistance.

The major gene for foliar resistance was linked to the gene(s) for resistance to seed infection or had a positive pleiotropic effect on resistance to seed infection. Similarly, the major gene for foliar resistance was linked to gene(s) for seed yield/plant in disease free environments and/or had a negative pleiotropic effects on the seed yield/plant. Selection for resistance and yield among inbreds with the same major resistance gene may be necessary to enhance the resistance level, and to reduce the negative effect on yield of the major resistance gene.

6.1 Introduction

As an efficient means to control Ascochyta blight in lentil, breeding for resistance has been the objective of several international and national breeding programs (Erskine *et al.*, 1994). A good understanding of the genetics of Ascochyta blight resistance in lentil is required to obtain maximum returns from these programs.

All reported studies, and Chapter 5 of the present study, have used segregation analysis to detect the genetic mechanisms of resistance and several inheritance models of major gene(s) were proposed. Analysing resistance as a qualitative trait implies that the variations found within resistant and susceptible classes are totally attributable to environmental effects. However, the presence of different resistance levels within the resistant and susceptible classes suggests that minor genes may also contribute to the resistance. Evidence supporting the role of minor genes in conditioning Ascochyta blight resistance would be useful in developing breeding methodologies aimed at further increasing the resistance level in lentil. For instance, accumulating the effects of minor genes will provide a buffer against possible changes in pathogen virulence. This buffer is required to if durable resistance is to be admired.

It has been well documented that minor genes contributing to disease resistance mainly controlled by major gene(s) such as resistance to southern corn *rust (Puccinia polysora)* and maize streak virus in maize (*Zea mays*) (Rodier *et al.*, 1995; Holland *et al.*, 1998), resistance to the P3 isolate of phytophthora blight (*Phytophthora drechsleri*) in pigeonpea (*Cajanus cajan*) (Gupta *et al.*, 1997), and resistance to apple (*Malus sylvestris*) scab fungus (*Venturia inaequalis*) (Patocchi *et al.*, 1999). The combined effects of these major and minor genes have been suggested as a means of obtaining durable resistance. For instance, Pretorius *et al.* (1994) suggested that Lr34 and Sr2, genes for resistance to leaf rust (*Puccinia recondita* f.sp. *tritici*) and stem *rust (Puccinia graminis* f.sp. *tritici*) of wheat (*Triticum aestivum*), respectively, (thought to be major genes), in concert with minor genes conditioned durable resistance.

The objectives of the study reported in this chapter were;

1. to investigate the contribution of minor genes to Ascochyta blight resistance in lentil lines where resistance was mainly conditioned by major genes and;
2. to study the association between the major resistance gene and genes for seed yield.

6.2 Materials and methods

6.2.1 Plant materials

The resistant inbred ILL 5684 (*Lens culinaris*) and W6 3241 (*L. orientalis*) were crossed to the susceptible inbred Titore (*L. culinaris*) to create two populations which were segregating for Ascochyta blight resistance. The line ILL 5684 was the most resistant available and W6 3241 was selected to represent resistance sourced from a wild lentil relative. The *in vitro* micropropagation methods developed in Chapter 3 were used to obtain large number of F_1 plants.

The F_1 plants were selfed to produce F_2 plants in a glasshouse at Lincoln University. One hundred seeds collected from the F_1 plants of each cross were sown in a glasshouse for a foliar resistance test. A total of 160 seeds from F_1 plants of each cross were sown *in vitro* for micropropagation (Chapter 3). Therefore, clones of genetically true to type individual plants of each F_2 seed line were obtained. The resultant plants were selfed to produce F_3 families.

From each F_3 family 15 plants were tested for foliar resistance and the results were used to classify F_2 individual clones into non-segregating resistant, non-segregating susceptible and segregating classes. A total of 25 non-segregating resistant and 25 non-segregating susceptible individual F_2 clones were randomly selected from each cross and were advanced to the F_5 generation using a modified single seed decent method. The seeds of each F_5 line were harvested in bulk to produce 25 lines of each of four sets of inbred lines (Titore x ILL 5684: resistant and susceptible sets; Titore x W6 3241: resistant and susceptible sets) for subsequent glasshouse testing.

6.2.2 Experimental design

Each set of inbreds (25 inbred lines) was tested for foliar disease resistance independently due to limited space. A randomised complete block design with three replicates of ten plants/plot (in two pots) was used for all the four tests. Seeds were collected and used for seed infection tests. In addition, another four tests were carried out using the same design to measure the seed yield/plant.

6.2.3. Inoculation and disease rating

Methods for Ascochyta inoculum preparation, and seedling inoculation are the same as those described in Chapter 5. The rating method used was the modified ICARDA method as described in Table 6.1. Plants scored one or three were regarded as resistant.

6.2.4 Seed infection test

Seeds were surface sterilised with 0.6 % NaOCl for 10 min, soaked in sterile filter paper and plated on V_8-juice (Campbell Soups Ltd.) agar media with 10 seeds/petri dish. The plates were incubated at 20 °C under 12 h continuous fluorescent light in an incubator. The number of seeds/inbred varied due to seed availability, but the minimum number was 20. From the day seven after incubation, the number of infected seeds was counted daily until day 21.

Table 6.1: Modified ICARDA 5- point disease rating scale for lentil Ascochyta blight.

Classifications		Scale	Disease intensity
R[1]	HR	1	No visible lesions
R	HR	3	Few scattered lesions after careful examination
R	MR	5	Lesions common & easily observed, but little defoliation
S	MS	7	Lesions very common, defoliation moderate
S	HS	9	Extensive lesions on all plant parts with stem girdling

[1]R = resistant, S = susceptible, H = highly, M = moderately.

6.2.5 Data analysis

The χ^2 test was used to fit the inheritance model of a major gene. The SAS function of general linear model was used to test for differences among the lines. A two-sample "t" test was used to test the difference between resistant and susceptible sets for yield. Genotypic variance and heritability were estimated for each class and cross combination respectively using the method of Hallauer and Miranda (1988).

6.3 Results

6.3.1 Segregation for major gene

In both crosses, the segregation ratios in F_2 generations fitted a dominant genetic model with a major allele for increased resistance at a single locus (Table 6.2). In the F_3 generations, the ratios of homozygous resistant, heterozygous resistant and homozygous susceptible families were also consistent with the one dominant gene model. Therefore, a dominant major gene is responsible for the resistance observed in these two crosses. Whether the major gene in ILL 5684 is the same as the major gene in W6 3241

Table 6.2: Foliar reaction to Ascochyta blight of the F_2 and F_3 plants from two lentil crosses.

Cross/ Parent	Number of F_2 plants						Number of F_3 families			
	Resistant		Susceptible			(3:1) χ^2	HR*	HtR*	HS*	(1:2:1)χ^2
Score	1	3	5	7	9					
Titore × ILL 5684	38	34	10	15	3	0.488	41	79	30	0.361
Titore × W6 3241	34	37	14	8	7	0.356	28	79	42	0.206
Titore	-	-	-	31	9	-	-	-	-	-
ILL 5684	10	30	-	-	-	-	-	-	-	-
W6 3241	28	12	-	-	-	-	-	-	-	-

*HR: homozygous resistant, HtR: Heterozygous resistant, HS: homozygous susceptible.

was not determined. This was because while attempts were made to cross the two lines no seeds were obtained from the cross between ILL 5684 and W6 3241.

6.3.2 Minor gene variation for foliar resistance

The frequency of plants which scored one was more in the resistant F_3 families of Titore × ILL 5684 than the frequency found in plants of the resistant parent (ILL 5684). Conversely, this frequency was lower in the resistant F_3 families of the cross between Titore × W6 3241 than the frequency found in plants of the resistant parent (W6 3241; Table 6.2). Similarly, for susceptible groups, the frequency of plants scored 7 or less was more in the F_3 families than in the susceptible parent (Titore) for the Titore × ILL 5684 cross and less than the susceptible parent in the Titore × W6 3241 cross, respectively (Table 6.2).

The average disease severity score of the resistant $F_{5:6}$ set of Titore × ILL 5684 was lower (i.e. more resistant) than the resistant parent, but that of the resistant $F_{5:6}$ set of Titore × W6 3241 was higher (i.e. less resistant) than the resistant parent (Table 6.3). Significant differences among the inbred lines were established for all four sets of inbred lines (Table 6.4).

Table 6.3: Means and ranges of reactions to Ascochyta blight of $F_{5:6}$ inbred lines homozygous for a major resistance gene for two lentil crosses.

		Titore (S)	ILL5684 (R)	W6 3241 (R)	Titore × ILL 5684		Titore × W6 3241	
					R[3]	S[3]	R	S
DS [1]	Mean	7.45	2.50	2.30	1.82	7.66	2.75	8.23
	Range	7-9	1-3	1-3	1-3	5-9	1-4	5-9
	$LSD_{0.05}$	-	-	-	0.23	0.26	0.30	0.25
SI % [2]	Mean		15.5	17.5	13.3	42.5	21.4	40.5
	Range		12.3-16.5	14.5-19.9	10.5-19.5	37.3-56.7	10.5-30.3	37.0-57.4
	$LSD_{0.05}$	-	-	-	2.73	3.26	3.50	3.87

[1]DS = Disease severity.

[2]SI % = Seed infection percentage.

[3]R: Resistant set, S: Susceptible set .

A large number of inbred lines were significantly different from the parents.

Among the resistant inbred lines of Titore × ILL 5684, 17 lines were significantly more resistant and two more susceptible than the resistant parent. The other six did not show significant differences from the parent. Among the resistant inbred lines from Titore × W6 3241, nine lines were significantly more susceptible and five more resistant than the resistant parent, and the other 11 did not show significant differences from the parent. No susceptible inbred lines from either cross were classified as resistant.

In Titore × ILL 5684, the genotypic variance for the set of resistant lines was smaller than that for the set of susceptible lines. In Titore × W6 3241, similar genotypic variances were found for both sets of lines (Table 6.5). The resistant set of Titore × W6 3241 had three times more genotypic variance than that of Titore × ILL 5684. In all four instances, however, genotypic variances were substantially higher than environmental variances.

The estimates of entry-mean heritability were higher in all four sets. As with the genotypic variances, the heritability estimates were higher in the Titore × W6 3241 sets than in the Titore × ILL 5684 sets.

Table 6.4: Analysis of variance of reaction to *Ascochyta lentis* of $F_{5:6}$ inbred lines homozygous for a major resistant gene from two lentil crosses.

			MS			
			Titore × ILL 5684		Titore × L6 3241	
	Source	df	Resistant	Susceptible	Resistant	Susceptible
DS	Between inbred	24	0.324	0.607	0.971	1.060
	Error	48	0.018	0.024	0.031	0.022
SI %	Between inbred	24	15.75	29.12	30.59	61.75
	Error	48	1.75	2.88	2.48	3.52

6.3.3 Seed infection

The resistant sets had significantly lower seed infection rates than the susceptible sets in both crosses (Table 6.3). There was a large range of seed infection

rate in each set of inbred lines (Table 6.3). Significant differences among inbreds were found in all sets (Table 6.4). The estimates of heritability were very similar in size to those for foliar infection (Table 6.5). Inbred lines with much lower seed infection rates than their resistant parents could be selected from both resistant sets. The correlations between foliar rating and seed infection were positive and significant. The correlation coefficients were 0.78 and 0.65 for the resistant sets of Titore × W6 3241 and Titore × ILL5684, respectively.

Table 6.5: Estimates of genetic parameters for reaction to Ascochyta blight using $F_{5:6}$ inbred lines homozygous for a major resistance gene from two lentil crosses.

		Titore × ILL 5684		Titore × L6 3241	
		Resistant	Susceptible	Resistant	Susceptible
DS	V_g	0.102	0.194	0.313	0.345
	V_e	0.018	0.024	0.031	0.022
	H^2	0.85	0.89	0.91	0.94
SI %	V_g	7.00	13.12	14.05	31.68
	V_e	1.75	2.88	2.48	3.54
	H^2	0.80	0.82	0.85	0.90

6.3.4 Associations between the major resistance gene and genes for yield

The two sets of inbred lines from each cross made it possible to detect the associations between major Ascochyta resistance genes and genes for other traits by testing the difference between the two sets for these traits. As could be expected, the resistant set yielded much more than the susceptible set under disease stress in both crosses (Table 6.6). However, in a disease free environment the average yield of the resistant set was significantly lower than that of the susceptible set in both crosses. Inbred lines within a set were variable in yield. The yield ranges of the inbred lines were similar among the resistant and susceptible sets (Table 6.6). The correlations between yield under disease pressure and yield in the absence of disease in the resistant sets were not significant.

6.4 Discussion

A dominant gene conferred resistance to Ascochyta blight in both ILL 5684 and W6 3241. This was consistent with the results of Ahmad *et al.* (1997b), Ford *et al.* (1999) and those presented in Chapter 5. However, at this stage it is not possible to know whether it is the same gene in both cultivars.

It was also found that minor genes contributed to resistance in the two crosses used in this study. Firstly, the disease severity in each set of inbred lines was continuously distributed. Secondly, there were significant differences among inbred lines within each set. Such differences were the results of transgressive segregation arising through combinations of different loci controlling resistance. Thirdly, the genotypic variance of each set was significantly different from zero.

Table 6.6: Mean seed yield/plant (mg/plant) under disease stress and without disease for families of F_5 inbred lines homozygous for a major resistance gene from two lentil crosses.

Environment		Titore × L 5684		Titore ×W6 3241	
		R[1]	S	R	S
Stress	Mean	0.78**	0.31	0.41**	0.16
	Range	0.71 - 0.86	0.23 - 0.42	0.33 - 0.52	0.11 - 0.29
Non-stress	Mean	0.83**	0.98	0.48**	0.64
	Range	0.74 - 0.95	0.84 - 1.12	0.32 - 0.49	0.55 - 0.78

[1]R = resistant, S = Susceptible.

**: The difference between the resistant and susceptible sets was significant at the $p \leq 0.01$ level.

Generally, the effects of the minor genes in the cross Titore × ILL 5684 contribute positively to resistance, while those in the cross Titore × W6 3241

contribute negatively to resistance. Selecting for resistance in the resistant lines is effective (in Titore × ILL 5684) and necessary (in Titore × W6 3241). The minor genes alone did not seem to confer sufficient (lack of) resistance, as indicated by the observation that no lines within the susceptible set could be classified as resistant. Similarly, no lines within the resistant set could be classified as susceptible. The effect of the minor genes was to increase or decrease the effects of the major gene. However, the limited options within the foliar disease scaling method used prevented an accurate quantification of the resistance level and consequently may have underestimated the effects of the minor genes.

To some extent, the scaling system used reflects the level of discrimination needed to evaluate the comprehensive resistance resulting from major genes rather than from minor genes. Although the method is simple, it does not allow the accurate quantification of the genetic control of resistance components, which are more likely to be controlled by minor genes.

Though the major genotypes of the F_2 plants were identified based on foliar disease severity, the resistant set had significantly lower seed infection rate than the susceptible set in both crosses. Consequently, the foliar disease rating and the percentage seed infection rate were positively correlated. This suggests that the major gene for foliar resistance has a pleiotropic effect on seed infection rate or is linked to the genes for seed infection rate. Published results showed that this is not always the case. For example, Andrahennadi *et al.* (1996) showed that cultivars with the same foliar disease rating had very different seed infection rates. Seed infection rates of the cultivars with neighbouring foliar ratings overlapped. Ahmed and Morrall (1996) reported that the cultivar Indian head showed higher foliar infection but lower seed infection than ILL 5684 and ILL 5588. They attributed this to differences in the timing of maturity. The later maturing cultivar (Indian head) may produce new pods after losing infected leaves early in an epidemic. On the other hand, in early maturing cultivars, the pods may be exposed to saprophytic infection. The high correlation between the foliar resistance and seed infection rate found in the present study may be because all of the inbred lines in each set contained the same major gene.

The significantly lower yield of the resistant sets under a disease free environment suggested that the major gene for resistance to Ascochyta blight is linked with genes for reduced yield/plant or has a negative pleiotropic effect on it.

Therefore, selection for resistance conferred by major gene caused an unfavourable yield/plant response under a normal environment. The adverse effect of selection for disease resistance on yield has been reported in ryegrass (*Lolium perenne*) for crown rust (*Puccinia coronata*) resistance (Reheul and Chesquiere, 1996). They reported that a one unit increase in crown rust resistance in perennial ryegrass gave an annual loss of 1.4 % of dry matter yield.

The main objective of breeding for disease resistance is to obtain cultivars with a high level of resistance and consequently a high yield under disease stress. Therefore, yield under disease pressure (stress) is the ultimate measurement of disease resistance. However, an ideal cultivar should yield better both under stress and in normal environments.

From a selection breeding point of view, yield under stress and under normal environments can be regarded as different traits. Because the yield under a stress may not reflect the yield with no stress, both should be used as selection criteria in a breeding program. This requires yield testing in both environments and doubles the resource requirements if they are negatively correlated as was found in present study. However, there was a large genetic variation for yield among the inbred lines in the resistant set. Therefore, selection for high yield among the lines would be an efficient way to reduce the adverse effect of the major resistance gene on yield.

In this study, the effect of a major resistance gene on yield (or other agronomic traits) was easily detected since the genotypes of the major gene could be determined from phenotypic observations. Unfortunately, this may be an exception rather than the rule in disease resistance studies. In most cases, major genotypes cannot be distinguished based on phenotypic observations. To identify major genotypes and reduce unfavourable linkage, neutral markers linked to major gene(s) are urgently required. Recently, Ford *et al.* (1999) identified three RAPD markers linked to an Ascochyta blight resistant gene in ILL 5588.

Only an association between a major gene for resistance and genes for yield/plant was observed in this study. Many other traits are important for the success of a cultivar. In Chapter 9 the effects of the major gene for resistance on plant height and days to flowering are reported.

CHAPTER 7

GENERATION MEAN ANALYSIS OF ASCOCHYTA BLIGHT RESISTANCE IN LENTIL

Summary

Four crosses were investigated using generation mean analysis for reaction to Ascochyta blight in lentil. Genetic mechanisms of both foliar and seed resistance were genotype-dependent. Resistance was dominant to susceptibility in all crosses except for Indian head × Titore. The simple additive-dominant model was suitable only for foliar resistance in the cross ILL 5684 × Titore. Using seed infection rate to measure the resistance, a more complicated gene action was detected. Six basic generations were sufficient for detecting the gene action for foliar resistance. However, more generations were required to detect the gene actions for seed resistance in ILL 5588 and W6 3261. Either different genes were responsible for foliar and seed resistance or the same genes were expressed differently. For foliar resistance, a simple recurrent selection scheme for resistance can be followed to isolate plants with higher resistance from a segregating population derived from crosses between parents containing different resistance genes.

7.1 Introduction

Ascochyta blight caused by *Ascochyta lentis* is a major disease in many lentil production areas (Erskine *et al.*, 1994). Ascochyta blight is favoured by cold and wet environments and occurs on shoots, stems, pods and seeds (Nene *et al.*, 1988, Pederson and Morrall, 1994). It has a considerable effect on seed quality and yield (Morrall and Sheppard, 1981; Gossen and Morrall, 1983; Morrall, 1997). Chemical control was found to be effective (Beauchamp *et al.*, 1986 a; b; Bedi, 1990), but its use is limited because of its high cost. Unlike other major crop plants, lentils are

mainly cultivated in developing countries and on infertile soils. Breeding for improving host resistance holds the greatest potential to reduce the effect of the disease.

To breed for Ascochyta blight resistance, in lentils, it is necessary to identify resistant genetic sources and to have a good understanding of the genetics of the resistance in these resources. Several workers have studied the genetics of Ascochyta blight resistance in lentils and different models have been suggested (Tay, 1989; Tay and Slinkard, 1989; Andrahennadi, 1994; 1997; Ahmad *et al.*, 1997b; Ford *et al.*, 1999). In the present study, a series of experiments have been conducted to investigate different aspects of the genetic mechanisms of Ascochyta blight resistance. Inheritance models of major genes were described in Chapter 5. The contribution of minor genes was confirmed in Chapter 6. However, it is difficult to fully separate the contributions of major genes and minor genes in practice. Therefore, an investigation of the overall genetic effects is helpful in understanding the genetic mechanism of Ascochyta blight resistance. Generation mean analysis has several advantages over other designs for studying the inheritance of a quantitative trait. For instance, the experimental error is relatively small. This makes the experiment size needed to achieve same level of precision smaller relative to other methods (Hallauer and Miranda, 1988). This is important in lentil, because it is difficult to obtain seed from artificial crossing. In addition, the populations evaluated in generation-mean analysis provide materials that can be used in an applied breeding program (Campbell *et al.*, 1997; Coates and White, 1998).

The objective of this chapter is to investigate the inheritance of resistance to Ascochyta blight in four crosses of resistant and susceptible lentil parents using generation mean analysis.

7.2 Materials and methods

7.2.1 Plant materials

Four resistant lentil inbreds (P_1), ILL 5588, ILL 5684, Indian head and W6 3261, were crossed to the susceptible cultivar Titore (P_2) in a glasshouse using the

procedure of Ahmad *et al.* (1995b). The F_1 plants were selfed to produce an F_2 and were then backcrossed to the susceptible parent to produce BC_1 families and to the resistant parent to produce BC_2 families. Six basic generations were tested for foliar resistance in the greenhouse.

7.2.2 Experimental design

The six basic generations of each cross were evaluated separately in a glasshouse using a randomised complete block design with three replications. The number of plants per generation for each plot was not the same (see Table 7.1 for details). Seed was collected separately from each plant and was used for a seed infection test. For the seed infection test seed was only collected from one replicate of the foliar resistance test.

7.2.3 Disease severity testing

For foliar disease infection, inoculum prepared from infected seeds was used at a concentration of 7.5×10^4 conidia/ml (see Chapter 5 for the details). 15 d old seedling were inoculated and kept under a plastic tent for 24 h before they were moved to a shade house. Disease severity was recorded 10 d after inoculation using the modified ICARDA scoring system as described in Table 6.1 (Chapter 6).

For seed infection test, seeds were surface sterilised with 0.6 % NaOCl for 10 min, soaked on sterile filter paper and plated onto V_8-juice (Campbell Soups Ltd.) agar medium in petri dish with 10 seeds/petri dish. The plates were incubated at 20 ^oC under 12 h fluorescent light in an incubator. The number of seeds/plant varied but, the minimum number used was 20. From 7 d after incubation started, the number of infected seeds was counted daily until 15 d.

7.2.4 Data analysis

For foliar infection, a randomised complete block analysis of variance was conducted using the general linear model (GLM) procedure of the SAS statistical package (SAS Institute Inc., 1990). To take account of the heterogeneity of within plot variances, the within plot variance was used as a weighting factor.

The genetic analysis was done in three stages with three different models,

1. the additive-dominance model;
2. six parameter model and;
3. The best parsimonious fit model.

The genetic models were fitted by the weighted least square method as follow

$$Y = CM + \varepsilon$$

Where Y is a k ×1 vector of the observed generation means: M is a p × 1 vector of unknown parameters to be estimated: C is a k × p matrix of known constants relating the generation means to the model parameters, and ε is a k × 1 vector of random errors with covariance matrix D. D = diag (σ^2_1/n_1, σ^2_2/n_2, ...σ^2_k/n_k). σ^2_k and n_k are the variances and sample size for the generation kl, respectively.

The unbiased and constant estimates of σ^2_1, σ^2_2 ...σ^2_k are the corresponding sample variances s^2_1, s^2_2...s^2_k. Let S= diag (s^2_1, s^2_2,..., s^2_k) and N= diag (n_1, n_2, ..., n_k) then the estimated D is

$$\hat{D} = SN^{-1}$$

The least square solution for M is

$$\hat{M} = \left[C'S^{-1}NC\right]^{-1} C'S^{-1}NY$$

The goodness of fit test is given by

$$\chi^2_{k-p} = (Y - C\hat{M})' SN^{-1} (Y - C\hat{M})$$

Using the six-parameter model, the mean, additive (a), dominant (d), the pooled additive × additive (aa), the pooled additive × dominant (ad) and the pooled

dominant × dominant (dd) effects were estimated. The estimated values of the gene effects were tested by t-test. The standard errors of estimated genetic effects are the diagonal elements of the estimated covariance matrix:

$$[C'S^{-1}NC]^{-1}$$

The effects with non-significant t values were deleted and a new model was fitted. If all six genetic effects are significant, there is no way to test the model with the six basic generations. All computations were done using the interactive matrix language (IML) of the SAS program (SAS Institute, 1990).

7.3 Results

7.3.1 Foliar resistance

Generation means and within plot variances are given in Table 7.1. As expected, segregating generations (F_2, BC_1, BC_2) had bigger within plot variances than non-segregating generations (P_1, P_2, F_1). Except for the cross, Indian head × Titore, the deviations of the F_1 from the mid-parent values were towards the resistant parent. This is an indication of dominance of resistance over susceptibility. Analysis of variance showed that the differences among generations were significant in all crosses (Table 7.2).

In the cross, ILL 5588 × Titore, the simple additive-dominant model is rejected by the joint scaling test (Table 7.3), as indicated by the fact that the χ^2 is larger than the table value at the 0.01 significance level. In the six-parameter model, the additive × additive effect (aa) and the dominant × dominant effect (dd) were not significant. The best model was the 'm-a-d-ad' four-parameter model. The genetic effects estimated from this four-parameter model are given in Table 7.3. The additive (a) and dominant (d) effects played an equal role, the additive-by-dominant effect (ad) is smaller. The negative value for 'a' reflected that the resistant parent was regarded as P_1. The negative value for 'd' indicated that resistance was partially or completely

dominant to susceptibility. The negative value for 'ad' indicated that 'ad' contributed to increased resistance, in other words, the additive-by-additive effect was favourable.

Table 7.1: Generation means and standard deviations for four lentil crosses evaluated for reaction to *Ascochyta lentis* [1].

G[1].	Sample size	Titore × W6 3261		Titore × 5684		Titore × 5588		Titore × Indian head	
		DS[2]	SI %[2]	DS	SI %	DS	SI %	DS	SI %
P_1	5	2.12	17.6	3.00	25.7	2.56	15.5	1.89	18.5
		(0.11)	(1.53)	(0.07)	(0.84)	(0.16)	(1.50)	(0.07)	(2.53)
P_2	5	8.02	48.7	8.13	48.7	7.82	48.7	7.76	48.7
		(0.21)	(2.53)	(0.14)	(2.53)	(0.19)	(3.60)	(0.12)	(1.09)
F_1	5	3.27	18.16	4.15	26.9	3.73	16.23	7.50	45.4
		(0.23)	(3.1)	(0.10)	(0.62)	(0.17)	(1.80)	(0.22)	(1.96)
F_2	20	3.85	20.08	4.23	24.2	3.31	17.9	6.73	32.1
		(0.30)	(4.41)	(0.24)	(0.71)	(0.25)	(4.20)	(0.30)	(3.32)
BC_1	15	3.87	17.2	4.33	21.2	3.56	14.36	6.76	38.5
		(0.25)	(4.18)	(0.21)	(1.45)	(0.21)	(4.41)	(0.17)	(3.76)
BC_2	15	7.55	46.88	7.67	30.1	7.27	46.45	7.71	35.5
		(0.29)	(2.47)	(0.25)	(1.37)	(0.26)	(1.53)	(0.28)	(2.42)

[1]G = generation, [2]DS = foliar disease SI% = Seed infection percentage.

Values in brackets are standard deviations.

Table 7.2: Analysis of variance for foliar disease severity of six basic generations of four lentil crosses.

Source	Df	Mean squares			
		Titore × W6 3261	Titore × 5684	Titore × 5588	Titore × Indian head
Generations	5	8.12***	10.53***	6.67***	12.13***
Error	10	0.24	0.35	0.15	0.14

***: $P < 0.001$.

In the cross, ILL 5684 × Titore, the joint scaling tests failed to reject the simple additive-dominant model, indicating that there was nothing else except three parameters, m, 'a' and 'd' to interpret the resistance (Table 7.3). The factor 'd' showed a slightly greater role than 'a'. Resistance is therefore dominant to susceptibility.

In the cross, Indian head × Titore, the simple additive-dominant model is rejected by the joint scaling test (Table 7.3). In the six-parameter model, 'aa' and 'dd' were not significant. The best model was the 'm-a-d-ad' four-parameter model. The estimates of genetic effects from this parsimonious model indicated that 'a' and 'd' had similar effects, and 'ad' was about five times more important than 'a' and 'd'. The positive value for 'd' indicated that resistance is partially or completely recessive to susceptibility. The positive value for 'ad' indicated that it contributes to reduced resistance.

In the cross, W6 3261 × Titore, the joint scaling test rejected the simple additive-dominant model (Table 7.3). In the six-parameter model, 'ad' and 'dd' were not significant. The estimates of genetic effects from the parsimonious model (m-a-d-aa) indicated that 'a' was the most important, and resistance is dominant to the susceptibility. The factor 'aa' is favourable for resistance. However, the significant χ^2 suggested that this model was not a good fit of the observed pattern.

Table 7.3: Weighted least square estimates and standard deviations of genetic parameters for reaction to *Ascochyta lentis* in four lentil crosses evaluated for foliar disease severity.

Model	Effect	Titore × W6 3261	Titore × 5588	Titore × 5684	Titore × Indian head
Additive-dominant	m	5.25**	5.12**	5.35**	4.90**
	Overall mean	(0.11)	(0.11)	(0.08)	(0.07)
	a	-3.07**	-2.53**	-2.55**	-2.71**
	Additive effect	(0.11)	(0.11)	(0.08)	(0.07)
	d	-1.37**	-2.33**	-2.83**	3.39**
	Dominant effect	(0.24)	(0.21)	(0.13)	(0.19)
	χ^2	50.28**	7.47*	0.337	336.9**
Six-parameter	m	-2.37 ns	5.95**	4.71**	7.29**
		(1.43)	(1.23)	(1.19)	(1.37)
	a	-2.95**	-2.63**	-2.55**	-2.95**
		(0.12)	(0.12)	(0.08)	(0.07)
	d	19.20**	-5.23**	-1.33 ns	-2.23**
		(3.37)	(2.91)	(2.80)	(3.13)
	aa	7.44**	-0.76 ns	0.64 ns	-2.34 ns
	a-by-a effect	(1.43)	(1.20)	(1.18)	(1.37)
	ad	-1.46**	1.70*	-0.18 ns	11.5**
	a-by-d effect	(0.81)	(0.72)	(0.68)	(0.68)
	dd	-13.60**	2.18 ns	-0.86 ns	2.26 ns
	d-by-d effect	(2.03)	(1.75)	(1.67)	(1.85)

Table 7.3 (Continued)

Model	Effect	Titore × W6 3261	Titore × 5588	Titore × 5684	Titore × Indian head
Best	m	6.41**	5.12**	5.35**	4.96**
		(0.57)	(0.11)	(0.08)	(0.07)
	a	-3.04**	-2.62**	-2.55**	-2.96**
		(0.12)	(0.12)	(0.08)	(0.07)
	d	-2.78**	-2.36**	-2.83**	2.47**
		(0.72)	(0.21)	(0.13)	(0.20)
	aa	-1.22*	-		
		(0.59)			
	ad		1.46*		11.4**
			(0.71)		(0.63)
	dd				
	χ^2	45.95**	3.209	0.337	3.620

ns: $P > 0.05$, *: $0.01 < P \leq 0.05$, **: $P \leq 0.01$.

7.3.2 Seed infection

The generation means and their standard deviations are given in Table 7.1. As with foliar disease severity, the deviations of F_1 from the mid-parent values were towards the resistant parent in all crosses except Indian head × Titore. This was an indication of dominance of resistance over susceptibility in these three crosses. The reverse was true for Indian head × Titore.

The joint scaling test rejected the simple additive-dominant model in all four crosses (Table 7.4). In the crosses, ILL 5588 × Titore and W6 3261 × Titore, all effects were significant in the six-parameter model. However, the estimates of 'd' were large and positive. This was not acceptable since the resistance was clearly dominant over susceptibility. The best model, which gave the smallest χ^2 value, was

the 'm-a-d-ad' four-parameter model (Table 7.4). The inheritances in these two crosses were too complicated to be resolved using the six basic generations.

In the cross ILL 5684 × Titore, 'aa' and 'ad' were not significant in the six-parameter model. The best parsimonious model was the 'm-a-d-dd' model. In this model 'd' and 'dd' showed a much greater role than 'a'. Resistance is dominant to susceptibility. The presence of 'dd' results in reduced resistance. The opposite signs of 'd' and 'dd' suggested that the inter-loci interaction was of a complementary type (Table 7.4).

In the cross, Indian head × Titore, 'dd' was not significant in the six-parameter model. The estimates of genetic effects from the best parsimonious five-parameter

Table 7.4: Weighted least square estimates and their standard deviations of genetic parameters for reaction to *Ascochyta lentis* in four lentil crosses evaluated with seed infection rate.

Model	Effect	Titore × W6 3261	Titore × ILL 5684	Titore × ILL 5588	Titore × Indian head
Additive-dominant	m	33.4**	30.9**	33.7**	33.5**
		(1.42)	(0.89)	(1.48)	(1.30)
	a	-17.0**	-15.5**	-19.2**	-14.1**
		(1.41)	(0.96)	(1.48)	(1.30)
	d	-10.1**	-5.88**	-15.2**	8.85**
		(3.06)	(1.21)	(2.81)	(2.31)
	χ^2	24.617**	78.590**	30.106**	26.108**
Six-parameter	m	-14.7 ns	31.4**	-17.9 ns	14.0ns
		(20.19)	(5.07)	(15.83)	(16.08)
	a	-15.5**	-11.5**	-16.6**	-15.1**
		(1.48)	(1.33)	(1.60)	(1.37)
	d	106**	-24.3 ns	109.0**	41.0ns
		(46.07)	(13.85)	(38.63)	(38.04)

Table 7.4 (*Continual*)

Model	Effect	Titore × W6 3261	Titore × ILL 5684	Titore × ILL 5588	Titore × Indian head
	aa	47.8**	5.8 ns	50.0**	19.6 ns
		(20.14)	(4.90)	(15.75)	(16.02)
	ad	-28.3**	5.2 ns	-31.0**	36.2**
		(10.15)	(4.8)	(10.02)	(9.36)
	dd	-73.4**	19.8 *	-75.0**	-9.6 ns
		(27.12)	(8.96)	(23.76)	(22.79)
Best	m	33.7**	37.8**	32.4**	20.3**
		(1.42)	(1.19)	(1.51)	(5.95)
	a	-15.3**	-10.7**	-16.7**	-15.1**
		(1.47)	(1.11)	(1.59)	(1.37)
	d	-13.2**	-41.7**	-15.4**	25.3**
		(3.15)	(4.28)	(2.82)	(7.01)
	aa			-	13.4*
					(6.20)
	ad	-38.3**		-41.8**	34.9**
		(9.23)		(9.43)	(8.81)
	dd	-	30.7**	-	-
			(3.52)		
	χ^2	7.383**	2.483	10.470**	0.180

ns: P>0.05, *: $0.01 < P \leq 0.05$, **: $P \leq 0.01$.

model (m-a-d-aa-ad) indicated that 'ad' was the most important effect followed by 'd', while 'a' and 'aa' had least effect and were similar. The susceptibility is over-

dominant over the resistance (Table 7.4). Both types of epistasis result in reduced resistance.

7.4 Discussion

Both additive and dominant effects were important for the resistance to Ascochyta blight in the four genotypes evaluated by foliar disease severity and seed infection rate. Dominance favoured resistance in all crosses except Indian head × Titore.

The relative importance of the different gene effects was genotype-dependent. For foliar disease severity, the additive and dominant effects were similar in all four crosses. For seed infection rate, the dominant effect played a more important role than the additive effect in crosses, ILL 5684 × Titore and Indian head × Titore. The relative importance could not be determined for the crosses, ILL 5588 × Titore or W6 3261 × Titore, since no suitable models were fitted.

A single dominant gene model was previously suggested for the foliar resistance of ILL 5588 and W6 3261 (Ahmad *et al.*, 1997b; Chapter 5). In the present study significant epistatic effects ('aa' for ILL 5588 × Titore and 'ad' for W6 3261 × Titore, respectively) were detected. Therefore, the genetic mechanisms underlying the resistance to seed infection are more complicated. Two-gene models for foliar resistance were proposed in Chapter 5. Two dominant genes, one for high resistance and one for moderate resistance were proposed as being responsible for foliar resistance in ILL 5588. The resistance of W6 3261 was under the control of one partially dominant gene with a large effect and one dominant gene with less effect. Because generation mean analysis assumes that all genes have the same effects, these two-gene models could not be confirmed directly by this study. However, it was clear that more than one gene is responsible for the resistance.

For seed resistance, Andrahennadi (1994; 1997) and Vakenlabharanam *et al.* (1997) proposed a single dominant gene model. The present results did not support this simple model. Tay (1989) suggested that two dominant genes and one recessive gene conferred seed resistance to ILL 5588 using small F_2 populations.

For the cross, Indian head × Titore, 'ad' effect was the only significant inter-loci interaction effect detected for foliar disease severity, while both the 'aa' and 'ad' - effects were significant for the seed infection rate. For both the foliar disease severity and the seed infection rate, resistance was recessive to susceptibility. Two recessive genes with additive effects were suggested for the foliar resistance of Indian head (Chapter 5), and two duplicated recessive genes for seed resistance (Andrahennadi, 1994; 1997). The predominant epistatic effect found in the present study partially supported Andrahennadi's results.

For the cross, ILL 5684 × Titore, the simple additive-dominant model was sufficient to model the inheritance of foliar disease severity, while the 'dd' effect was required for seed infection rate. This is partially consistent with previous results. For instance, a single dominant gene model for foliar resistance (Chapter 5), and two dominant complementary genes for the seed resistance (Tay, 1989) were previously suggested for ILL 5584.

If parents used in a cross are in the association phase, the dominant effect is always smaller than the additive effect. However, if parents are in the dispersion phase, the estimate of the dominant effect is always higher than that of the additive effect (Mather and Jinks, 1982). According to this, Titore, the susceptible cultivar, had the genes for seed resistance in Indian head × Titore and ILL 5684 × Titore, and genes for foliar resistance in ILL 5684 × Titore. The presence of a dominant gene in Titore was reported by Ahmad *et al.* (1997b) and in Chapter 5.

The inheritance models of foliar disease severity were simpler than that of seed infection in all the crosses used. This may be an artefact of the measurement scale. It is well known that the results of generation mean analysis is scale-dependent. Therefore, it could not be expected that the same gene action would be found for both foliar and seed resistance, even if the same genes and gene actions were responsible for both foliar and seed resistance. In addition, highly susceptible plants produce few or no seeds and consequently the sample sizes for susceptible plants were smaller than those for resistant plants and this may have reduced the precision of the seed infection test.

The presence of dominance and unfixable epistasis in Ascochyta blight resistance suggests that selection for resistance would be more effective if the dominance and epistatic effect were reduced after a few generations of selfing.

CHAPTER 8

AN ANALYSIS OF ASCOCHYTA BLIGHT RESISTANCE IN LENTIL CROSS ILL 5588 × TITORE USING A MIXED-GENETIC MODEL

Summary

The inheritance of resistance to Ascochyta blight in lentil (*Lens culinaris* Medikus) was studied using the cross of ILL 5588 × Titore. Foliar disease severity and seed infection rate were used to measure the resistance. The inheritance model was determined by using a recently proposed procedure for analysing mixed inheritance of major genes and polygenes. Foliar resistance was controlled by two additive-dominance-epistasis genes. Both genes were from the resistant parent ILL 5588, and dominance was towards resistance. Of the four types of epistasis, only the additive A and dominant B was towards resistance.

Seed resistance as measured as seed infection rate was controlled by one dominant major gene and additive-dominance-epistasis polygenes. The major resistance gene and most of the polygenes for resistance were also from the resistant parent, and the dominance was toward resistance. The additive – by -additive component of the interloci interaction of polygenes favoured resistance, while the additive–by-dominant, and the dominant–by-dominant components were favoured susceptibility. More than 70 % of the phenotypic variation in segregating populations was due to the major gene.

8.1 Introduction

Ascochyta blight is one of the most serious lentil diseases in the main production countries (Erskine *et al.*, 1994). Great economic losses have been recorded but no

efficient control method is available. Breeding for host resistance has good potential to control the disease (Erskine *et al.*, 1994; Ye *et al.*, 2000). Several authors have studied the genetic regulation of Ascochyta blight resistance in lentil and consequently several major resistance genes were identified (see Chapter 5). In Chapter 6, it was shown that both major and minor genes are involved in the determination of Ascochyta blight resistance in two lentil crosses between resistant and susceptible cultivars. Generation mean analysis suggested that the inheritance of Ascochyta blight is more complicated than indicated by classical segregation analysis (Chapter 7).

When both major and minor genes are responsible for the genetic determination of a trait, the inheritance of this trait is said to be complex inheritance or mixed major gene and polygene inheritance (Edwards, 1960; Elston and Stewart, 1971; Morton and McLean, 1974). Classical quantitative genetic methods are not appropriate for analysing mixed inheritance, because they are based on the assumption of many genes with equivalent minor effects (Mather and Jinks, 1982; Falconer, 1989). Therefore special methods have been developed to analyse mixed inheritance. Complex segregation analysis was developed by geneticists studying human and animals to determine the underlying genetic structure of a trait using pedigree (Elston and Stewart, 1971; Morton and McLean, 1974; Cannings *et al.*, 1978; Bonney, 1984; Hosechele, 1988; Elston, 1993). Though this form of analysis is mainly used in human genetic studies, it is also applicable to any diploid cross-pollinated plant species for which it is feasible to maintain a pedigree (Tourjee *et al.*, 1994; 1995). Elkind and Cahaner (1986) proposed a method based on the mixed linear model methodology. This model requires F_3 families derived from F_2 plants heterozygous for a single major gene and consequently the major genotypes of an individual should be known. If this is the case, the effects of the major gene, polygenes and their interaction can be determined.

In Chapter 6, inbred lines homozygous for a major resistance gene were used to confirm the presence of minor genes. The advantage of this approach lies in its simplicity of data analysis. The standard analysis of variance was used to partition total variation into the variation due to genetic effects and that due to environmental effects. Another advantage of this analysis is that the associations between the major gene for resistance with the genes for other traits can be easily tested by comparing the differences

between the means of two sets of inbred lines with and without the major gene for resistance, respectively. However, as with Elkind and Cahaner's (1986) method, this method can only be used when the major genotypes of the F_2 individuals can be easily determined. Without neutral markers tightly linked to major gene, it is always possible that the major genotypes can be incorrectly determined. Consequently, these two methods may under - or over - estimate the effects of minor genes.

Recently, a procedure based on the analysis of the six basic generations of a cross between two inbred lines has been developed to analyse mixed-inheritance models (Wang and Gai, 1997; Gai and Wang, 1998). This procedure is a combination of the conventional generation mean analysis and the maximum likelihood method for parameter estimation. To use this procedure, it is not necessary to know the genotypes of the major gene. Instead, they are inferred from the best - fit model. Moreover, the genetic parameters for both major genes and minor genes can be estimated based on the best - fit model.

The purpose of the work reported in this chapter was to produce an inheritance model of Ascochyta blight resistance in the cross ILL 5588 × Titore using this mixed-inheritance model, and to determine the relative effects of major genes and minor genes as contributing factors to this variation.

8.2 Materials and methods

8.2.1 Plant materials

The Ascochyta blight resistant lentil cultivar ILL 5588 (P_1) was crossed with the susceptible cultivar 'Titore' (P_2), in a glasshouse using the procedure of Ahmad *et al.* (1997b). The crosses BC_1 and BC_2 were produced by backcrossing the F_1 with Titore and ILL 5588, respectively. The F_2 population was produced by selfing the F_1 plants. Twenty plants of each parental cultivar and the F_1, 40 plants of BC_1, 80 plants of BC_2 and 120 plants of the F_2 were inoculated with *Ascochyta lentis* in the glasshouse to evaluate

their foliar resistance. Seeds were collected separately from each plant and used to test seed infection by the pathogen.

8.2.2 Inoculation procedure

A pathogenic isolate of *Ascochyta lentis* was obtained from infected seed of cv. Invincible (isolate Rakaia). A random sample of infected seeds were surfaced sterilised in 0.6 % NaOCl for 10min, soaked in sterile filter paper and plated on V8-juice (Campbell Soups Ltd.) agar medium with five seeds per plate. After 7 d of growth under near ultraviolet light at 18 °C on a laboratory bench seeds which had produced a characteristic diurnal colonies of A. *lentis* were selected and a small plug of these colonies was transferred onto fresh media for further growth and colonisation. Inoculum was prepared from 15 d old cultures, grown under similar conditions as those for the seed plates. Spores were washed from the culture plates using distilled water and filtered through a muslin cloth. Two drops of Tween 20 were added per 100 ml. The concentration of the spore suspension was measured using a haemocytometer and adjusted to 7.5×10^4 conidia/ml by dilution. Seedlings with four - five expanded leaves (15 d after sowing) growing in pots were inoculated with the suspension (1 ml/seedling) using an atomiser. After inoculation, pots were kept in a controlled environment cabinet (18 °C and high humidity) for 24 h and then moved to a green house bench for disease development to occur.

8.2.3 Foliar disease rating

Foliar disease severity was rated using a modified ICARDA 1 - 9 scoring system as described in Table 6.1 (Chapter 6).

8.2.4 Seed infection rate

Seeds were surface sterilised with 0.6 % NaOCl for 10 min, soaked in sterile water on filter paper and plated on V_8-juice agar medium with 10 seeds per petri dish. The plates were incubated at 20 °C under 12 h fluorescent light 12 hr in an incubator.

The number of seeds/plant varied but the minimum number used was 20. From the seventh day after incubation, the number of infected seeds was counted daily until day 21.

8.2.5 Data analysis

The procedure described by Gai and Wang (1998) was used. Briefly, this procedure consisted of three steps. First, the segregating populations were assumed to consist of component distributions controlled by a major gene and modified by both polygenes and the environment. With this assumption the distributions of the six basic generations were formulated. Second, the complete likelihood function of all observations from the six basic generations was constructed based on these distributions. The component parameters were then estimated by maximising this likelihood function via the expectation maximum algorithm. The best-fitted model was chosen based on Akaike's Information Criterion (AIC value) and tests for goodness of fit. Third, the genetic parameters (gene effects, genetic variances and heritability of the major gene and minor genes were estimated by the use of the relationships between the component parameters (Appendix Table 8A.1, 8A.2) and the genetic effects in a manner similar to the generation mean analysis.

In this procedure, the genetic effects of the minor genes and the effects of the environment for segregating populations are assumed to follow a normal distribution, and the variances within P_1, P_2 and F_1 are the same. In addition, in each segregating population, the variances within each major genotype group are the same. The genetic models tested in this study are given in Table 8.1 with the corresponding number of component distributions and number of independent parameters (first-order genetic parameter and the second-order parameters) to be estimated.

8.3 Results

8.3.1 Foliar resistance

The frequency distribution of foliar disease severity is given in Table 8.2. The F_1 and BC1 populations tended towards resistance. In the populations F_2 and BC_2, both resistant and susceptible plants were present. Therefore, major gene(s) are involved in the disease reaction in this cross. However, the resistance performance in the F_1 and BC1 was not the same as in the resistant parent, and new categories appeared in the BC2 and F_2 populations.

The observed disease severities of the six generations were used to fit a series of genetic models using the maximum likelihood method. The AIC value and maximum likelihood estimates of the mean and variance parameters under each model for foliar disease severity and seed infection rate were given in Table 8.3. Based on the AIC value, the best model for describing the inheritance of foliar resistance to Ascochyta blight was B1, and the second and third were B2 and B3, respectively. Therefore, the inheritance of foliar resistance observed in the cross of ILL 5588 × Titore was best fitted by an inheritance model in which resistance is controlled by two additive-dominance-epistatic major genes. This conclusion was supported by the three statistics which tested the goodness of fit (Table 8.4).

Based on the maximum likelihood estimates of the mean parameters under this model, the genetic effects of each gene were estimated using the least squares method and were given in Table 8.5. The additive and dominant effects were negative for both genes. This indicates that both the resistance conferring alleles come from the resistant parent, and that dominance was towards resistance (less disease severity). Of the four types of epistasis, only the additive A and dominant B was towards resistance.

8.3.2 Seed resistance

The frequency distribution of seed infection rate is given in Table 8.6. The F_1 and BC_1 populations tend towards resistance. In the F_2 and BC_2 populations both resistant and susceptible plants were present. Therefore, major gene(s) were also involved in the inheritance of seed infection in this cross. However, the resistance performance in the F_1

and BC1 was not the same as in the resistant parent, and new categories appeared in both the BC2 and F_2 populations.

The observed seed infection rates of the plants from the six generations were used to fit a series of genetic models using the maximum likelihood method. The AIC value and the maximum likelihood estimates of the mean and variance parameters under each model for seed infection were given in Table 8.7. Based on the AIC value, the best model for describing the inheritance of seed resistance was D1, and the second and third were D2 and D3, respectively. Therefore, the inheritance of seed resistance was best modelled by one major gene and additive-dominance-epistasis polygenes. This conclusion was supported by the three statistics used for testing goodness of fit (Table 8.8).

Based on the maximum likelihood estimates of the parameter means under this model, the genetic effects of each gene were estimated using the least squares method and they were given in Table 8.9. The additive effects of the major gene and polygenes were negative. This indicates that the major resistance gene was from the resistant parent, and most of the polygenes for resistance were also from the resistant parent. The dominant effects of the major gene and polygenes were negative, also indicating that dominance was towards resistance. The additive by additive component of the inter-loci interaction of polygenes favoured resistance, while the additive by dominant, and the dominant by dominant components favoured susceptibility.

Of the phenotypic variations in the BC1, BC2 and F_2 populations 71 %, 61 % and 74 % were from the major gene, while only 10 %, 19 % and 15 % were from the polygenes respectively. Combined together, the genetic variations in these three populations accounted for 81 %, 86 % and 89 % of the phenotypic variations, respectively. Averaged overall 18 % of the genetic effect was from the polygenes.

8.4 Discussion

Two additive-dominance-epistasis genes were shown to be responsible for foliar resistance to Ascochyta blight in the lentil cross of ILL 5588 × Titore. Ford *et al.* (1999) suggested that the foliar resistance of ILL 5588 was under control of one major dominant gene. In Chapter 5, it was shown that one dominant gene for high resistance and one dominant gene for moderate resistance was responsible for foliar resistance in ILL 5588. Although the assumptions underlying the methods used here are different from those underlying segregation analysis used in Chapter 5, and consequently the conclusions are not directly comparable, two major genes for the foliar resistance in ILL 5588 are clearly demonstrated by both methods.

However, this model was not much better in terms of AIC and the three statistics for testing goodness of fit than the second and third best models. They are two additive and dominant genes with additive effect being equal, or unequal to dominant effect, respectively. Nevertheless, two major genes are involved in the determination of foliar resistance, because all the three best models are in the two-gene group.

Seed resistance was under the control of one major gene and additive-dominant-epistasis polygenes, with the major gene having a much large effect than the polygenes. However, Similar to the foliar resistance in this cross, this best model for seed resistance was not much better than the second and third best models in terms of AIC and the three statistics for testing goodness of fit. Nevertheless, the inheritance of seed infection rate is one major gene and polygenes mixed inheritance, because all the three best models are in this group.

The inheritance of seed resistance measured as seed infection rate in ILL 5588 was previously reported to be conferred by three major genes (Tay, 1989), one major dominant gene and two complementary dominant genes. However, the smaller sample sizes used by Tay (1989) made his conclusion less conclusive. The importance of sample size in detecting inheritance models for lentil Ascochyta blight resistance was highlighted in Chapter 5. The presence of polygenes implies that the correct classification of plants in segregating populations for segregation analysis is difficult or impossible and consequently the results from segregation analysis may be misleading.

Using classical generation mean analysis, it was shown that the inheritance model of seed resistance was more complicated than that of foliar resistance (Chapter 7). This is supported by the results of this Chapter. The reason may be that the seed infection rate has a higher discrimination power than the visual scores used for quantifying foliar disease severity. Moreover, seed infection is the result of accumulated effects of all the possible infections which occurred during the plant's life time, while foliar infection was observed after only one round of infection.

The method used in this Chapter has several shortcomings. First, as with segregation analysis, significant errors may exist in the original data because observations of individual plants were used. This could be overcome by using selfing progenies of the segregating populations or by cloning the individual plants. Second, only one major gene was assumed to be involved for the mixed model. In reality, more than one major gene may be present. Third, the major gene is assumed to be independent of polygenes and consequently the variances within each major genotype in each segregating population are assumed to be the same. Clearly, removing these assumptions means more parameters have to be estimated and makes computation formidable. Nevertheless, this approach provides more insight into the genetic mechanism than classical segregation analysis and generation-mean analysis.

Table 8.1: Genetic models for Ascochyta blight resistance in lentil tested, with their corresponding numbers of component distributions, and numbers of independent parameters to be estimated.

Model	Code	Description	Component distributions	Independent parameters	First-order genetic parameters[1]	Second-order parameter[2]
Single gene	A1	Additive and dominant	3	4	m, a, d	σ^2
	A2	Additive	3	3	m, a	σ^2
	A3	Dominant	3	3	m, a	σ^2
Two genes	B1	Additive, dominant and epistasis	9	10	m, a_1, a_2, d_1, d_2, aa, ad_1, ad_2, dd	σ^2
	B2	Additive and dominant	9	6	m, a_1, a_2, d_1, d_2	σ^2
	B3	Additive, dominant	9	4	m, a_1, a_2	σ^2
	B4	Equal additive	9	3	m, a (= a_1 = a_2)	σ^2
	B5	Dominance	9	4	m, a_1, a_2	σ^2
Polygene	C1	Additive, dominant and epistasis	6	10	m, [a], [d], [aa], [ad], [dd]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$
	C2	Additive and dominant	6	7	m, [a], [d]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$
A major gene and polygene	D1	Mixed one major gene and additive-dominant-epistasis polygene	10	14	m, a, d, [a], [d], [aa], [ad], [dd]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$
	D2	Mixed one major gene and additive-dominant	10	9	m, a, d, [a], [d]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$
	D3	Mixed one additive major gene and additive-dominant polygene	10	8	m, a, [a], [d]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$

Table 8.1 *(Continued).*

D4	Mixed one dominant major gene and additive-dominant polygene	10	8	m, a (=d), [a], [d]	$\sigma^2, \sigma_1^2, \sigma_2^2, \sigma_3^2$

[1] 'a', 'd', 'aa', 'ad' and 'dd' are the additive, dominant, additive by additive, additive by dominant, and dominant by dominant effects of the major genes. [a], [d], [aa], [ad], [dd] are the additive, dominant, additive by additive, additive by dominant, and dominant by dominant effects of the minor genes. Subscripts are used where there is more than one gene of the same type.

[2] σ^2: Variance for the distributions of parental and F_1 populations; σ_1^2: Variance for the distributions of the sub-populations of BC_1, BC_2 or F_2 population consisted of AA major genotypes; σ_2^2: Variance for the distributions of the sub-populations of BC_1, BC_2 or F_2 population consisted of Aa major genotypes; σ_3^2: Variance for the distributions of the sub-populations of BC_1, BC_2 or F_2 population consisted of aa major genotypes.

Table 8.2: The frequency distribution of foliar Asochyta blight severity in the six basic generations of the lentil cross ILL 5588 × Titore.

Generation	Disease severity rating									n
	1	2	3	4	5	6	7	8	9	
P_1	8	10	2	-	-	-	-	-	-	20
P_2	-	-	-	-	-	3	12	4	1	20
F_1	3	6	7	4	-	-	-	-	-	20
BC_1	5	8	19	5	3	-	-	-	-	40
BC_2	13	23	6	-	5	7	14	8	4	80
F_2	12	30	30	15	24	2	4	2	1	120

Table 8.3: The AIC values and maximum-likelihood estimates under various genetic models for foliar Ascochyta blight severity. Bold figures indicated the best fitted model.

Model	AIC[1]	Mean[2]										Variance			
		μ_1	μ_2	μ_3	μ_4	μ_5	μ_6	μ_7	μ_8	μ_9	μ_{10}	σ^2	σ_1^2	σ_2^2	σ_3^2
A1	372.54	1.79	3.38	6.72								0.49			
A2	409.84	1.87	3.09	7.32								1.04			
A3	409.80	1.86	3.09	7.37	2.60	2.30	2.34	9.11	2.13	1.90		1.03			
B1	**360.69**	**1.81**	**3.30**	**6.91**	**2.42**	**2.43**	**2.57**	**8.09**	**1.96**	**2.73**	**6.88**	**0.24**			
B2	363.19	1.80	3.30	6.93	2.43	2.55	2.57	8.17	1.95	2.65	6.65	0.24	0.47	0.36	0.69
B3	363.21	1.80	3.30	6.93	2.39	2.59	2.57	8.19	1.93	2.71	6.60	0.24	0.50	0.36	0.71
B4	420.62	1.75	2.97	7.30	2.61	2.82	2.02	10.12	1.67	2.34		2.08			
B5	373.29	1.73	3.45	6.75	2.39	2.59	1.68	12.99	1.95	1.77		0.57			
C1	390.16	1.81	3.30	6.91	2.32		2.06		1.81			0.24	0.89	0.76	0.87
C2	392.04	1.80	3.29	6.93	2.38		2.10		1.76			0.24	0.49	0.77	0.84

Table 8.3: *(Continued).*

D1	364.18	1.80	3.35	6.88	2.56	2.45	2.66	7.68	1.71	2.03		0.28	0.26	0.36	0.67
D2	365.47	1.80	3.33	6.90	2.21	2.80	2.66	7.81	1.92	1.96		0.29			
D3	391.94	1.80	3.29	6.93	2.49	2.47	2.21	9.93	1.92	2.46	8.46	0.24	0.27	4.19	2.59
D4	373.20	1.73	3.45	6.73								0.57			

[1] AIC: Akaike's Information Criterion, [2] μ_1, μ_2, and μ_3 are means of the distributions of P_1, P_2 and F_1 populations; μ_4 and μ_8 are the means of the distributions of the sub-populations of BC_1 and F_2 populations consisting of the AA major genotypes; μ_5, μ_7, and μ_9 are the means of the distributions of the sub-populations of BC_1, BC_2 and F_2 population consisting of the Aa major genotypes; μ_6 and μ_{10} are the means of the distributions of the sub-populations of the F_2 population consisting of the aa major genotypes.

Table 8.4: Tests of the goodness of fit for different genetic models for foliar Ascochyta blight severity in six generations of lentil cross ILL 5588 × Titore. Bold figures indicated significantly derivation from the model.

Model	Generation	U1	P	U2	P	U3	P
A1	P_1	2.39	0.122	0.06	0.806	**25.26**	0.000
	F_1	**8.93**	0.003	**12.63**	0.000	**6.97**	0.008
	P_2	**7.51**	0.006	3.51	0.061	**9.72**	0.002
	BC_1	**33.61**	0.000	**30.42**	0.000	0.15	0.699
	BC_2	2.41	0.121	0.20	0.655	**17.78**	0.000
	F_2	**13.26**	0.000	**13.67**	0.000	0.47	0.493
B1	P_1	0.48	0.488	0.19	0.663	0.87	0.351
	F_1	0.04	0.841	0.31	0.578	2.10	0.147
	P_2	0.10	0.752	0.32	0.572	1.09	0.296
	BC_1	0.43	0.512	0.30	0.584	0.11	0.740
	BC_2	0.69	0.406	0.63	0.427	0.00	1.000
	F_2	**3.97**	0.046	2.64	0.104	1.50	0.221
C1	P_1	**3.48**	0.062	**3.19**	0.074	0.87	0.351
	F_1	**3.04**	0.081	**3.31**	0.069	2.10	0.147
	P_2	2.10	0.147	2.32	0.128	1.09	0.296
	BC_1	1.08	0.299	2.00	0.157	**3.67**	0.055
	BC_2	1.08	0.299	0.82	0.365	**22.03**	0.000
	F_2	**9.49**	0.002	**7.04**	0.008	1.73	0.188
D1	P_1	1.99	0.158	0.75	0.386	**3.96**	0.047
	F_1	**9.19**	0.002	**9.48**	0.002	0.33	0.566
	P_2	0.59	0.442	0.40	0.527	0.20	0.655
	BC_1	**30.31**	0.000	**29.47**	0.000	0.15	0.699
	BC_2	2.17	0.141	2.57	0.109	0.51	0.475
	F_2	**23.35**	0.000	**24.62**	0.000	1.29	0.256

[1] The U1, U2 and U3 are statistics used to test the goodness of fitness.

Table 8.5: The estimates of genetic effects for foliar Ascochyta blight severity in lentil cross ILL 5588 × Titore under the two additive-dominance-epistasis gene model (B1) [1].

Generation	m	a_1	a_2	d_1	d_2	aa	ad	da	dd
P_1	1	1	1	0	0	1	0	0	0
F_1	1	0	0	1	1	0	0	0	1
P_2	1	-1	-1	0	0	-1	0	0	0
BC_{11}	1	1	0.5	0	0.5	0.5	0.5	0	0
BC_{12}	1	0	0.5	1	0.5	0	0	0.5	0.5
BC_{21}	1	0	-0.5	1	0.5	0	0	-0.5	0.5
BC_{22}	1	-1	-0.5	0	0.5	-0.5	-0.5	0	0
F_{21}	1	1	0	0	0.5	0	0.5	0	0
F_{22}	1	0	0	1	0.5	0	0	-0.5	0.5
F_{23}	1	-1	0	0	0.5	0	-0.5	0	0
	4.36	-1.39	-1.96	-0.88	-1.34	0.88	-0.14	1.04	0.16

Table 8.6: The frequency distribution of seed infection rate by *Ascochyta lentis* in the six basic generations of the cross ILL 5588 × Titore.

Generation	Percentage Infection												n
	0-5	5-10	11-15	16-20	21-25	26-30	31-35	36-40	41-45	46-50	51-55	56-	
P_1	2	2	10	5	1	-	-	-	-	-	-	-	20
P_2	-	-	-	-	-	-	-	3	1	8	5	2	19
F_1	1	4	7	4	4	-	-	-	-	-	-	-	20
BC_1	3	11	18	5	3	-	-	-	-	-	-	-	40
BC_2	1	8	14	10	6	1		2	12	14	5	3	76
F_2	10	6	25	12	3	4	5	9	7	20	11	6	118

Table 8.7: The AIC values and maximum-likelihood estimates under various genetic models for seed infection of lentil by *Ascochyta lentis*. Bold figures indicated the best fitted model.

		Mean										Variance			
Model	AIC	μ_1	μ_2	μ_3	μ_4	μ_5	μ_6	μ_7	μ_8	μ_9	μ_{10}	σ^2	σ_1^2	σ_2^2	σ_3^2
A1	1457.15	17.93	16.88	43.85								42.37			
A2	1606.36	18.73	15.46	47.74								97.15			
A3	1459.79	17.26	17.26	43.90								49.97			
B1	1428.88	17.99	16.64	45.00	15.96	18.67	17.73	40.73	19.18	16.97		22.18			
B2	1534.75	18.01	16.43	45.17	17.97	16.47	14.74	51.80	19.25	21.28	47.01	12.69	19.69	41.70	51.90
B3	1606.19	18.64	15.46	48.06	18.78	15.32	15.60	47.52	21.28	16.37		96.41			
B4	1649.49	17.49	14.83	47.60	18.83	18.83	13.50	52.82	16.68	20.22		101.44			
B5	1460.14	17.25	17.25	44.03	17.25	17.25	11.18	67.77	19.48	15.25		49.94			
C1	1527.64	18.06	16.48	45.03	16.77		13.73		18.14			16.70	82.48	68.77	79.56
C2	1535.15	18.01	16.43	45.17	17.22		14.01		17.57			16.68	41.61	70.05	77.27
D1	**1409.74**	**18.06**	**16.48**	**45.03**	**17.44**	**16.22**	**17.13**	**42.23**	**19.59**	**23.59**	**38.24**	**12.68**	**19.48**	**29.07**	**30.47**
D2	1419.76	18.03	16.49	45.19	17.53	16.99	17.14	42.61	19.50	22.90	36.94	12.64	20.17	29.28	61.65
D3	1419.85	18.03	16.48	45.18	17.26	17.26	17.11	42.75	19.33	23.43	36.67	12.64	23.12	29.47	63.62
D4	1423.73	18.00	16.75	44.86	18.47	16.34	17.72	40.07	17.07	17.55		20.60			

Table 8.8: Tests of goodness of fitness for different genetic models for seed infection rate by *Ascochyta lentis* in six generations of lentil cross ILL 5588 × Titore. Bold figures indicated significant derivation from the model.

Model	Generation	U_1	P	U_2	P	U_3	P
A_1	P_1	2.20	0.138	1.03	0.310	**13.63**	0.000
	F_1	**5.47**	0.019	**7.32**	0.007	**4.49**	0.034
	P_2	**4.76**	0.029	**2.76**	0.097	**5.86**	0.015
	B_1	**17.81**	0.000	**16.21**	0.000	1.08	0.300
	B_2	2.21	0.138	1.10	0.294	**9.89**	0.002
	F_2	**7.63**	0.006	**7.84**	0.005	1.24	0.266
B_1	P_1	2.00	0.158	1.38	0.241	**2.98**	0.084
	F_1	**5.60**	0.018	**5.74**	0.017	1.17	0.280
	P_2	1.30	0.255	1.20	0.273	1.10	0.294
	B_1	**16.16**	0.000	**15.74**	0.000	1.08	0.300
	B_2	2.09	0.149	2.29	0.131	1.26	0.263
	F_2	**12.68**	0.000	**13.31**	0.000	1.65	0.200
C_1	P_1	1.24	0.265	1.10	0.295	1.44	0.231
	F_1	1.02	0.313	1.16	0.283	2.05	0.152
	P_2	1.05	0.306	1.16	0.281	1.55	0.214
	B_1	1.04	0.308	1.00	0.317	1.34	0.248
	B_2	1.04	0.308	1.41	0.235	**12.02**	0.001
	F_2	**5.75**	0.017	**4.52**	0.034	1.87	0.172
D_1	P_1	1.24	0.265	1.10	0.295	1.44	0.231
	F_1	1.02	0.313	1.16	0.283	2.05	0.152
	P_2	1.05	0.306	1.16	0.281	1.55	0.214
	B_1	1.22	0.270	1.15	0.284	1.06	0.304
	B_2	1.35	0.246	1.32	0.251	1.00	0.317
	F_2	**2.99**	0.084	2.32	0.128	1.75	0.186

Table 8.9: The estimates of genetic parameters for seed infection rate by *Ascochyta lentis* of lentil cross ILL 5588 × Titore under the mixed one major gene and additive-dominance-epistasis gene model (D1).

Effect	Estimate	Variance & Heritability[1]	BC_1	BC_2	F_2
a	-10.2	σ_p^2	67.18	87.67	117.10
d	-9.97	σ_e^2	12.68	12.68	12.68
[a]	-3.22	σ_{mg}^2	47.70	58.60	86.63
[d]	-25.4	σ_{pg}^2	6.80	16.39	17.79
[aa]	-8.7	h_{mg}^2	0.71	0.67	0.74
[ad]	1.09	h_{pg}^2	0.10	0.19	0.15
[dd]	11.6	h^2	0.81	0.86	0.89

[1] σ_p^2: Phenotypic variance, σ_{mg}^2: Major-gene genetic variance, σ_{pg}^2 : Minor-gene genetic variance, h_{mg}^2: Major-gene heritability, h_{pg}^2 : Minor-gene heritability, and h^2 : Heritability.

Appendix

Table 8A1. The relationships between population means and genetic effects under a model of two additive-dominance-epistasis genes.

Generation	a_1	a_2	d_1	d_2	aa	ad	da	dd
P_1	1	1	0	0	1	0	0	0
F_1	0	0	1	1	0	0	0	1
P_2	-1	-1	0	0	-1	0	0	0
BC_{11}	1	0.5	0	0.5	0.5	0.5	0	0
BC_{12}	0	0.5	1	0.5	0	0	0.5	0.5
BC_{21}	0	-0.5	1	0.5	0	0	-0.5	0.5
BC_{22}	-1	-0.5	0	0.5	-0.5	-0.5	0	0
F_{21}	1	0	0	0.5	0	0.5	0	0
F_{22}	0	0	1	0.5	0	0	-0.5	0.5
F_{23}	-1	0	0	0.5	0	-0.5	0	0

Table 8A2. The relationships between population means and genetic effects under a model of one major-gene and additive-dominance-epistasis polygenes.

Generation	m	a	d	[a]	[d]	[aa]	[ad]	[dd]
P_1	1	1	0	1	0	1	0	0
F_1	1	0	1	0	1	0	0	1
P_2	1	-1	0	-1	0	1	0	0
BC_{11}	1	1	0	0.5	0.5	0.25	0.25	0.25
BC_{12}	1	0	1	0.5	0.5	0.25	0.25	0.25
BC_{21}	1	0	1	-0.5	0.5	0.25	-0.25	0.25
BC_{22}	1	-1	0	-0.5	0.5	0.25	-0.25	0.25
F_{21}	1	1	0	0	0.5	0	0	0.25
F_{22}	1	0	1	0	0.5	0	0	0.25
F_{23}	1	-1	0	0	0.5	0	0	0.25

CHAPTER 9

GENETIC RELATIONSHIPS AMONG ASCOCHYTA BLIGHT RESISTANCE AND OTHER AGRONOMIC TRAITS

Summary

Two sets of recombinant inbred lines of the cross ILL 5684 × Titore, one with and the other without the major gene for Ascochyta blight resistance, were tested in the glasshouse with and without artificial disease inoculation. Foliar disease severity, seed yield/plant, plant height and time to flowering were measured. The major gene for resistance was found to be either linked to seed yield/plant or had a pleiotropic effect on seed yield/plant. The resistance gene was independent of plant height and time to flowering. Within each set of inbred lines, there were significant differences among inbreds for all the observed traits. The estimates of heritability based on inbred mean were high for seed yield/plant and time to flowering, and moderate for plant height for both sets of inbred lines and under both growing conditions. For the set of lines containing the major resistance gene: 1) Type A genetic correlation estimates suggested that disease severity was not correlated with seed yield/plant and plant height, but was weakly and negatively correlated with time to flowering when measured under disease pressure. 2) Seed yield/plant was strongly and positively correlated with plant height, but moderately and negatively correlated with time to flowering. Plant height was weakly and positively correlated with time to flowering under both sets of testing conditions. 3) Inbred × environment interactions were not important and selection could be made with or without artificial inoculation. Selection within the set of inbreds with the major resistance gene is required and feasible for the improvements of yield and other traits and for the utilisation of resistance conferred by minor genes.

9.1 Introduction

Ascochyta blight is an important disease in many lentil production regions (Muehlabure *et al.*, 1995). Chemical control is effective but uneconomic and has not been accepted by farmers (Morrall, 1997). Breeding for resistance has been suggested as a means to control Ascochyta blight in lentil. Several international and national breeding programmes are under way to breed for Ascochyta blight resistance (Erskine *et al.*, 1994; Ye *et al.*, 2000).

Major gene(s) and minor genes are involved in the genetic determination of Ascochyta blight resistance, with the major gene(s) being the main source of resistance (Chapter 6). Therefore, an efficient breeding strategy would be selection for major gene(s) resistance in early generations and selection for resistance conditioned by minor genes in later generations. However, Ascochyta blight resistance is only one of the traits of interest to lentil breeders. Other traits such as yield, plant height and maturity are important for the success of any new cultivar (Muehlabure *et al.*, 1995). Therefore, multi-trait selection is necessary at least in later generations to obtain new cultivars. For efficient multi-trait selection, genetic relationships between important traits must be well understood.

The objectives of the study reported in this chapter were to study the associations between a major gene for resistance to Ascochyta blight, and seed yield/plant, plant height and time to flowering and to investigate the genetic relationships among disease severity and these traits using sets of recombinant inbred lines with and without a major resistance gene.

9.2 Materials and Methods

9.2.1 Plant material and experimental design

The production of the resistant and susceptible sets of recombinant inbreds from the cross ILL 5684 × Titore, disease resistance and agronomic traits testing and experimental design are fully described in Chapter 6. Each set of inbreds consisted of

25, F_5 lines which were either susceptible or resistant to Ascochyta blight.

A randomised complete block design with three replications of 10 plants per plot (in two pots) was used for all the tests. Two test conditions were used either with or without inoculation with Ascochyta *lentis*.

9.2.2 Statistical analyses and genetic parameter estimation

For each environment, the following linear model was used to carry out the analyses of variance and covariance and to estimate variance components;

$$y_{ijk} = \mu + g_i + b_j + (bg)_{ij} + \varepsilon_{ijk},$$

where y_{ijk} is the k-th observation of the i-th inbred in the j-th replication; μ is the grand mean; g_i is the effect of the i-th inbred; b_j is the effect of the j-th replication; $(bg)_{ij}$ is the effect of inbred-by-replication interaction (in practice, usually a plot-environmental effect); ε_{ijk} is the error effect.

With the assumption that all effects are random, the variance components for each effect were estimated by the maximum likelihood method. The inbred-mean variance is estimated as;

$$\sigma_I^2 = \sigma_g^2 + \sigma_{gb}^2/b + \sigma_e^2/bn,$$

where σ_I^2 is the total phenotypic variance of the inbred mean, σ_g^2 is the variance due to the inbred, σ_{gb}^2 is the variance due to inbred-by-replication interaction, and σ_e^2 is the variance due to the error (within-plot variance), b is the number of replications and n is the number of plants per inbred-replication combination.

The inbred-mean heritability was estimated as;

$$H^2 = \frac{\sigma_g^2}{\sigma_I^2}$$

The genetic correlations between traits measured in the same individuals were estimated from analysis of cross-products of plots using the following equation;

$$r_{Aij} = \frac{\sigma_{ij}}{\sqrt{\sigma_{gi}^2 \sigma_{gj}^2}},$$

where σ_{ij} is the covariance between traits i and j from an analysis of cross-products, and σ_{gi}^2 and σ_{gj}^2 are inbred variances for traits i and j estimated from the ANOVAs. Standard error estimates for r_{Aij}'s were obtained according to Mode and Robinson (1959).

For combined analysis across the two test conditions (environments), the following linear model was used;

$$y_{ijkl} = \mu + g_i + e_j + b_{k(j)} + (ge)_{ij} + (gb)_{ik(j)} + \varepsilon_{ijk},$$

where e_j is the environment effect, $(ge)_{ij}$ is the effect of the inbred-by-environment interaction, $(gb)_{ik(j)}$ is the effect of the inbred-by-replication interaction in the jth environment, and ε_{ijkl} is the error.

The genetic correlations between the same trait measured in different environments (Type B) were estimated with;

$$r_{Bi} = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{ge}^2},$$

where σ^2_g and σ^2_{ge} are inbred and inbred-by-environment interaction variances estimated from the combined analysis of variance across environments (Yamada, 1962). Due to the heterogeneity of inbred variance components between the two environments, a correction term, C, was used to adjust r_{Bi} (Yamada, 1962), thus;

$$C = (\sigma^2_{g1} - \sigma^2_{g2})/2$$

where σ^2_{g1} and σ^2_{g2} are the square root of the inbred variance components from separate analyses of variance for each environment. The correction term C was subtracted from σ^2_{ge} and resulted in σ^2_{ge*}. The adjusted Type B genetic correlation estimate, is

$$r_{Bi}* = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_{ge*}}$$

standard error estimates for the unadjusted r_{Bi}'s were obtained according to Namkoong (1979) and were used as standard error estimates for the adjusted correlations.

The type B genetic correlations between disease severity measured under disease pressure and other traits measured under normal condition were estimated using the following equation (Burdon, 1977);

$$r_B = \frac{\sigma_{p12}}{\sqrt{\sigma^2_{g1}\sigma^2_{g2}}},$$

where σ_{p12} is the phenotypic covariance between disease severity measured under disease pressure and another trait measured under disease free condition, σ^2_{g1} is the

genetic variance of disease severity and σ^2_{g2} is the genetic variance of another trait estimated under disease free condition. Standard error estimates for r_{Aij}'s were obtained according to Mode and Robinson (1959).

9.3 Results

9.3.1 The influences of Ascochyta blight on agronomic traits

The level of disease pressure applied to the plants in this trial was high because large numbers of conidia were inoculated onto plants and the nevironmental conditions subsequently applied were likely to be highly conductive to disease development. Therefore, the difference between trait performances of the susceptible set of plants with and without disease pressure is likely to reflect the maximum influence of Ascochyta blight on the traits. The disease pressure reduced yield/plant, plant height and time to flowering (Table 9.1). The yield loss due to Ascochyta infection was 68.4 % averaged across the 25 susceptible lines. The reductions in plant height and time to flowering were 9.4 % and 4.6 % respectively. Comparing to the mean yield of the 25 susceptible lines and the mean yield of the 25 resistant lines under normal, disease free, condition, the mean yield loss was 15.3 % if resistant lines were used under disease free condition. However, when disease pressure is high, the use of resistant lines increased yield by 151.6 %.

9.3.2 Associations between the major gene for resistance and genes for other traits

Under disease free (uninoculated) conditions, the average seed yield/plant of the resistant set of inbred lines was significantly lower than the yield of the susceptible set of lines (15.3 %) (Table 9.1). This suggests that the major gene for resistance is either/both linked to genes for decreasing seed yield/plant, or has a negative pleiotropic effect on seed yield/plant.

On average, the resistant set of inbred lines was shorter than the susceptible set of inbred lines, but the difference in height between them was not significant. This

suggested that the major resistance gene is not linked to genes for plant height, and has no pleiotropic effect on plant height (Table 9.1).

As with plant height, there was no significant difference between the susceptible and resistant inbred sets of lines for time to flowering. However, the resistant set of lines flowered a little (not significantly) later than the susceptible set. This suggested that the major gene for resistance is independent of the genes for time to flowering, and it has no pleiotropic effect on time to flowering (Table 9.1).

Table 9.1: Means and ranges of yield/plant, plant height and time to flowering of two sets of inbreds under disease pressure (inoculated) and disease free conditions.

Trait	Set[1]	Statistics[2]	Inoculated	Uninoculated
Yield	Res.	Mean	0.78	0.83
(g/plant)		Range	0.70-0.87	0.73-0.94
	Sus.	Mean	0.31	0.98
		Range	0.23-0.42	0.79-1.17
Plant height (PH)	Res.	Mean	45.3	48.5
(cm)		Range	26.1-56.5	28.6-70.8
	Sus.	Mean	44.2	48.8
		Range	29.6-57.5	31.2-73.5
Time to flowering (TF)	Res.	Mean	52.8	55.3
(days)		Range	40-68	41-70
	Sus.	Mean	50.8	53.2
		Range	45-70-	40-72

[1]Res. = Resistant, Sus. = Susceptible.

[2] Mean across 25 F_5 lines and three replications; Range of the inbred means over three replications.

9.3.3 Inbred-mean heritability and genetic gain

Specific inbred lines within a set varied in their seed yield, plant height and time to flowering. Therefore, selection for these traits should be effective. The ranges for these traits among lines within a set were similar for the resistant and susceptible sets (Table 9.1).

Analysis of variance across the two test conditions indicated that condition, inbred and their interaction were all significant (Table 9.2). Analysis of variance was carried out at each condition (Table 9.3). The estimates of genetic variance, error variance, phenotypic variance, inbred-mean heritability and predicted genetic gain were obtained for each trait for each set under each of test conditions (Table 9.4). Seed yield/plant and time to flowering had high heritabilities. Plant height had a moderate heritability. The estimates for each trait, for each set of inbred lines, in each testing condition were very similar. Reasonably high genetic gains could be obtained by selecting within the sets for all of the traits. For the resistant set, the gains accruing from selection under disease pressure or under normal conditions were almost the same for seed yield/plant and time to flowering. The gains from selecting for plant height would be expected to be higher if the selection was under disease pressure.

Table 9.2: Combined analysis of variance of seed yield/plant (g), plant height (cm) and time to flowering of lentil inbreds resistant to Ascochyta blight across two test conditions.

Source	Degrees of freedom (DF)	Mean Square (MS)		
		Yield	PH	TF
Condition	1	0.094	384.0**	234.38**
Inbred	24	0.264**	101.85**	75.22**
Inbred × Condition	24	0.076**	24.74**	13.09**
Error	96	0.031	12.89	4.21

**: significant at 0.01 level.

Table 9.3: Analysis of variance of yield/plant (g), plant height (cm) and time to flowering of sets of lentil inbreds either inoculated or uninoculated with *Ascochyta lentis*[1].

Condition	Set	Source	DF	MS		
				Yield	PH	TF
Inoculated	Res.	Inbred	24	0.248**	40.46**	29.72**
		Error	48	0.026	12.41	4.53
Uninoculated	Res.	Inbred	24	0.275**	34.99**	31.33**
		Error	48	0.033	13.38	4.08
	Sus.	Inbred	24	0.528**	45.31**	23.18**
		Error	48	0.058	10.66	4.81

[1] ANOVA was not done for the susceptible set under inoculation because many plants died.

** significant at 0.01 level.

Table 9.4: Genetic parameters of seed yield/plant, plant height and time to flowering estimated for Ascochyta blight resistant and susceptible sets of lentil inbreds either inoculated or uninoculated[1].

Trait	Set	Condition	σ_e^2	σ_g^2	σ_l^2	H[2]	ΔG[2]
Yield (g/plant)	Res.	Inoculated	0.026	0.074	0.100	0.74	0.073
		Uninoculated	0.033	0.081	0.114	0.71	0.079
	Sus.	Uninoculated	0.058	0.157	0.215	0.73	0.154
PH (cm)	Res.	Inoculated	12.410	9.362	21.772	0.43	9.198
		Uninoculated	13.380	7.205	20.585	0.35	7.079
	Sus.	Uninoculated	10.660	11.548	22.208	0.52	11.346
TF(days)	Res.	Inoculated	4.523	8.400	12.922	0.65	8.253
		Uninoculated	4.080	9.081	13.161	0.69	8.922
	Sus.	Uninoculated	4.810	6.122	10.932	0.56	6.015

[1] Parameters were not estimated for the susceptible set under inoculation because many plants died, [2] Genetic gain predicted by assuming that one inbred was selected from the 25 inbreds tested.

9.3.4 Genetic correlation

Type A correlation is an indication of the commonality of gene effects of the two traits measured in the same individual. The Type A genetic correlations between disease severity and seed yield/plant, plant height and time to flowering were weak,

with only the correlation between disease and time to flowering being significantly different from zero. This suggested that minor genes for Ascochyta blight resistance had no or little effect on these traits.

The estimates of Type A genetic correlations among seed yield/plant, plant height and time to flowering had the same pattern under both test conditions, with the normal uninoculated environment giving a higher and more reliable estimation (Table 9.5). Seed yield/plant was strongly and positively correlated with plant height, but moderately and negatively correlated with time to flowering. Plant height and time to flowering were weakly and positively correlated.

The Type B correlations between disease severity and seed yield/plant, plant height and time to flowering were weaker though they were very similar to the corresponding Type A correlations (Table 9.6).

Table 9.5: Estimates of genetic correlation between traits measured in the same individuals (r_A) and their standard deviations (in parentheses) for the resistant set inbreds either inoculated with *Ascochyta lentis* or left uninoculated[1].

Condition	Traits	Disease Severity (DS)	Yield	PH	TF
Inoculated	DS		-0.16 (0.11)	-0.24 (0.20)	**-0.36 (0.14)**
	Yield			**0.66 (0.11)**	**-0.43 (0.10)**
	PH				0.21 (0.13)
Uninoculated	DS[2]	-	-	-	-
	Yield			**0.78(0.11)**	**-0.51(0.21)**
	PH				0.24(0.17)

[1] Figures in bold are significant at $p \leq 0.05$ level, [2] not measurable.

9.3.5 Inbred x environment interaction

The type B genetic correlations between measurements of the same traits in the two environments describe the rank-changing genotype-by-environment interaction. The type B correlations of yield/plant, plant height and time to flowering were all not significantly different from +1, indicating that there were not rank-changing genotype-by-environment interactions (Table 9.6). However, the combined analysis of variance showed significant genotype-by-environment interactions (Table 9.2). Thus, the interaction revealed by the analysis of variance was quantitative interaction only.

Table 9.6: Estimates of genetic correlation between traits measured *in Ascochyta lentis* inoculated and uninoculated conditions (r_B^*) and standard deviations (in parentheses) for the set of resistant inbreds[1].

Traits	Disease Severity (DS)	Yield	PH	TF
DS	-	-	-	-
Yield	-0.20(0.19)	**0.86(0.14)**	-	-
PH	**-0.32(0.24)**	-	**0.75(0.23)**	-
TF	**-0.38(0.25)**	-	-	**0.81(0.21)**

[1] Figure in bold is significant at $\alpha \leq 0.05$ level.

9.4 Discussion

Selection for one trait will cause correlated changes in other traits (Falconer, 1989). Depending on the direction of the genetic correlations between traits, correlated selection responses should either be utilised or avoided. Breeding for Ascochyta blight resistance in lentil does, and will in the future, rely on the resistance conferred by major genes due to the simplicity of selection. However, the major gene

contained in ILL 5684 was shown to be either linked to the gene for seed yield/plant, or it has a pleiotropic effect on it, or both. Consequently, the presence of the resistant allele of the major gene reduced mean yield by 15.3 %. Fortunately, considerable genetic variation for yield was present within the population of 25 resistant inbred lines. This provides a chance to improve yield by selection within the population of inbred lines. The existence of effects of major genes for disease resistance on yield has also been reported in other species (Reheul and Chesquiere, 1996).

The major gene for resistance was independent of genes for plant height and time to flowering. This is useful for lentil breeders because short plants and earliness in flowering are also required traits in elite lines (Muehlabure *et al.*, 1995).

When selection is carried out within a population of inbreds, which carry the major resistance gene, care should also be taken to ensure that resistance is not reduced due to selection for yield and other traits. Disease resistance conferred by minor genes was not correlated with seed yield/plant and plant height in this study. This suggests that selection for seed yield/plant or plant height can be done at no cost to Ascochyta blight resistance. The moderately negative correlation between disease severity and time to flowering implies that selection for earliness in flowering would increase the resistance conditioned by minor genes. Therefore, it is feasible that selection for all three of these important agronomic traits will not reduce the resistance to Ascochyta blight.

The genetic correlation between seed yield/plant and plant height was positive, and high. This is not unexpected as Ramgiry *et al.*, (1989), Esmail *et al.*, (1994) and Jian *et al.*, (1995) have reported similar results at the phenotypic level. Seed yield/plant was moderately and negatively correlated with time to flowering. This is consistent with the result of Esmail *et al.* (1994) at the phenotypic level. Plant height and time to flowering was weakly and positively correlated. These genetic relationships suggest that it may be hard to obtain cultivars that are short and high-yielding. However, it is important to realise these data are for individual plants and not for an established crop where height reducing genes frequently have been shown to increase yields (Muehlabure *et al.*, 1995).

In terms of heritability, genetic correlations between traits, and the higher Type B genetic correlations between the same traits measured in different

environments, these data indicate that selection for seed yield/plant, plant height and time to flowering can be carried out equally well under conditions of high Ascochyta blight pressure (artificial inoculation) or under disease free conditions. However, because selection for Ascochyta blight resistance conditioned by minor genes is required to enhance the resistance conferred by the major gene, selection for all the traits under disease pressure is likely to be the most appropriate.

As all the tests were performed in the glasshouse, the results now need to be verified in the field.

CHAPTER 10

BREEDING FOR RESISTANCE TO LENTIL ASOCHYTA BLIGHT: AN OVERVIEW

Summary

Ascochyta blight, caused by *Ascochyta lentis*, is one of the most important diseases of lentil. Breeding for resistance has been suggested as an efficient means to control this disease. This chapter summarises existing studies of the genetics of resistance to Ascochyta blight in lentil, and genetic variations among pathogen populations (isolates). Breeding methods for control of the disease are then discussed. Six pathotypes of *Ascochyta lentis* have been identified and many resistant cultivars/lines have been identified in both cultivated and wild lentils. Resistance to Ascochyta blight is mainly under the control of major genes, but minor genes also play a role. Current breeding programmes are based on crossing resistant and high yielding cultivars and multi-location testing. Gene pyramiding, exploring slow blighting and partial resistance, and using genes contained in wild relatives will be the methods of the future. Identification of more sources of resistance genes, good characterisation of the host-pathogen system and identification of molecular markers tightly linked to resistance genes are suggested to be the key areas for future study.

10.1 Introduction

Ascochyta blight, caused by *Ascochyta lentis*, is an important disease of lentil. It has been reported to be the major disease in many lentil producing countries including Argentina, Australia, Canada, Ethiopia, India, New Zealand, Pakistan and The Russian Federation (Kaiser and Hannan, 1986; Erskine *et al.*, 1994). The disease has considerable effect on both seed quality and yield (Morrall and Sheppard, 1981). Gossen and Morrall (1983) estimated that foliar infection has caused yield losses of

up to 40%, but in Canada economic losses from infected seed may reach more than 70 %. In some cases, seed infection is so severe that the lentils are unmarketable (Kaiser, 1981; Gossen and Morral, 1983).

In spite of extensive agronomic and chemical control studies, no effective or efficient method has been found to control Ascochyta blight in lentil. Breeding for host resistance is expected to be the most effective, efficient and environmentally friendly method to control the disease (Erskine *et al.*, 1994).

This Chapter summarises our understanding of the genetics of Ascochyta blight resistance in lentil incorporating my findings in previous chapters, and genetic variations among pathogen populations (isolates). Following this, methods for breeding for resistance and corresponding backup techniques are discussed.

10.2 Genetic variations among isolates of *Ascochyta lentis*

A good understanding of the genetic variations of pathogenicity and virulence patterns in the pathogen is useful for designing a breeding programme and infection test methods. For example, when there are many physiological races of the pathogen, race-specific resistance usually conferred by major genes can be overcome easily by the pathogen. Therefore, exploring partial resistance conditioned by polygenes and/or combining resistance genes for different races is necessary to obtain durable resistance. However, there is only limited information on variation in *A. lentis*. Tests done by Kaiser *et al.* (1994), using 24 isolates from different countries, showed only small differences among isolates in ability to attack two cultivars. Ahmed and Morrall (1996) investigated the reactions of lentil lines and cultivars (differentials) to seven isolates of *A. lentis* in the field using artificial inoculation, and percentage seed infection. Differences (based on initial disease severity) occurred among differentials in their reactions to the isolates. The differentials and isolates were significantly different in both years for seed infection percentage and the area under the disease progress curve, but, the isolate × differential interaction was non-significant in both years for seed infection percentage in both years, and was significant for the area under the disease progress curve in only one year.

Ahmed *et al.* (1996b) investigated the virulence patterns of 84 isolates of *A. lentis* from western Canada, and isolates from 13 other countries on 10 lentil lines and cultivars (differentials). Western Canadian isolates collected in 1978 and 1985 were less virulent than 1992 collections. Cluster analysis grouped the isolates into weak, intermediate and high virulence forms. The virulence pattern did not relate to specific geographic locations. Host differential × isolate interactions were generally lacking, indicating the absence of cultivar specificity. However, the cultivar Laird was moderately resistant to the 1978 isolates but susceptible to the 1992 isolates.

Nasir and Bretag (1997b) examined the pathogenic variability of 39 isolates from Australia using 22 lentil cultivars. Based on the different reaction of six cultivars (ILL 358, ILL 7537, ILL 7515, ILL 5588, ILL 5244 and Eston), they identified six pathotypes. Most of the isolates were virulent to some of the cultivars. There were three isolates that infected all the cultivars, and five isolates that were avirulent to all the cultivars. This study clearly showed that resistance is pathotype-specific.

10.3 Measuring the reaction to disease

The different genotypic reactions to *A. lentis* are the basis of breeding for resistance. To measure the reaction, a suitable infection test and disease rating scale needs to be designed for the desired breeding objectives, and to distinguish the variations in patterns of pathogenicity.

10.3.1 Infection test methods

Results from infection tests are the basic information upon which breeding programmes for resistance have been designed and success achieved. Therefore, the reliability of the infection test used is fundamentally important in the success of a breeding programme aimed at improving resistance. The choice of a suitable test method depends on the breeding objectives and the pathogenic variability of the pathogen. For instance, to screen for highly resistant lines, a higher dose of inoculum is necessary. However, to identify partial resistance, the dose of inoculum should not

be so high that small differences in resistance are obscured. Similarly, race-specific resistance can only be identified by the use of single races in sequential tests. Furthermore, suitable test methods may be different from one stage to another in a breeding process. For example, glasshouse test using single race inoculum is normally used at the earlier stages to gain an understanding of the genetic regulation of resistance, and/or to facilitate the selection of lines with multiple resistance genes. However, the putative resistant lines have to be tested in the field and exposed to as full a spectrum of pathogen races as possible before they are recommended to the producers.

Natural infection test in the field

The natural field infection test is the cheapest method. Its main advantage is that all lines (individuals) are exposed to environmental conditions that are similar to those, which the resultant resistant cultivars will face. The main disadvantage is that interactions between different biotic and abiotic factors can mask the expression of resistance. Without artificial inoculation, resistance can only be tested in epidemic years. In breeding for resistance, natural infection test is normally used in the late stage to verify the resistance of the lines selected in earlier stages. Another use of this method is to identify putative resistant cultivars/lines when natural epidemics happen.

Artificial infection test in glasshouse

Glasshouse testing has several advantages over field testing. Firstly, off-season testing can be done and testing can also be done at any development stage of the plants. Secondly, environmental conditions can be adjusted to enhance the development of disease. Thirdly, possible interference caused by other microorganisms and other factors can be reduced. Fourthly, the inoculum can be more evenly distributed and consequently reduce the risk of escapes. The main disadvantage of glasshouse screening is the high unit price and limited space.

Artificial infection test in the field

To overcome some of the potential problems of the natural infection test and the glasshouse infection test, artificial epidemics can be generated in the field. This

can be achieved by inoculation with inoculum prepared in the lab, scattering disease debris and inter-planting susceptible genotypes (spreaders) for increasing pathogen populations and providing irrigation for increasing relative humidity. This method is used in the breeding programme at ICARDA because of its successful application in breeding for resistance to Ascochyta blight in chickpea (Singh *et al.*, 1993).

Test in the laboratory

Recently a method based on a detached leaf test has been developed at Lincoln University (Russel and Hill, 1998). The correlation of disease severity or disease incidence values obtained from this method and the disease severity values for intact plants was high. The resistant or susceptible category for each cultivar in relation to each race was not changed by assay on young detached leaflets, but for older leaflets (from the 1st or 2nd oldest leaves) a susceptible rating sometimes was altered to a resistant rating (which may indicate a genuine resistance form in aged leaves). The advantage of this method is that the disease reaction can be tested using several leaflets while the intact plant is protected for seed production. Clearly, this method will find use in breeding for resistance, particularly at the earlier generations when the number of seeds per line is limited.

Sharma *et al.* (1995) developed a so-called cut twig method to screen *Ascochyta rabiei* resistance in chickpea without the destruction of the whole plant. It involves inoculating single cut branches with spores. They showed that this method could be used in a breeding programme with good success by testing the resistance of 542 chickpea plants in F_2 and backcrossed progenies. As with the detached leaf method, this procedure should be very useful in the earlier generations of a breeding programme. However, no attempt has been made to test its applicability in lentil. Mosconi *et al.* (1996) again in Ascochyta blight of chickpea, tested, and found promising, the possibility of using culture filtrate to measure the resistance of different genotypes.

10.3.2 Rating scale

Rating scales used to measure the disease reaction also depend on the objectives of the breeding program. To select for resistance conditioned by a major

gene, a simple scale is normally sufficient. However, to select for partial resistance conferred by polygenes, scales that are more precise are necessary.

Visual scales

Visual scales have been commonly used to measure blight reaction in lentils. At ICARDA, a 9-point scaling system based on visual judgement of disease severity was developed and can be used to describe the disease reaction in field plots. This system is given in Table 10.1.

There are other visual scales used by different authors. For instance, the rating scale used by Ahmed and Morrall (1996), and Ahmed *et al.* (1996) was as follow: 0 = no lesion; 1 = < 5% leaf lesions; 3 = 5- 10% leaf lesions; 4 = 11-25% leaf and stem lesions; and 5 = > 25% leaf and stem lesions,, and dieback. The rating scale used by Nasir and Bretag (1997a; b) and Ford *et al.* (1999) was as follow: 1 = no visible lesions; 3 = small flecks on leaves; 5 = many lesions on leaves with or without chlorotic zones, flecks on stems; 7 = extensive lesions on leaves and defoliation, many stem lesions with sporulation; and 9 = collapsed leaves, girdled stems and plant death. These last two scale systems were used for evaluating single plant infections.

Other scales

Area under the disease progress curve (AUDP) has also been used to quantify host resistance (Ahmed and Morrall, 1996). A high correlation between AUDP and the initial disease severity, measured using a visual scale, was found by Ahmed and Morrall (1996). However, a high disease pressure was required to obtain a good correlation. This may imply that they both measure the resistance conferred by major genes and consequently are highly correlated.

Incubation period (days from inoculation to first symptom appearance) and latent period (days from inoculation to appearance of fruiting structures) have been used by Pedersen and Morrall (1994) and Ahmed *et al.* (1996a). However, it is hard to use these two characteristics to discriminate resistance among cultivars because of their limited ranges

Table 10.1. ICARDA visual scale for Ascochyta blight reaction in lentil.

Scale	Disease intensity
1	No visible lesions
2	Very few lesions after careful examination
3	Few scattered lesions after careful examination
4	Lesions defoliation on some plants, not damaging
5	Lesions common and easily observed on all plants but defoliation and damage not great
6	Lesions and defoliation common
7	Lesions very common, defoliation moderate
8	All plants with extensive lesions, defoliation high and stem girdling
9	Extensive lesions on all plant parts accompanied by stem girdling

Percent seed infection has been used in Canada to measure the resistance of lentil cultivars/lines. The rationale is to alleviate the problem of subjectivity of foliar rating (Slinkard and Vandenberg, 1993). However, the difficulties with this method are: it is time consuming and expensive; it is not suitable for earlier generations of breeding because the limited number of seeds per line; the resistance of early maturing materials may be underestimated due to the saprophytic infection from late maturing materials. Nevertheless, percent seed infection is the ultimate measurement of resistance in practice, and it can be used in the late stage of breeding when seed supply is not a problem and lines can be grouped according to their maturity.

10.4 Genetics of Ascochyta blight resistance

Breeding for resistance has been suggested as an efficient means to reduce/avoid the economic loss caused by Ascochyta blight in lentil based on the

observation that resistance differences exist in both cultivated and wild lentils (see next section), and the success in other crops for major fungal diseases. To be successful and efficient in breeding, genetic resources that contain different genes for resistance must be available, the genetic regulation of the resistance needs to be well understood and techniques for accumulating the effects of different genes need to be established.

10.4.1 Genetic resources of Ascochyta blight resistance

Genotypic differences for Ascochyta blight resistance is present in both cultivated and wild lentil. Disease symptoms on the different accessions ranged from small flecks (resistant) to extensive lesions on both leaves and stems with death of some plants (highly susceptible) (Nasir and Bretag, 1998). A detailed summary was given in Chapter 2.

10.4.2 Inheritance of Ascochyta blight resistance

The genetics of blight resistance in lentils has been studied using segregation analysis. Several resistance genes have been discovered and are summarised in Table 10.2

Table 10.2: Genetic regulation mechanisms of Ascochyta blight resistance in lentil.

Resistant Parent	Susceptible Parent	Organ	Inheritance model	Reference
Indian head	PI 345635	seed	two duplicated recessive	Andrahennadi, 1994
Indian head	Titore	foliar	two additive recessive	Chapter 5
W6 3241 (*L. orientalis*)	Invincible	foliar	one dominant gene	Ahmad *et al.*, 1997b
W6 3241	Titore	foliar	one dominant gene	Chapter 5
W6 3261 (*L. orientalis*)	Invincible	foliar	one dominant gene	Ahmad *et al.*, 1997b Parh, 1998
W6 3261	Titore	foliar	one dominant gene	Ahmad *et al.*, 1997b
Resistant Parent	Susceptible Parent	Organ	Inheritance model	Reference

Table 10.2 (*continued).*

W6 3222 (*L. odemensis*)	Titore	foliar	two dominant complementary	Ahmad *et al.* 1997b
W6 3261	Titore	foliar	one partial dominant with large effect & one dominant with less effect	Chapter 5
W6 3261	Olympic	foliar	one dominant gene	Ahmad *et al.*, 1997b
W6 3192 (L. *eroides*)	Titore	foliar	two dominant complementary	Ahmad *et al.*, 1997b Chapter 5
Laird	Eston	seed	one recessive gene	Tay and Slinkard, 1989
Laird	Titore	foliar	one recessive gene	Chapter 5
ILL5588	Eston	foliar	one dominant gene	Ford *et al.*, 1999
ILL5588	Eston	seed	one dominant gene	Andrahennadi, 1997 Vakulabharanam *et al.*, 1997
ILL5588	Eston	seed	two dominant &one recessive	Tay, 1989
ILL5588	Eston	seed	one dominant & one recessive	Sakr, 1994
ILL5588	Titore	foliar	one dominant for high and one dominant for moderate resistance	Chapter 5
ILL5684	Eston	seed	one dominant gene	Tay and Slinkard, 1989
ILL5684	Titore	foliar	one dominant gene	Chapter 5
ILL5684	Eston	seed	two dominant genes	Tay, 1989
ILL5684	Eston	seed	two dominant genes	Tay, 1989

Using recombinant inbreds from two crosses between resistant (ILL 5684 and W 6 3241) and susceptible (Titore) lines, which are homozygous for the major resistance gene, Chapter 6 showed that minor genes contributed to the resistance. Though the accumulated effect of minor genes was much less than the effect of a major gene, they would provide more durable resistance than major genes at least from a theoretical point of view. When attempting selection within populations of plants with the same major genotype, the use of a 1 - 9 scoring system is less likely to be successful. A novel system, which distinguishes minor differences between

infection levels, is required. It is possible that the detected relative small effect of minor genes was due to the masking effect of the major gene. In other words, minor genes may confer much more resistance than has been discovered till now. If this is the case, sufficient resistance may still exist when the major gene resistance is broken down.

Using generation mean analysis with six basic generations of four crosses, Chapter 7 showed that genetic regulation of resistance is more complicated with significant inter-loci interaction in three of four crosses studied. Dominant effects played an important role in all crosses. The genetic mechanisms underlying seed resistance measured as percent seed infection were more complicated that those for foliar resistance.

Based on these studies, it is clear that Mendelian segregation analysis, though useful, can only provide an oversimplified genetic model, and that more careful dissection of genetics of Ascochyta blight resistance in lentils is required.

10.5 Breeding for resistance

10.5.1 Current breeding method

Breeding for resistance to Ascochyta blight in lentil has been initiated without much knowledges of the genetics of resistance and pathogenic variations. In many contries, it is still at a very preliminary stage, that is, large scale screening of collections of germplasm to identify resistant resources. Multi-location testing of promising cultivars from germplasm screening co-ordinated by ICARDA has led to the registration of several resistant cultivars in several countries (Russell, 1994; Singh *et al.*, 1994; Erskine *et al.*, 1996). A well-organised breeding programme in Canada has produced several cultivars with good resistance. Because the knowledge about the genetic variation of the pathogen and the genetic regulations of host resistance is currently very limited, well-designed breeding programmes are not yet possible. However, the recent identification of resistance genes and their relationships in several cultivated lines (Chapter 5) and the confirmation of the presence of pathotypes (Nasir and Bretag, 1997b) provided the basis to design a breeding programme aimed at transferring and combining these genes. Currently, breeding for resistance should

use the same method as that for other traits except that several tests for resistance are added. A combined bulk population and pedigree selection has been used successfully in lentil and chickpea breeding (Singh, 1994; Muehlabuer *et al.*, 1995) at ICARDA. A modified version of this procedure has been used with good success in breeding for Ascochyta blight resistance in chickpea (Singh, 1993). It could be used in breeding for Ascochyta blight in lentil as well. This procedure consists of seven steps.

1) Lines with Ascochyta blight resistance to multiple pathotypes are crossed to locally adapted superior cultivars. They are then backcrossed to the adapted parent (if necessary). F_1s are grown in optimal conditions to produce seeds.
2) F_2's are tested for resistance in the field in epiphytotic form by artificial inoculation. Selected resistant (rating 1-4) and partially resistant (5-6) plants are bulk harvested.
3) F_3 and F_4 bulks are advanced in disease-free conditions and selected for growth habit, branch number, flowering time, maturity and seed size. The selected plants in the F_4 generation are harvested individually.
4) F_5/F_6 progenies are tested for resistance. The effects of minor genes are more obvious due to several generations of recombination.
5) Selected lines are evaluated in preliminary yield trials. High yielding lines are selected.
6) Multi-location testing of the selected lines. The locations should cover the target cultivation region of the new cultivars. Tests for yield and other important traits and tests for Ascochyta blight resistance may need to be done separately. One or two superior lines with good combinations of traits can be selected.
7) On-farm test of the selected lines and cultivar registration.

The above breeding procedure relies on multi-location testing to obtain cultivars with broad resistance. This method is justified because only resistance to the prevalent pathotypes in the target region is required by producers. However, there is no guarantee that the resistant cultivars identified will have durable resistance.

10.5.2 Future breeding methods

With the development of novel techniques and the accumulation of our understanding about the host-pathogen system, more efficient breeding methods no doubt will be applicable in breeding for resistance to Ascochyta blight in lentil.

Multiple resistance by gene pyramiding

When there are different pathotypes and corresponding resistance genes, one method to breed for resistance is to combine different resistance genes into a single cultivar. This is so-called gene pyramiding. By combining genes conferring resistance to different pathotypes, the cultivar can be used in more diversified environments where different pathotypes are likely to be dominant. Additionally, multiple resistance genes may have additive effects. Even if they do not show additive effects, the presence of more genes implies that pathotypes have to be virulent to all the genes before a resistant cultivar will lose resistance (Crute, 1998). This procedure has been suggested for breeding for durable resistance in many crops. A breeding programme for improving resistance to Ascochyta blight of chickpea in ICARDA adopted this method (Singh, 1993). This method should also be used in lentil. Firstly, the different pathotypes have recently been identified by Nasir and Bretag (1997b). They found that the pathotype "6" can infect all the lines tested including the cultivars previously identified as resistant such as ILL 5588. Therefore, resistance to *A. lentis* conferred by major genes is pathotype-specific as well. Secondly, the introduction of resistant cultivars with major resistance genes in production (such as Laird and ILL 5588) will no doubt increase the chance of the development of pathotypes which can overcome the resistance (Burdon, 1993). In fact, the large-scale cultivation of the moderately resistant cultivar, Laird has been speculated as the cause of the increased aggressiveness of Canadian isolates of *A. lentis* (Ahmed and Morrall, 1996)

Exploring slow blighting and partial resistance

Some cultivars/lines may not be highly resistant in late stages but do have considerable resistance in early stages. The relatively slow development of disease has been identified in rust diseases in cereals where it has been used successfully in breeding for resistance (Wilcoxson, 1981). In breeding for Ascochyta blight

resistance in chickpea, slow blighting has been explored and consequently a cultivar with partial but durable resistance was released in eight countries (Singh, 1993). Though there are no reports on the existence of slow blighting in lentils, it is possible that this type of resistance can be identified in lentil after more carefully examining the disease reactions of more germplasm.

Using wild relatives

As discussed above, genes for Ascochyta blight resistance have been identified in wild lentil species, and it seems that more resistance resources are in the gene pool of wild relatives. Therefore, to transfer the resistance genes from wild species into elite cultivars will be an important strategy in breeding for resistance. Viable hybrids between the cultivated and wild species are essential to the utilisation of genes in wild relatives by conventional breeding. The only wild species that can easily be intercrossed with cultivated lentil is *Lens orientalis,* the progenitor of cultivated lentil (Ladizinsky 1979; 1993). A recent report showed that viable hybrids could be obtained from the combinations between the cultivated and all wild lentil species by applying GA_3 after pollination (Ahmad *et al.*, 1995b). Upon improvement, this technique may lead to an efficient method to transfer useful genes including resistance genes from all wild species into cultivated species

In vitro culture has been used in two ways to promote the utilisation of wild relatives in lentil. Cohen *et al.*, (1984) established a two stage *in vitro* technique for the development of inter-specific hybrid embryos. Fourteen days old fertilised ovules were cultured on MS medium supplemented with zeatin, followed by release of the embryos from the ovular integuments. These embryos later developed into viable and vigorous plants. Ladizinsky *et al.*, (1985) also obtained vegetatively normal *L. culinaris* × *L. ervoides* hybrids using embryo culture techniques.

Several biological characteristics of wild lentil, such as very small flower size, low number of seeds per pod, and possible post fertilisation abortion, make artificial crossing difficult. Therefore, the success rate for artificial crossing is very low even if there is no post fertilisation abortion. Micropropagation of the limited F_1 materials has been explored as a way to enlarge the F_1 population and eliminate the requirement of large-scale pollination to obtain enough hybrid material for further genetic study

and breeding. In the context of multiplying hybrids by *in vitro* culture, different systems may be required for different purposes. To produce plants for genetic analysis, an *in vitro* propagation method aimed at producing genetically true-to-type plants is useful. A system specially intended for this purpose was developed based on single node culture in Chapter 3. To enhance the production of novel genotypes that combine the favorable genes from both species, an efficient regeneration system is required. Two highly efficient multiplication systems based on seed culture and cotyledonary node culture were developed in Chapter 4. These can be used to multiply the limited hybrid seeds obtained by artificial crosses and consequently generate a large amount of breeding materials for future selection. The rationale of this approach is to introgress small chromosome fragments of wild relatives and reduce the number of backcrosses (Larkin, 1987). Larkin *et al.* (1990) have provided experimental support to such a proposal using tissue culture of wheat chromosome addition lines (with a rye or a barley chromosome).

With the refinement of *in vitro* culture techniques and an artificial crossing method, it becomes possible to use resistance genes contained in wild species. Backcrossing can then allow transfer of all useful genes of wild species to generate new lines with novel gene(s). These new lines can be released as new cultivars and/or used as initial materials to combine different resistance genes.

Marker assisted introgression (MAI)

Genes conferring resistance are conventionally introgressed into an elite background by repeated backcrossing. If, instead of tracking the gene itself, a marker tightly linked to the gene is used to trace its segregation in the selection process, then this method is referred as maker-assisted introgression (MAI). MAI has several obvious advantages. 1) The tedious and troublesome test for resistance is not required, and selection can be done in normal environments; 2) Selection can be done at an earlier stage and hence generation intervals may be reduced; 3) The transfer of both dominant and recessive genes is feasible; 4) The results from both theoretical study and practical application have shown that MAI reduces the necessary population size and the number of generations required for developing commercial varieties (Hillel *et al.*, 1990; Melchinger, 1990; Lee, 1995; Michelmore, 1995; Visscher *et al.*,

1996; Hospital *et al.*, 1997; Mohan *et al.*, 1997; Bent and Yu, 1998;). This is because flanking markers can be used to identify the backcross lines that are heterozygous for target genome regions. Advancing only these selected lines will also reduce linkage drag and allow for selection of genotypes with the maximum percentage of the recurrent parent genome.

For resistance conferred by major genes, classical Mendelian linkage analysis can be used to identify linked markers. Marker genotypes and genotypes for resistant genes are determined for plants in a segregating population and the linkage between marker and trait is confirmed by estimating recombination rate. However, to generate DNA markers is costly and time-consuming. Better strategies are required to facilitate the identification of markers linked to a target trait. Ideally, few DNA samples are used to screen markers and most of the DNA markers used for genotyping individual plants are linked to the target trait. Near-isogenic lines (NILs) produced from backcrossing programs to introgress resistance genes are ideal materials for identifying linked markers (Penner *et al.*, 1993). As NILs are genetically identical except for the locus conferring resistance, markers represented by bands, which appear only on DNA from resistant NILs, must link to the gene for resistance. Unfortunately, NILs for Ascochyta blight in lentils are unavailable. Bulk segregation analysis proposed by Michelmore *et al.*, (1991) does not require special materials and has become a standard practice in this field. Two DNA pools formed by mixing DNA from highly resistant individuals and highly susceptible individuals are used to detect polymorphism. Markers represented by bands, which appear only for one of the DNA pools, are likely to be linked to the gene for resistance or susceptibility. Then the linkage and recombination rates between these markers and resistant gene are confirmed and estimated using Mendelian linkage analysis. Using this method, Ford *et al.*, (1999) identified seven RAPD markers linked to the resistance locus conferring Ascochyta blight resistance in ILL 5588. Five of the seven RAPD markers were within 30 cM of the resistance locus and the closest flanking markers were approximately 6 and 14cM away from the resistance locus. However, to use MAI, the linkage between the resistance gene and markers must be stable across generations and populations, and the technique for detecting the marker(s) must be efficient so that large populations can be screened. In these terms, there are inherent difficulties in the application of RAPDs in MAI, such as band

reliability between runs and multiple step protocols. To overcome these problems, sequence-characterised amplified regions (SCARs) or allele-specific associated primers (ASAPs) (Weeden *et al.*, 1992) have been described. The SCARs and ASAPs use longer primers in pairs to specifically amplify a DNA fragment linked to a gene of interest. These SCARs and ASAPs are obtained by sequencing the ends of a RAPD, originally amplified by a single short primer. Once developed, SCARs and ASAPs shorten the protocol necessary to identify individuals with the markers, as dye or fluorimetric quantification replaces electrophoretic detection of amplified DNA fragment. SCARs have been described for many plant host-pathogen systems (Adam-Blondon *et al.*, 1994; Deng *et al.*, 1997; Paran and Michelmore, 1993; Witsenboer *et al.*, 1995). Based on the RAPD markers identified, SCARs need to be developed to make MAI feasible for breeding for Ascochyta blight resistance.

Transgenic technology

Transgenic technology provides plant breeders with new tools in breeding for resistance. Its main advantages are :1) linkage drag, which refers to the inheritance of undesirable genes with the desirable gene, can be avoided and consequently repeated backcrossing to the elite parent is not required and considerable time saving is achieved; 2) Resistance genes from different sources including artificial constructs can be used. This should make plant breeders less reliant on natural variation for resistance genes; 3) By inducing or enhancing plant protective mechanisms, durable and/or multiple resistance may be achieved by a single engineering effort. Because its great potential in improving disease resistance, extensive studies using plant species that can be easily transformed have been conducted. Therefore, several promising procedures have been demonstrated and used in economically important crops (Bushnell *et al.*, 1998; Bent and Yu, 1999). In view of the results achieved for other species, transgenic techniques should also be explored in lentil breeding, particularly in breeding for Ascochyta blight resistance.

Several authors have tried genetic transformation in lentil (Warkentin and McHughen, 1993; Chowrira *et al.*, 1995; Maccarrone *et al.*, 1995; Oktem, 1999). These studies clearly showed that transformation could be achieved as confirmed by GUS assay, but regeneration of transgenic plants was very difficult. This is not unexpected. On one hand, the regeneration systems available may not be efficient

enough. According to experiences from other species, to achieve successful production of transgenic plants, the regeneration system needs to be highly efficient. This can only be achieved by optimising the factors affecting regeneration including the use of competent genotypes, suitable sources of explants and medium composition. One the other hand, only one attempt has been made to produce transgenic lentil plants. Even with good regeneration systems, the production of transformed plants is not a simple matter, and many experiments are required to refine the whole process. The regeneration system based on cotyledonary node culture was refined in Chapter 3. Comparing the efficiency of this refined protocol with that of the regeneration system used for successful transformation of pea (Jordan and Hobbs, 1993), it is expected that this system should be suitable for transformation. Oktem (1999) obtained transgenic shoots from cotyledonary nodes containing GUS gene transferred by particle bombardment. Electroporation of DNA into intact nodal meristem has resulted in the production of transgenic plants (Chowrira *et al.*, 1995). Therefore, production of transgenic lentil plants and consequently the application of transgenic techniques is soon likely to become feasible.

10.5.3 Factors that need to be considered in breeding for resistance

Correlated selection response

The changes in other traits caused by selection for a trait is termed the correlated selection response (Falconer, 1989). When two traits are positively correlated, the correlated selection response is desirable from a breeder's point of view and consequently does not bring any difficulty. However, when traits are negatively correlated, care must be taken to reduce the unfavourable response in other traits. The correlations among Ascochyta blight resistance and other agronomic traits have not been fully investigated. Chapter 5 showed that the major gene for resistance in ILL 5588 and W6 3241 had adverse effects on seed yield/plant. According to the experiences of breeding for resistance in other legume species, some other possible adverse effects of selection for Ascochyta blight resistance may be; 1) anti-nutritional factors are increased, which is undesirable both for human and animal consumption of grain; 2) digestibility is reduced, and; 3) maturity is delayed (Singh, 1993; Porta-

Puglia *et al.*, 1994). Conversely, correlation between traits can also be used in breeding via indirect selection. The most obvious application of indirect selection in breeding for resistance is to use glasshouse (growth chamber) test results to select for improved resistance performance in the field.

Genotype-by-environment interaction

Genotype-by-environment (GE) interaction is of concern if the resultant cultivar is to be used across a large area. There are two reasons why GE interaction is more important in breeding for resistance than for other traits. Firstly, pathogens may vary in their aggressiveness under different environments. Furthermore, if different physiological races exist they may be differentially prevalent in different environments. Secondly, the growth, development, and physiological status of host genotypes may be different under different environments. There is a paucity of information regarding the GE interaction in lentil. However, the different levels of aggressiveness among isolates from different locations, and the recent identification of pathotypes suggest that GE interaction would be important if tested.

10.6 Conclusion

Considerable progress has been made in the last two decades in understanding the Ascochyta blight pathogen (*A. Lentis*) and the resistance of its host to this disease. Resistance sources are available both in cultivated and wild lentil species. Several major resistance genes have been identified, and minor genes have also been shown to play a role in the resistance. Different pathotypes are present in the pathogen population and resistance conferred by major genes seems to be pathotype-specific. However, many challenges remain both for plant pathologists and for breeders. From a breeding point of view, more studies in the following areas are urgently required to sustain breeding progress.

1. Identify more resistance resources: Identification and incorporation of new sources of resistance genes into the breeding programme is always necessary. Large

scale screening of germplasm for resistance is required. What is important for the future screening of germplasm is to characterise the resistance reactions more carefully. It is necessary to evaluate the resistance several times and/or to test against different isolates. Three types of germplasm should be selected: 1) highly resistant with rating 1 - 2, 2) germplasm with slow blighting and 3) germplasm with multiple resistance.

2. Careful characterisation of the host-pathogen interaction system: To identify and incorporate new sources of resistance genes into the breeding programme, it is required to have a good understanding about the pathogenic variability of *A. lentis*. A recent study has led to the recognition of six different pathotypes (Nasir and Bretag, 1997b). However, since the numbers of cultivars and isolates used were small, it is expected that more pathotypes may be present. Molecular marker based methods have been successfully used to provide additional information on the pathogenic variability and have the potential to fingerprint pathotypes in many host-pathogen systems including Ascochyta blight in chickpea (Weising *et al.*, 1991; Morjane *et al.*, 1997; Udupa *et al.*, 1997; 1998). It can be expected that a better understanding of the pathogenic variability of *A. lentis* will soon be obtained by using these novel techniques. Armed with the knowledge of pathogen populations, the host genotypes can be easily determined using different pathotypes to challenge host genotypes, and consequently gene pyramiding becomes feasible (Crute, 1988).

3. Identification of molecular markers tightly linked to different resistance genes: with the development of modern molecular techniques, more and more marker systems become available and their costs are reduced. It is likely that it will be possible to develop rapid and cost-effective techniques for screening large populations for markers linked to resistance genes. Once developed, the benefits of marker assisted selection (introgression) will be available.

CHAPTER 11

GENERAL DISCUSSION

11.1 Background

Lentil is one of the most important grain legumes and ranks fifth in terms of production area (FAO, 1995) but first in terms of consumer's preference in many countries (Saker *et al.*, 1991). A threat to lentil production is Ascochyta blight, caused by *Ascochyta lentis*, present in almost all lentil-growing countries (Erskine *et al.*, 1994). *Ascochyta lentis* can affect all plant parts and can survive year after year in crop residues and infected seeds (Pedersen *et al.*, 1993). Breeding for host resistance has been suggested as the most efficient, effective and environmentally-friendly approach to reduce or avoid the losses from this disease (Erskine *et al.*, 1994; Ye *et al.*, 2000). This suggestion is based on the experiences with other major crops and the fact that resistant germplasm is available in cultivated and wild lentils (Bayaa *et al.*, 1991; 1994).

Success in any breeding programme requires, the availability of desirable genetic resources, the better understanding of the genetics of the target traits and suitable techniques to utilise the desirable genes. Extensive screening of lentil germplasm has identified many cultivars/lines which are resistant to Ascochyta blight. This forms the basis of breeding for resistance (see Chapter 2 for a summary). However, the genetic mechanisms underlying Ascochyta blight resistance in lentil are not well understood. Some useful techniques which can speed up the breeding processs such as techniques to facilitate the transfer of resistant gene from wild relatives to superior cultivated cultivars, has not yet established. Therefore, the overall objective of this study was to provide genetic information and useful techniques for designing and implementing resistance breeding programs.

As with other naturally self-pollinated species, artificial hybridisation is necessary both for creating useful gene combinations for selecting superior cultivars or breeding materials and for creating materials for genetic analysis. However, the success rate of hand pollination is very low in lentil due to its tiny and fragile flowers,

and post fertilisation abortion (for inter-specific crossing). Therefore, methods are required to multiply the limited F_1 hybrids obtained from artificial crossing. *In vitro* culture has been shown to be an efficient multiplication method in many crops including legumes (Ahuja, 1993; Kyte and Kleyn, 1996). When developing *in vitro* multiplication protocols, the utilisation of the resultant plants must be taken into consideration. Efficient procedure for producing genetically true-type plantlets is necessary for genetic study using the resultant plantlets, even if this is at the expense of multiplication efficiency. No *in vitro* protocol reported in the literature, which could be safely used for this purpose. An efficient procedure for this purpose was established based on stimulating elongation of the axilary buds (Chapter 3). The application of this multiplication approach produced about 20 plants from a single seed in 3 months or about 100 plants in 4 months. Though no genetic and/or molecular methods were used to confirm the genetic stability of plantlets from this protocol, somoclonal variation was unlikely to be induced, because no cytokinins and very low concentration of GA_3 /or IBA were used for shoot elongation. This system was used extensively in this study to get sufficient F_1 plants and consequently other advanced generations (i.e. F_2) for carrying out genetic analysis.

In the context of producing many plants from interspecific hybrids, *in vitro* regeneration has been proposed for promoting somatic recombination between chromosomes of parental species (Larkin and Scowcroft, 1981; Larkin, 1987; Larkin *et al.*, 1990). This method has the potential to successfully introgress small chromosome fragments of wild relatives and reduces the number of backcrossing generations required. Larkin *et al.* (1990) have provided experimental support to such a proposal using tissue culture of wheat chromosome addition lines (with a rye or a barley chromosome). An efficient multiplication system is also required for successful genetic transformation, which is a more direct method to obtain resistant cultivar than the backcross-based methods. Two systems, one based on multiple shoot induction from intact seedlings and the other based on cotyledonary node culture (Chapter 4) were developed for this purpose. The shoot regeneration rates and rooting frequencies of the induced shoots of these two systems were much higher than any *in vitro* protocols reported previously. Compared with the cotyledonary node system for pea transformation, it is expected that the systems developed here have good potential for achieving successful genetic transformation in lentil.

With the establishment of a procedure for multiplying F_1 hybrids, it is relatively easy to obtain sufficiently large populations of advanced generations and consequently materials for genetic analysis. All previous studies of the genetics of Ascochyta blight resistance in lentils used Mendelian segregation analysis, and consequently different inheritance models of major genes were proposed as summarised in Chapter 2. Unlike for other species, where several segregating populations are commonly used, all the studies in lentil used F_2 populations only, and these F_2 populations were usually small, presumably due to the difficulty of obtaining sufficient F_1 plants. It is necessary to confirm these proposed models first. Furthermore, the allelic relationships among major genes need to be tested before a suitable breeding strategy can be developed, if the major gene resistance is confirmed. For instance, resistance conferred by allelic genes cannot be combined in the same plants. To achieve these purposes, segregation patterns of F_2, BC_1, and BC_2 populations of the crosses between four resistant cultivars (lines) and two susceptible lines with reported resistant genes were fitted to different inheritance models (Chapter 5). In total, ten major genes were identified from the cultivars used. The inheritance models for major genes found were: one dominant gene for high resistance and one dominant gene for moderate resistance (ILL 5588), a single dominant gene (ILL 5684 and W6 3241), two complementary dominant genes (W3 3192 and Titore), two recessive genes with additive effect (Indian head), one recessive gene (Laird) and one partial dominant gene with large effect and one dominant gene with less effect (W3 3261). The gene in ILL 5684 is allelic to the one in ILL 5588 for high resistance.

There are several reasons why minor genes may be involved in the resistance. Firstly, many varieties show different resistance levels and no complete resistance has been identified. Secondly, in the resistant and susceptible classes formed to facilitate the determination of a major gene model, the frequencies of plants with different scores were different. Therefore, resistance to Ascochyta blight in lentils may be the result of both major gene(s) and minor genes. The contributions of minor genes were confirmed for the first time by creating recombinant inbreds with the same major genotypes but different minor genotypes from two crosses. The results and their implications were discussed in Chapter 6.

The existence of both major gene(s) and minor genes in resistance to Ascochyta blight implies that the results from the Mendelian segregation analysis

(Chapter 5 and all previous studies) were only approximate. Therefore, the generation mean analysis and a procedure based on a mixed model (assuming one major and polygenes) were used to gain further understanding of the genetics of Ascochyta blight resistance and were reported in Chapter 7 and 8.

Generation mean analysis uses the information from different generations to detect the overall genetic effect and partition the genetic effect into additive, dominant and epistatic effects. Generation mean analysis was done for four crosses in this study (Chapter 7). The six basic generations are sufficient to model foliar resistance, whereas they are not sufficient for seed resistance measured as seed infection rate in two crosses. The underlying genetic mechanisms of seed resistance were more complicated than that of foliar resistance. Dominance played an important role in all crosses for both seed and foliar resistance. Except for the foliar resistance in the cross ILL 5684 x Titore, at least one type of inter-gene effect (epstastic) contributed to the increased/reduced resistance.

The mixed model analysis of the cross ILL 5588 × Titore suggested that foliar resistance was controlled by two additive-dominance-epistasis genes. Both genes were from the resistant parent ILL 5588, and dominance was towards resistance. Only the additive A and dominant B was toward to resistance. Seed resistance was controlled by one dominant major gene and additive-dominance-epistasis polygenes. The major resistant gene and most of the polygenes for resistance were from the resistant parent as well, and the dominance was towards resistance. The additive –by-additive component of the interloci interaction of polygenes favoured resistance, while the additive-by-dominant, and the dominant-by-dominant components favoured susceptibility. More than 70% of the phenotypic variations in segregating populations were due to the major gene (Chapter 8).

From the plant breeding point of view, Ascochyta blight resistance is only one of the traits of interest and a cultivar must be superior for multiple traits. Ideally, selection for resistance should not bring too many negative effects on other traits. The major gene in ILL 5684 for foliar resistance to Ascochyta blight is linked to the genes for seed yield/plant or it has a pleiotropic effect on seed yield/plant, while it is independent of the genes for plant height and days to flowering. Fortunately, within each set there were significant differences among inbreds for all the three traits, and the estimates of heritability based on inbred mean were high for seed yield/plant and

days to flowering, and moderate for plant height. Thus, selection within-set was feasible (Chapter 9). For the set with the major resistance gene, 1) disease severity was not correlated with seed yield/plant and plant height, but weakly and negatively correlated to days to flowering when measured under disease pressure. 2) Seed yield/plant was strongly and positively correlated with plant height, moderately and negatively correlated with days to flowering, and plant height was weakly and positively correlated with days to flowering under inoculated and uninoculated conditions. 3) Inbred × environment interaction is not important and selection can be done with or without artificial inoculation. Thus, selection should be carried out within the set of inbreds with the major resistance gene to utilise the resistance conditioned by minor genes and to improve seed yield/plant and other traits (Chapter 9).

Based on the results from this study and previous studies, a breeding method suitable for the current situation was developed, and breeding methods applicable in the future were discussed in Chapter 10.

11.2 Contributions of this study

1. An efficient *in vitro* multiplication system for producing genetically true-to-type plants was developed and used to enlarge the populations of F_1 hybrids. The rationale of this procedure is widely applicable to any self-pollinated species for which there are difficulties in obtaining enough hybrid seeds for plant improvement programmes.

2. Two highly efficient *in vitro* regeneration systems were developed based on the multiple shoot formation from intact seedlings and cotyledonary nodes. These two systems have good potential for genetic transformation via *Agrobacterium*, which has not yet been achieved in lentil, partially due to the low efficiency of regeneration.

3. For the first time the genetic regulation of the foliar resistances to Ascochyta blight in ILL 5684, Indian head and Laird were studied, and corresponding major gene inheritance models were proposed. Previously only two reports (Ahmad *et al.*, 1997b; Ford *et al.*, 1999) have studied the inheritance of foliar resistance. The added information produced here should promote breeding for resistance. New major gene models for the foliar resistance of ILL 5588 and W3 3261 were suggested; and the allelic relationships between these major genes were established.

4. For the first time, minor genes were confirmed to contribute to the resistance to Ascochyta blight in lentil. The implication of this is that selection for resistance in the population of plants with the same major genotypes is effective and/or necessary. By doing this, it will be possible to obtain recombinant inbreds with resistance higher than the resistant parent.

5. It was shown that the inheritance of seed resistance measured using seed infection rate is more complicated than that of foliar resistance. Dominance was found to be important in the regulation of foliar and seed resistance, and epistasis was involved in the inheritance of seed infection in all the crosses used in this study.

6. For the first time, the effects of a major resistance gene on seed yield/plant, plant height and days to flowering were studied. Under disease free environment, resistant cultivars with a major gene may yield less than cultivars without the resistant gene. Genetic variations for all these three traits within the resistant inbred population are high with moderate to high estimates of inbred mean heritability. Resistance conferred by minor genes is not correlated with seed yield/plant and plant height, but moderately correlated with days to flowering.

7. The importance of sample size and using F_3 plants in detecting inheritance models were highlighted.

8. A new method for analysing mixed inheritance, which provides more insights into the genetic mechanisms of a quantitative trait, was applied to study the genetics of plant disease resistance for the first time.

9. A method for breeding for Ascochyta blight resistance in lentil was developed.

11.3 Suggested further studies

1. ***Testing whether the resistance genes identified also confer resistance to other pathotypes***: Ten major resistance genes were identified in this study using only one isolate of *A. lentis*. A recent study has led to the recognition of six different pathotypes (Nasir and Bretag; 1997b). It may be expected that more pathotypes will be identified in the future. It is well known that a major gene conferring resistance to one pathotype may/may not confer resistance to other pathotypes. To guide the design of a breeding strategy for obtaining durable resistance, the interaction between resistance genes and pathotypes needs to be understood. Therefore, all the resistance genes identified in lentil, including those reported in this thesis, need to be evaluated using more pathotypes.

2. ***More careful investigation of the contributions of minor genes***: Although the effects of minor genes in the inheritance of Ascochyta blight resistance have been established in this study, as discussed in Chapter 6, the measurement scale used may underestimate the effects of minor genes. Studies using other measurements, such as latent period, the area under the disease progress curve, and infected leaf area, are needed.

3 ***Genetic relationships among Ascochyta resistance and other agronomic traits***: Resistance is unlikely to be the only objective of a breeding programme and multitrait selection is essential. To make multitrait selection efficient the genetic

correlations among Ascochyta blight resistance and other agronomic traits are required. Although there are some studies of the genetic correlations among agronomic traits, no studies have taken Ascochyta blight resistance into account. Moreover, this study found that the major genes for resistance in ILL 5588 and W6 3241 had adverse effects on seed yield/plant (Chapters 6 and 9). Similar investigations should be done for other major genes as well.

4. ***Identification of markers linked to different resistance genes***: Transfer of resistance from resistant sources into elite genetic background to create new cultivars is a time-consuming process. This is particularly true, when genes from wild species are used, since repeated backcrossing is required to eliminate linkage drag. Indirect selection using markers tightly linked to the resistance has several advantages (Chapter 10). Many techniques for generating markers have been developed and novel techniques are likely to become available in the future. At present, AFLP and SSR-PCR, which was shown to be more reliable and efficient than RAPD, may be good choices of marker-generating procedures. However, as with RAPDs, these two procedures generate dominant markers, which cannot distinguish homozygous resistant plants from heterozygous plants. Conversion of the identified dominant markers into sequence characterised amplified regions (SCARs) may be necessary to simplify the detection of resistance gene markers and facilitate the selection of resistant recombinant inbreds.

5. ***Genetic transformation***: Genetic transformation has shown good success in generating disease- and insect- resistant breeding materials. With the two *in vitro* regeneration systems developed in this study, successful genetic transformation via *Agrobacterium* may be expected and should be attempted in future.

6. ***Establish efficient techniques for transferring resistance genes from wild lentils***: No attempts have been made in this study to increase the production of interspcific hybrid seeds and to overcome post fertilisation abortion. This clearly is important if wild lentil are to be used in breeding. Embryo culture has proved to be useful in rescuing interspecific hybrids in lentil (Cohen *et al.*, 1984; Ladizinsky *et al.*,

1985). GA_3 treatment after pollination improved seed setting of interspecific crosses (Ahmad *et al.*, 1995b). Studies are needed in these areas to successfully transfer resistant genes from wild lentils, particularly, *L. nigricans* and *L. odemensis*.

REFERENCES

Abbo S, Ladizinsky G and Weeden NF 1992. Genetic analysis and linkage study of seed weight in lentil. *Euphytica*. 58: 259-266

Abo-Elwafa A, Murai K and Shimada T 1995. Intra- and inter-specific variations in Lens revealed by RAPD markers. *Theor. Appl. Genet.* 90: 335-340

Agrawal RL 1998. Fundamentals of plant breeding and hybrid seed production. 394 pp.; Science Publishers, Inc.; Enfield, New Hampshire; USA.

Ahmad M, Fautrier AG, Burritt DJ and McNeil DL 1995a. Genetic diversity and relationships in Lens species and their F1 interspecific hybrids as determined by SDS-PAGE. *NZ. J. Crop Hort. Sci.* 25: 99-108.

Ahmad M, Fautrier AG, McNeil DL, Burritt DJ and Hill GD 1995b. Attempts to overcome postfertilization barrier in interspecific crosses in the genus Lens. *Plant Breeding*. 114:558-560.

Ahmad M, Fautrier AG, McNeil DL, Hill GD and Burritt DJ 1997a. In vitro propagation of Lens species and their F1 interspecific hybrids. *Plant Cell, Tiss.Org. Cult.* 47:169-176

Ahmad M, Fautrier AG and McNeil DL 1997b. Identification and genetic characterisation of different resistance sources to Ascochyta blight within the genus Lens. *Euphytica* 97: 311-315.

Ahmad M, McNeil DL, Fautrier AG, Armstrong KF and Paterson AM 1996. Genetic relationships in Lens species and parentage determination of their interspecific hybrids using RAPD markers. *Theor. Appl. Genet.* 92: 1091-1098

Ahmed S and Beniwal SPS 1991. Ascochyta blight of lentil and its control in Ethiopia. *Tropical Pest Management*. 37: 368-373

Ahmed S and Morrall RAA 1996. Field reactions of lentil lines and cultivars to isolates of Ascochyta fabae f. sp. Lentis. *Can. J. Plant Pathol.* 18: 362-369.

Ahmed S, Morrall RAA and Sheard JW 1996a. Distribution of mating types of

Ascochyta fabae f. sp. Lentis. *Can. J. Plant. Pathol.* 18: 347-353.

Ahmed S, Morrall RAA and Sheard JW 1996b. Virulence of Ascochyta fabae f. sp. lentis on lentils. Can. J. Plant Pathol. 18: 354-361.

Ahuja MR (eds). 1993. Micropropagation of woody plants. Dordrecht, Boston, Kluwer Academic Publishers.

Alam MM, Ilyas MB and Iftikhar K 1993. Screening of chickpea and lentil germplasm against their Ascochyta blight diseases. *Pakist. J. Phytopathology.* 5: 110-114

Adam-Blondon AF, Seviganc M, Bannerot H and Dron M 1994. SCAR, RAPD and RFLP markers linked to a dominant gene (Are) conferring resistance to anthracnose in common bean. *Theor. Appl. Genet.* 88:865-870.

Anderson VL and Kempthorne O 1954. A model for the study of quantitative inheritance. *Genetics.* 39: 883-898

Andrahennadi CP 1994. Genetics and linkage of isozyme markers and resistance to seedborne ascochyta infection in lentil. M.Sc. Thesis, Department of Crop Science and Plant Physiology, University of Saskatchewan, Canada

Andrahennadi C P 1997. RAPD markers for Ascochyta blight resistance, phylogenetic studies and cultivar identification in lentil. PhD thesis, University of Saskatchewan, Saskatoon, Canada.

Andrahennadi CP, Slinkard AE and Vandenberg A 1996. Ascochyta resistance in lentil. *Lens Newsl.* 23: 5-7

Asins MJ and Carbonell EA 1988. Detection of linkage between restriction fragment length polymorphism markers and quantitative traits. *Theor. Appl. Genet.* 76: 623-626

Bahraei S 1991. Karyotype study on lentils (Lens culinaris Med.) of Iranian origin. *Seed and Plant.* 7: 1-7

Bajaj YPS 1995. Biotechnology in agriculture and forestry 34: Plant protoplasts and genetic engineering VI. Springer-Verlag; Berlin, Germany.

Bajaj YPS and Dhanju MS 1979. Regeneration of plants from apical meristem tips of

some legumes. *Curr. Sci.* 48: 906-907

Bayaa B, Erskine and Hamdi A 1991. Screening wild lentil for resistance to vascular wilt and Ascochyta blight disease. Proceeding 4th Arab Congress of Plant Protection, Cairo, December 1991

Bayaa B, Rescan and Hammed A 1994. Response of wild lentil o Ascochyta Faber f. sp. Lentils from Syria. *Genetic and Crop Evolution.* 41: 61-65

Beauchamp CJ, Morrall RAA and Slinkard AE 1986a. The potential for control of ascochyta blight with foliar-applied fungicides. Can. J. Plant Pathol. 8: 254-259.

Beauchamp CJ, Morrall RAA and Slinkard AE 1986b. Effects of scheduling applications of benomyl, caprafol and chlorothalonil on ascochyta blight of lentil. *Can. J. Plant Pathol.* 8: 260-268

Bedi S 1990. Sources of inoculum and management of Ascochyta fabae f. sp. lentis on lentil. Thesis, MSc., University of Saskatchewan, saskatoon, Canada.

Bent AF and Yu IC 1999. Applications of molecular biology to plant disease and insect resistance. *Adv in Agron.* 66: 251-299

Berger K and Schaffner W 1995. In vitro propagation of the leguminous tree Swartzia madagascariensis. *Plant Cell Tiss. Org. Cult.* 40: 289-291

Bhuyan AK, Pattnaik S and Chand PK 1997. Micropropagation of curry leaf tree [Murraya koenigii (L.) Spring.] by axillary proliferation using intact seedlings. *Plant Cell Rep.* 16: 779-782

Bondartzeva- Monteverde VN and Vassilievsky NI 1940. A contribution to the biology and morphology of some Ascochyta on Leguminosae. Acta Institute of Botanical and Academic Sciences, USSR., Ser. II pp 354:376.

Bonney GE 1984. On the statistical determination of major gene mechanisms in continuous human traits: regressive models. *Am. J. Med. Genet.* 18: 731-749

Burdon JJ 1993. Genetic variation in pathogen populations and its implications for adaptation to host resistance. In Jacobs Th and Parlevliet JE (eds) Durability of

disease resistance. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Burdon RD 1977. Genetic correlations as a concept for studying genotype-environment interaction in forest tree breeding. *Silvae Genet.* 26: 168-175

Bushnell WR, Somers D A, Giroux RW, Szabo LJ and Zeyen RJ 1998. Genetic engineering of disease resistance in cereals. *Can. J. Plant. Pathol.* 20: 137-220.

Campbell KW, Hamblin AM and White DG 1997. Inheritance of resistance to Aflatoxin production in the crosses between corn inbreds B73 and LB31. *Phytopathology.* 87: 1144-1147

Cannings C, Thompson EA and Skolnick MH 1978. Probability functions on complex pidegrees. *Adv. Appl. Prob.* 10: 26-61

Carbonell and Asins 1996. Statistical models for QTL analysis. In Jian SM, Sopory SK and Veillex RE (eds) In vitro haploid production in higher plants. Vol 2: 255-285

Carbonell EA, Gerig TM, Balansard E. and Asins MJ 1992. Interval mapping in the analysis of nonadditive quantitative trait loci. *Biometrics.* 48: 305-315

Chague V, Mercier JC, Guenard M, de Courcel A and Vedel F 1997. Identification of RAPD markers linked to a locus involved in quantitative resistance to TYLCV in tomato by bulked segregant analysis. *Theor. Appl. Genet.* 95: 671-677

Chowrira GM, Akella V and Lurquin PF 1995. Electroporation-mediated gene transfer into intact nodal meristem in planta: generating transgenic plants without in vitro tissue culture. *Mol. Biotech.* 3: 17-23

Christine BR and Shattuck VI. 1992. The diallel analysis: design, analysis, and use for plant breeders. *Plant Breed. Rev.* 9-35

Coates ST and White GD 1998. Inheritance of resistance to Gray Leaf Spot in crosses involving selected resistant inbred lines of corn. *Phytopathology* 88: 972-982

Cohen D, Ladizinsky G, Ziv M and Muehlbauer FJ 1984. Rescue of interspecific Lens hybrids by means of embryo culture. *Plant Cell Tiss. Org. Cult.* 3: 343-347

Cowen NM 1988. The use of replicated progenies in marker based mapping of QTLs.

Theor. Appl. Genet. 75: 857-862

Crute IR 1988. The elucidation and exploitation of gene-for-gene recognition. Plant Pathol. 47: 107-113

Demenasis FM and Bonney GE1989. Equivalence of the mixed and regressive models for genetic analysis. I. Continuos traits. *Genet. Epidemiol.* 6: 597-617

Deng Z, Huang S, Xiao S and Gmitter FG 1997. Development and characterisation of SCAR markers linked to the citrus tristeza virus resistance gene from Poncirus trifoliata. Genome 40: 697-704

Distabanjong K and Geneve RL 1997. Multiple shoot formation from cotyledonary node segments of eastern redbud. *Plant Cell, Tiss.Org. Cult.* 47: 247-254

Dudley JW 1993. Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. *Crop Sci.* 33: 660-668

Edwards JH 1960. The simulation of Mendelism. *Acta Genet. Stat. Med.* 10:63

Edwards MD and Page NJ 1994. Evaluation of marker-assisted selection through computer simulation. *Theor. Appl. Genet.* 88: 376-382.

Edwards MD, Stuber CW and JF Wendl 1987. Molecular-marker-based investigations of quantitative trait loci in maize I. Numbers, genomic distribution and types of gene action. *Genetics.* 116: 113-125

Elkind Y and Cahanr A 1986. A mixed model for the effects of single gene, polygenes and their interaction on quantitative traits. I. The model and experimental design. *Theor. Appl. Genet.* 72: 377-383

Elston RC 1993. Some recent developments in the theoretical aspects of segregation analysis. In Majumder PP (eds) Human Population Genetics. Plenum Press, N.Y.

Elston RC and Rao DC 1978. Statistical modelling and analysis in human genetics. *Ann. Rev. Biophys. Bioeng.* 7: 253-286

Elston RC and Stewart J 1971. A general model for the genetic analysis of pedigree data. *Hum. Hered.* 21: 523-542

Erskine W and Bayaa B 1991. Screening for resistance to ascochyta blight. ICARDA Annual report. Pp 98-102

Erskine W and Bayaa B 1993. Screening for resistance to ascochyta blight. ICARDA Annual report. Pp 98-99

Erskine W, Bayaa B and Saxena MC 1996. Registration of ILL 5588 lentil germplasm resistant to vascular wilt and ascochyta blight. Crop Sci. 36:1080

Erskine W, Tufail M, Russell A, Tyagi MC, Rahman MM and Saxena MC 1994. Current and future strategies in breeding lentil for resistance to biotic and abiotic stresses. *Euphytica.* 73: 127-135

Esmail AM, Mohamed AA, Hamdi A and Rabie EM 1994. Analysis of yield variation in lentil (Lens culinaris Medik). *Ann. Agri. Sci. Moshtohor.* 32: 1073-1087

Eujayl I, Baum M, Erskine W and Pehu E 1995. Development of a genetic linkage map for lentil based on RAPD markers. 2nd European Conference on Grain Legumes, Copenhagen, Denmark

Eujayl I, Baum M, Erskine W, Pehu E and Muehlbauer FJ 1997. The use of RAPD markers for lentil genetic mapping and the evaluation of distorted F2 segregation. *Euphytica.* 96: 405-412

Eujayl I, Baum M, Powell W, Erskine W and Pehu E 1998a. A genetic linkage map of lentil (Lens sp.) based on RAPD and AFLP markers using recombinant inbred lines. *Theor. Appl. Genet.* 97: 83-89

Eujayl I, Erskine W, Baum M and Pehu E 1999. Inheritance and linkage analysis of frost injury in lentil. *Crop Sci.* 39: 639-642

Eujayl I, Erskine W, Bayaa B, Baum M and Pehu E 1998b. Fusarium vascular wilt in lentil: inheritance and identification of DNA markers for resistance. *.Plant Breeding.* 117: 497-499

Facolner DS 1989. Introduction to quantitative genetics. Longman, London.

FAO yearbook 1995. Production. 1995. FAO statistics series No 130, Food and

Agricultural Organisation of the United Nations, Rome, Italy

Ferguson ME and Robertson LD 1996. Genetic diversity and taxonomic relationships within the genus Lens as revealed by allozyme polymorphism. *Euphytica.* 91: 163-172

Ford R, Pang ECK and Taylor P 1999. Genetics of resistance to ascochyta blight of lentil and the identification of closely linked RAPD markers. *Theor. Appl. Genet.* 98: 93-98

Ford R, Pang ECK and Taylor PWJ 1997. Diversity analysis and species identification in Lens using PCR generated markers. *Euphytica.* 96: 247-255

Frances A, Tay J and Cotez M 1987. Desinfection de semillas en lentjas. Investigatciones y progresso Agropecuario, Quilamapu, Chile. 31: 22-26

Franklin CI, Trieu TN, Gonzales RA and Dixon RA 1991. Plant regeneration from seedling explants of green bean (Phaseolus vulgaris L.) via organogenesis. *Plant Cell, Tiss. Org. Cult.* 24: 199-206

Gai JY and Wang JK 1998. Identification and estimation of a QTL model and its effect. *Theor. Appl. Genet.* 97: 1162-1168

Gamborg OL, Miller RA and Oijima K 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Cult.* 10: 135-142

Garcia-Dorado A and Gallego A 1992. On the use of the classical tests for detecting linkage. *J. Hered.* 83: 143-146

Gardner CO and Eberhart SA 1966. Analysis and interpretation of the variety cross diallel and related populations. *Biometrics.* 22: 439-452

Ghaemi M and Sarrafi A 1993. Analysis of anther culture to measure genetic variability for embryogenesis in tetraploid wheat (Triticum turgidum var.). *J. Genet. Breed.* 47: 295-298.

Ghanem SA 1995. In vitro embryogenesis of lentil under saline conditions. Bulletin of Faculty of Agriculture, University of Cairo. 46: 113-125.

Giovannoni JJ, Wing RA, Ganal MW and Tanksley SD 1991. Isolation of molecular

markers from specific chromosomal intervals using DNA pools from existing mapping populations. *Nucleic Acids Res.* 19: 6553-6558

Gossen BD and Morrall RAA 1983. Effects of ascochyta blight on seed yield and quality of lentils. *Can. J. Plant Pathol.* 5: 168-173

Gossen BD, Sheppard JW, Beauchamp CJ and Morrall RAA 1986. Ascochyta lentis renamed Ascochyta fabae f.sp. lentis. *Can. J. Plant Pathol.* 8: 154-160

Griffing B 1950. Analysis of quantitative gene action by constant parent regression and related techniques. *Genetics.* 35: 303-321

Gulati A and Jaiwal PK 1994. Plant regeneration from cotyledonary node explants of mungbean (Vigna radiata (L.) Wileczek). *Plant Cell Rep.*13: 523-527

Gupta AK, Singh IS, Reddy MV and Bajpai GC 1997. Genetics of resistance to P3 isolate of Phytophthora blight in pigeonpea. *Euphytica* 95: 73-76.

Hackett CA 1994. Selection of markers linked to quantitative trait loci by regression techniques. In Van Ooijen JW (eds) Biometrics in plant breeding: application of molecular markers. Proc 9th Meet of the Eucapia section, Biometrics in plant breeding, 6-8, July 1994, Wageningen, The Netherlands.

Halbach T, Kiesecher H, Jacobsen HJ and Kathen A de 1998. Tissue culture and genetic engineering of lentil (Lens culinaris Medik.). In 3rd European Conference on Grain Legumes. Valladolid, Spain.

Haley CS and Knott SA 1992. A simple regression method for mapping quantitative trait loci in line crosses using flanking markers. *Heredity.* 69: 315-324

Hallauer AR and Miranda JB 1988. Quantitative genetics in maize breeding, 2nd ed. Iowa State University Press, Ames.

Hanson WD1959. Minimum family size for the planning of genetic experiments. *Agron. J.* 51: 711-715.

Havey MJ and Muehlbauer FJ 1989a. Variability for restriction fragment lengths and phylogenies in lentil. *Theor. Appl. Genet.* 77: 739-843

Havey MJ and Muehlbauer FJ1989b. Linkage between restriction fragment length, isozyme and morphological markers. *Theor. Appl. Genet.* 77:395-401

Hayman BI 1954. The analysis of variance of diallel crosses. *Biometrics.* 10: 235-244

Hayman BI 1957. Interaction, heterosis and diallel crosses. *Genetics.* 42: 336-355

Hayman BI 1958. The separation of epistatic form additive and dominance variation I generation means. *Heredity.* 12: 371-390

Hernandez-Fernandez MM, Carrozzo G and Blanco A. 1984. Inheritance of somatic embryogenesis in alfalfa (Medicago savita L.). *Genome.* 32: 318-321

Hillel J, Schaap T, Haberfeld A and Jeffreys Y 1990. DNA fingerprint applied to gene introgression breeding programs. *Genetics.* 124: 783-789

Hoffman DL, Soltis DE, Muehlbauer FJ and Ladizinsky G 1986. Isozyme polymorphism in lens (leguminosae). *Syst. Bot.* 11: 392-402.

Hoffman DL, Muehlbauer FJ and Ladizinsky G 1988. Morphological variation in Lens (Leguminosae). *Syst. Bot.* 13: 87-96

Holland JB, Uhr DV, Jeffers D, Goodman MM 1998. Inheritance of resistance to corn rust in tropical-by-corn-belt maize populations. *Theor. Appl. Genet.* 96: 232-241.

Hosechele I 1988. Statistical techniques for detection of major genes in animal breeding data. *Theor. Appl. Genet.* 76: 311-319

Hosptial F, Moreau L, Lacoudre F, Charcosser A, and Gallais A 1997. More on the efficiency of marker-assisted selection. *Theor. Appl. Genet.* 95: 1181-1189

Hyland FC and Quaas RL 1991. Benefits from marker-assisted selection under varying assumptions. *J. Dairy Sci.* 74: 282

Iqbal SM, Bakhsh A, Malik RA 1990. Identification of resistant sources to ascochyta blight in lentil. *Lens Newsl.* 17: 26-27

Iqbal SM, Hussian S, Tahir M and Malik BA 1992. Effect of fungicidal seed treatment on lentil germination and recovery of seedborne Ascochyta fabae. *Lens Newsl.* 19: 52-55.

Jain SK, Madaria SK, Rao SK and Nigam PK 1995. Analysis of yield factors in lentil. *Indian J. Agri. Res.* 29: 173-180

Jansen RC 1992. A general mixture model for mapping quantitative trait loci by using molecular markers. *Theor. Appl. Genet.* 85: 252-260

Jansen RC 1993. Interval mapping of multiple quantitative trait loci. *Genetics*. 135: 205-211

Jansen RC 1996. Complex plant traits: time for polygenic analysis. *Trends in Plant Sci.* 1: 89-94

Jansen RC and Stam P 1994. High solution of quantitative traits into multiple loci via interval mapping. *Genetics*. 136: 1447-1455

Jinks JL 1956. The F2 and backcross generations from a set of diallel crosses. *Heredity*. 10: 1-30

Jordan MC and Hobbs SLA 1993. Evaluation of a cotyledonary node regeneration system for Agrobacterium-mediated transformation of pea (Pisum sativum). *In Vitro Cell. Dev. Bio.* 291: 77-82

Kaiser WJ 1981. Disease of chickpea, lentil, pigeon pea, and tepary bean in continental United States and Puerto Rico.con. *Bot.* 35: 300-320

Kaiser WJ and Hannan, RM 1986. Incidence of seed-borne ascochyta lentis in lentil germplasm. *Phytopathology*. 76: 605-610.

Kaiser WJ and Hellier BC 1993. Didymella sp., the teleomorph of Ascochyta fabae f. sp. Lentis.on lentil straw. *Phytopathology* 83:692

Kaiser WJ, Hannan RM and Rogers JD 1994. Factors affecting growth and sporulations of Ascochyta fabae f. sp. Lentis. *Plant Dis.* 76: 605-610

Kaiser WJ, Wang BC and Rogers JD 1997. Ascochyta fabae and A. lentis: host specificity, teleomorphs (Didymella), hybrid analysis, and taxonomic status. *Plant Dis.* 81: 809-816

Kaneda Y, Tabei Y, Nishimura S, Harada KT and Kitamura K 1997. Combination of

thidiazuron and basal media with of salt concentrations increases the frequency of shoot organogenesis in soybean [Glycine max (L.) Merr.]. *Plant Cell Rep.* 17: 8-12

Kapoor S, Singh G, Gill AS and Singh B 1990 Lentil lines resistant to Ascochyta blight. *Lens Newsl.* 17: 26-28

Kearsey MS and Pooni HS 1996. The genetic analysis of quantitative traits. 381pp, London, Chapman and Hall.

Khatri HL and Singh K 1975. Lentil blight- a new record for India. Labdev. Bull.13: 73-74

Kim JH, LaMotte CE and Hack E 1990. Plant regeneration in vitro from primary leaf nodes of soybean (Glycine max.) seedlings. *Plant Physiol.* 136: 664-669

Knapp SJ 1991. Using molecular markers to map multiple quantitative trait loci: model for backcross, recombinant inbred and doubled haploid progeny. *Theor. Appl. Genet.* 81: 333-338

Knapp SJ, Bridges WC and Birkes D 1990. Mapping quantitative trait loci using molecular marker linkage maps. *Theor. Appl. Genet.* 79: 583-592

Knott SA and Haley CS 1992. Aspects of maximum likelihood methods for the mapping of quantitative trait loci in line crosses. *Genet. Res. Camb.* 60: 139-151

Kole C, Teutonico R, Mengistu A, Williams PH and Osborn TC 1996. Molecular mapping of a locus controlling resistance to Albugo candida in Brassica rapa. *Phytopathology.* 86: 367-369

Kothari SL, Chandra N and Sharma VK 1991. Multiple shoot formation from cotyledonary node and shoot apex cultures of Glycier max. Soybean Genet. *Lens Newsl.* 18: 148-149

Kyte L and Kleyn J 1996. Plants from test tubes: an introduction to micropropagation. 3rd ed. Portland, Or : Timber Press.

Ladizinsky G 1979. The origin of lentil and its wild genepool. *Euphytica.* 28: 179-187.

Ladizinsky G 1993. Wild lentils. *Crit. Rev. Plant Sci.* 12: 169-184

Ladizinsky G, Braun D and Muehlbauer FJ 1984. The biological species of the genus Lens. *Bot. Gaz.* 145: 253-261

Ladizinsky G, Cohen D and Muehlbauer FJ 1985. Hybridization in the genus Lens by means of embryo culture. *Theor. Appl. Genet.* 70: 97-101

Lande R 1994. Marker-assisted selection in relation to traditional methods of plant breeding. Plant Breeding in 90's.

Lande R and Thompson R 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. *Genetics.* 124: 743-756

Lander ES and Botsein D 1989. Mapping mendelian factors underlying quantitative traits using RFLPs linkage maps. *Genetics.* 121: 185-199

Larkin PJ 1987. Somaclonal variation: history, method and meaning. *Iowa State J. Res.* 61: 393:434

Larkin PJ and Scoweroft 1981. Somaclonal variation - a novel source of variability from cell culture for plant improvement. *Theor. Appl. Genet.* 60: 197-214

Larkin PJ, Spinder LH and Banks PM 1990. Cell culture of alien chromosome addition lines to induce somatic recombination and gene introgression. In Nijkamp HJJ, Van Derplas LHW and Van Aartrijk J eds Progress in plant cellular and molecular biology. Kluwer Academic Publishers. P.O.Box 17,3300, AA Dordrecht, the Netherland.

Lawson WR, Goulter KC, Henry RJ, Kong GA and Kochman JK 1996. RAPD markers for a sunflower rust resistance gene. *Aus. J. Agri. Res.* 47: 395-401

Lazar MD, Baenziger PS and Schaeffer GW 1984. Combining abilities and heritability of callus formation and plantlet regeneration in wheat (Triticum aestivum L.) anther cultures. *Theor. Appl. Genet.* 68: 131-134

Lee M 1995. DNA markers and plant breeding programs. *Adv. Agron.* 55: 265-344.

Lu C 1993. The use of thidiazuron in tissue culture. *In Vitro Cell Dev. Biol.* 29:92-96

Lui BH 1998. Computational tools for study of complex traits. In Paterson AH (eds)

Molecular dissection of complex traits. Pp 43-79.

Luo ZW and Kearsey MJ 1989. Maximum likelihood estimation of linkage between a marker gene and a quantitative locus. *Heredity*. 63:401-408

Luo ZW and Kearsey MJ 1991. Maximum likelihood estimation of linkage between a marker gene and a quantitative locus. II. Application to backcross and doubled haploid populations. *Heredity*. 66: 117-124

Maccarrone M, Veldink GA, Agro AF and Vliegenthart JFG 1995. Lentil root protoplasts: a transient expression system suitable for coelectroporation of monoclonal antibodies and plasmid molecules. *Biochim. Biophy. Acta Gen. Subj.* 1243: 136-142

Maisonneuve B, Bellec Y, Anderson P and Michelmore RW 1994. Rapid mapping of two genes for resistance to downy mildew from Lactuca serriola to existing clusters of resistance genes. *Theor. Appl. Genet.* 89: 96-104

Malick MA and Rashid A 1989. Induction of multiple-shoots from cotyledonary node of grain legumes, pea and lentil. *Biologia Planta*. 31: 230-232

Malik KA and Saxena PK 1992a. Thidiazuron induces high-frequency shoot regeneration in intact seedlings of Pea (Pisum sativum), chickpea (Cicer arietinum) and lentil (Lens culinaris). *Aust J. Plant Physiol.* 19: 731-740

Malik KA and Saxena PK 1992b. Regeneration in Phaseolus vulgaris L.: high-frequency induction of direct shoot formation in intact seedlings by N6-benzylaminopurine and thidiazuron. *Planta*. 186: 384-389

Martinez O and Currow RN 1992. Estimating the location and the sizes of the effects of quantitative trait loci using flanking markers. . *Theor. Appl. Genet.* 85: 423-434

Mather K 1949. Biometrical genetics. Methuen, London

Mather K and Jinks JL 1982. Biometrical genetics: the study of continuous variation. 3rd edn. Chapman and Hall, New York.

Mayer MS and Soltis PS 1994. Chloroplast DNA phylogeny of Lens (Leguminosae):

origin and diversity of the cultivated lentil. *Theor. Appl. Genet.* 87: 773-781

Melchinger AE 1990. Use of molecular markers in breeding for ologogenic disease resistance. *Plant Breeding*. 104:1-19

Michelmore RW 1995. Molecular approaches to manipulation of disease resistance genes. *Ann. Rev. Phytopathol.* 33: 393-427

Michelmore RW, Paran I and Kesseli RV 1991. Identification of markers linked to disease-resistance genes by bulked sergregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA*. 88: 9828-9832

Mitchell-Olds T and Bergelson J 1990. Statistical genetics of annual plant, Impatiens capensis. I. Genetic basis of quantitative variation. *Genetics*. 124: 407-415

Mitidieri IZ and De M 1974. Lentil (Lens culinaris Medik) spot caused by Ascochyta lentis B. and Vassil. In Agrentina. Rev. *Plant Pathol.* 58: 462.

Mittal RK 1997. Effect of sowing dates and disease development in lentil as sole and mixed crop with wheat. J. Mycol. *Plant Pathol.* 27: 203-209

Mode CJ and Robinson HF 1959. Pleiotropism and the genetic variance and covariance. *Biometrics*. 15: 518-537

Mohan M, Nair S, Bhagwat A, Krishna TG, Yano M, Bhatia CR and Sasaki T 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. *Mol. Breed.* 3: 87-103

Moreau L, Charcosser A, Hosptial F and Gallais A 1997. Marker-assisted selection efficiency in populations of finite size. *Genetics*.

Morjane H, Geistlinger J, Kahl G, Harrabi M and Halila H 1997. Genotypic diversity of Tunisian Ascochyta rabiei on micro- and macro-geographical scales. In Udupa SM and Weigand F (eds) DNA markers and breeding for resistance to ascochyta blight in chickpea. Proceedings of a symposium held at Aleppo, Syria, 11-12 April 1994. 79-97.

Morrall RAA 1997. Evolution of lentil diseases over 25 years in western Canada. *Can. J.*

Plant Pathol. 19: 197-207

Morrall RAA and Sheppard JW 1981. Ascochyta blight of lentils in western Canada: 1978-1980. *Can. Plant Dis. Surv.* 61: 7-13

Morton ME and McLean CJ 1974. Analysis of family resemblance. III. Complex segregation analysis of quantitative traits. *Am. J. Hum. Genet.* 26: 489-503

Mosconi C, Crino P, Saccardo F and Singh KB 1996. Effect of culture filtrate of Ascochyta rabiei on chickpea genotypes. *Petria.* 6: 137-148

Muehlbauer FJ, Weeden NF and Hoffman 1989. Inheritance and linkage relationships of morphological and isozume loci in lentil (Lens miller). *J. Hered.* 80: 298-303

Muehlbauer FJ, Kaiser WJ, Clement SL and Summmerfield RJ 1995. Production and breeding of lentil. *Adv. Agron.* 54: 283-332

Muench DG, Slinkard AE and Scoles GJ 1991. Determination of genetic variation and taxonomy in lentil (Lens Miller) species by chloroplast DNA polymorphism. *Euphytica.* 56: 213-218

Murashige T and Skoog F 1962. A revised medium for rapid growth and bioassays with Tabasco tissue culture. *Physiologica Plantarum.* 15: 473-497

Nair S, Bentur JS, Prasada RU and Mohan M 1995. DNA markers tightly linked to a gall midge resistance gene (Gm2) are potentially useful for marker-aided selection in rice breeding. *Theor. Appl. Genet.* 91: 68-73

Namkoong G 1979. Introduction to quantitative genetics in forestry. USDA Gen. Tech. Bull. No 1588. 342 p

Nandanwar RS and Narkhede MN 1991 Intraspecific variation in karyotype of lentil. *J. Maharashtra Agri. Universities.* 16: 24-27

Nasir M and Bretag TW 1996. Screening lentil for resistance to Australia isolates of Ascochyta blight. *Lens Newsl.* 23: 7-9

Nasir M and Bretag TW 1997a. Prevelence of Ascochyta fabae f. sp. Lentis. On lentil seed from Victoria, Australia. *Aus. Plant. Pathol.* 26: 117-120

Nasir M and Bretag TW 1997b. Pathogenic variability in Australian isolates of Ascochyta fabae f. sp. Lentis. *Aus. Plant Pathol.* 26: 217-220

Nasir M and Bretag TW 1998. Reactions of lentil accessions from 25 different countries to Australian isolates of Ascochyta lentis. *Genetic Resources and Crop Evolution.* 45: 297-299

Nene YL, Hanounik SB, Qureshi SH and Sen B1988. Fungal and bacterial foliar disease of peas, lentils, faba bean and chickpea. In World crops: Cool Season Food Legumes (Ed Summerfield RJ) pp. 577-589. Kluwer Academic Publishers, Dordrecht, The Netherlands

Oktem HA1999. GUS gene delivery and expression in lentil cotyledonary nodes using particle bombardment. *Lens Newsl.* 26: 3-6

Ordon F, Bauer E, Friedt W and Graner A 1995. Marker-based selection for the ym4 BaMMV-resistance gene in barley using RAPDs. *Agronomie.* 15: 481-485

Oss H van, Aron Y and Ladizinsky G 1997. Chloroplast DNA variation and evolution in the genus Lens Mill. *Theor. Appl. Genet.* 94: 452-457

Ou G, Wang WC and Nguyen HT 1989. Inheritance of somatic embryogenesis and organ regeneration from immature embryo cultures of winter wheat. *Theor. Appl. Genet.* 78: 137-142

Papp E 1980. Flowering biology of lentil. *Lens Newsl.* 8:8

Paran I, Kesseli R and Michelmore R 1991. Identification of restriction fragment polymorphism and random amplified polymorpic DNA markers linked to downy mildew resistance genes in lettuce using near isogenic lines. Genome 34: 1021-1027

Paran I and Michelmore RW 1993. Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor. Appl. Genet.* 85:985-993.

Parent JG and Page D 1995. Evaluation of SCAR markers to identify raspberry cultivars. *HortScience* 30:856 (abstract).

Parh D K 1998. Interspecific mapping of the ascochyta gene(s) of lentil (Lens culinaris

Medikus) using RAPD technology. MS thesis, Lincoln University, Canterbury, New Zealand.

Parh DK, Conner AJ, Jacobs JME and McNeil DL 1998. Shoot-tip necrosis and alleviation during in vitro culture of Lens culinaris. *SABRAO J. Breed. Genet.* 30: 97-101

Paterson AH, DeVerna JW, Lanini B and Tanksley SD 1990. Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes, in an interspecifices cross of tomato. *Genetics*. 124:735-742

Patil PB, Vrinten PL, Scoles GJ and Slinkard AE 1995. Variation in the ribosomal RNA units of the genera Lens and Cicer. *Euphytica*. 83: 33-42

Patocchi A, Gianfranceschi L and Gessler C 1999. Towards the map-based cloning of Vf: fine and physical mapping of the Vf region. *Theor. Appl. Genet.* 99: 1012-1017

Pedersen EA and Morrall RAA 1994. Effects of cultivar, leaf wetness duration, temperature, and growth stage on infection and development of Ascochyta blight of lentil. *Phytopathology* 84: 1024-1030

Pedersen EA, Morrall RAA, McCartney HA and Fitt BDL 1993. Gradients of Ascochyta Blight in Saskatchewan lentil crops. *Plant Dis.* 77: 143-149

Pedersen EA, Morrall RAA, McCartney HA and Fitt BDL 1994. Dispersal of conidia of Ascochyta fabae f. lentils from infected lentil plants by simulated wind and rain. *Plant Pathol.* 43: 50-55

Penner GA, Chong J, Levesque-Lemay M, Molnar SJ and Fedak G 1993. Identification of a RAPD marker linked to the Oat stem rust gene Pg 3. *Theor. Appl. Genet.* 85: 702-705

Polanco MC, Pelaez MI and Ruiz ML 1988. Factors affecting callus and shoot formation from in vitro cultures of Lens culinaris Medik. *Plant Cell Tiss. Org. Cult.* 15: 175-182

Polanco MC and Ruiz ML 1997. Effect of benzylaminopurine on in vitro and in vivo root development in lentil, Lens culinaris Medik. *Plant Cell Rep.* 17: 22-26

Porta-Pauglia A, Bernier CC, Jellis GJ and Kaiser WJ 1994. Screening techniques and

sources to foliar diseases caused by fungi ad bacteria in cool season food legumes. In Muehlbauer FJ and Kaiser WJ (eds). Expanding the Production and Use of Cool Season Food Legumes. Kluwer Academic Publishers, Dordrecht, the Netherlands

Prakash NS, Pental D and Bhalla SN 1994. Regeneration of pigeonpea (Cajanus cajan) from cotyledonary node via multiple shoot formation. *Plant Cell Rep.* 13: 623-627

Pretorius ZA, Kloppers FJ and Drijepondt SC 1994. Effects of inoculum density and tempture on three components of leaf rust resistance controlled by Lr34 in wheat. *Euphytica* 74: 91-96

Ramesh B and Salimuddin 1992. Intervarietal variation for chromatin content in lentil. *Lens.* 19: 3-8

Ramgiry SR, Paliwal KK and Tomar SK 1989. Variability and correlations of grain yield and other quantitative characters in lentil. *Lens.* 16: 19-21

Raziuddin, Ahmed H, Altaf CM and Hanan F 1990. Investigation on the karyotype of lentil (Lens culinaris Medik). *Sarhad J. Agri.* 6: 261-264

Rebetzke GJ, Burton JW, Carter TE and Wilson RE 1998. Genetic variation for modifiers controlling reduced saturated fatty acid content in soybean. *Crop Sci.* 38: 303-308

Reddy MV and Singh KB 1993. Rate-reducing resistance to Ascochyta blight in chickpeas. *Plant Dis.* 77: 231-233

Reddy VRK and Annadurai M 1992. Cytological effects of different mutagens in lentil (Lens culinaris Medik). *Cytologia.* 57: 213-216

Reheul D and Ghesquiere A 1996. Breeding perennial ryegrass with better crown rust resistance. *Plant breeding* 115: 465-469

Reisch B and Bingham ET 1980. The genetic control of bud formation from callus cultures of diploid alfalfa. *Plant Sci. Lett.* 20: 71-77

Rodier AJ, Assie JL, Marchan D and Herve Y 1995. Breeding maize lines for complete and partial resistance to maize streak virus (MSV). *Euphytica* 81: 57-70.

Roundhill SJ, Fineran BA, Cole ALJ and Ingerfeld M 1995. Structural aspects of Ascochyta blight of lentil. *Can. J. Bot.* 73: 485-497

Russell AC 1994. Three new pulse cultivars for New Zealand's arable industry. *Proc. Ann. Conf. Agron. Soci. New Zealand.* 24: 125-128

Russell ÀC 1996. Identification of lentil (Lens culinaris) with resistance to ascochyta blight caused by Ascochyta fabe f. sp. lentis in New Zealand. Dissertation, Post-graduate Diploma, Lincoln University, Canterbury, New Zealand

Russell AC and Hill GD 1998. A detached leaf assay for screening of Lens culinaris for resistance to Ascochyta blight (Ascochyta fabae f. sp. lentis). 3rd European conference on grain legumes. Valladolid, Spain.

Sakr B 1994. Inheritance and linkage of genetic markers and resistance to ascochyta blight in lentil. PhD thesis, Washington State University, Pullman, Washington, USA

Salimuddin and Ramesh B 1994a. Karyotype, nuclear and chromosomal DNA variation in Lens culinaris Med. *Cytologia.* 59: 7-15

Salimuddin and Ramesh B 1994b. Intervarietal nuclear DNA variation in lentil. *Indian J. Pulses Res.* 7: 116-118

Sarker A, Erskine W, Sharma B and Tyagi MC 1999. Inheritance and linkage relationships of days to flower and morphological loci in lentil (Lens culinaris Medikus subsp. culinaris). *J. Hered.* 90: 270-275

Sakr, B., 1994. Inheritance and linkage of morphological markers and resistance to Ascochyta blight in lentil. PhD thesis, Washington State University, Pullman, Wash., USA.

SAS Institute Inc.1990. SAS/STAT/User guide, Volume 2, version 6, Fourth Edition, Cary, NC: SAS Institute Inc

Sax K 1923. The association of size differences with seed coat pattern and pigmentation in Phaseolus vulgaris. *Genetics.*8: 552-560

Saxena PK and King J 1987. Morphogenesis in Lentil: Plant regeneration from callus

cultures on Lens culinaris Medik via somatic embryogenesis. *Plant Sci.* 52: 223-227

Saxena PK, Malik KS and Gill R 1992. Induction by thidiazuron of somatic embryogenesis in intact seedlings of peanut. *Planta* 187: 421-424

Schenk RU and Hilldebrandt AC 1972. Medium and techniques for induction and growth of monocotyledonous and dicotylendonous plant cell cultures. *Can. J. Bot.* 50: 199-204

Sha L, McCown BH and Peterson LA 1985. Occurrence and cause of shoot-tip necrosis in shoot cultures. *J. Amer. Soc. Hort. Sci.* 110:643

Sharma SK, Dawson IK and Waugh R 1995. Relationships among cultivated and wild lentils revealed by RAPD analysis. *Theor. Appl. Genet.* 91: 4, 647-654

Sharma SK, Knox MR and Ellis THN 1996. AFLP analysis of diversity and phylogeny of Lens and its comparison with RAPD analysis. *Theor. Appl. Genet.* 93: 751-758

Sharma YR, Singh G and Kaur L 1995. A rapid technique for ascochyta blight resistance in chickpea. *Interna.Chickpea Pigeonpea Newsl.* No. 2: 34-35

Shekhawat NS, Rathore TS, Singh RP, Deora N and Rao SR 1993. Factors affecting in vitro clonal propagation of Prosopis cineraria. Plant Growth Regulation 12: 273-280.

Shetty K and Asano Y 1992. Stimulation of in vitro shoot organogenesis in Glycine (Merrill.) by allantion and amides. *Plant Sci.* 81: 245-251

Simpson SP 1989. Detection of linkage between quantitative trait loci and restriction fragment length polymorphisms using inbred lines. *Theor. Appl. Genet.* 85: 110-111

Singh G, Singh K, Gill SA and Barar JS 1982. Screening of lentil varieties and lines for blight resistance. *India Phytopathology.* 35: 678-679

Singh IS, Singh JP, Singh AK and Chauhan MP 1994. Pant Lentil 4: a high yielding, rust-, wilt- and blight-resistant variety for the North-Western Plains of India. *Lens Newsl.* 21: 8-9

Singh KB 1993. Experiences, difficulties and prospects of disease resistance breeding in chickpea. In Jacobs T and Parlevliet JE (eds) Durability-of-disease-resistance. 241-

248. Kluwer Academic Publishers; Dordrecht; Netherlands

Singh KB, Hawtin GC, Nene Y L and Reddy MV 1981. Resistance in chickpeas to Ascochyta rabiei. *Plant Dis.* 65: 586-587

Singh RK and Raghuvanshi SS 1989. Plantlet regeneration from nodal segment and shoot tip derived explants of lentil. *Lens Newsl.* 16: 33-35

Slinkard AE and Vandenberg A 1993. The past, present and future of pulse crops in Sakatchewan. Soils and /crops Workshop'93. Extension Division, University of Saskatchewan, Saskatoon, pp 226-231

Smith CAB 1986. Chi-square test with small numbers. *Ann. Hum. Genet.* 50:163-167

Soller M and Beckmann 1990. Marker-based mapping of quantitative trait loci using replicated progenies. *Theor. Appl. Genet.* 80: 205-208

Soller M and Beckmann JS 1983. Genetic polymorphism in varietal identification and genetic improvements. . *Theor. Appl. Genet.* 67: 25-33

Stuber CW, Edwards MD and Wendel JF 1987. Molecular marker-facilitated investigations of quantitative trait loci. II. Factors influencing yield and its components. *Crop Sci.* 27: 639-648

Tadmor Y, Zamir D and Ladizinsky G 1987. Genetic mapping of an ancient translocation in the genus Lens. *Theor. Appl. Genet.* 73: 889-892.

Tahir M and Muehlbauer FJ 1994. Gene mapping in lentil with recombinant inbred lines. *J. Hered.* 85: 306-310

Tahir M and Muehlbauer FJ 1995. Association of quantitative trait loci with isozyme markers in lentil (Lens culinaris L.). *J. Genet. Breed.* 49: 145-150

Tahir M, Muehlbauer FJ and Spaeth SC 1994. Association of isozyme markers with quantitative trait loci in random single seed descent derived lines of lentil (Lens culinaris Medik.). *Euphytica.* 75: 111-119

Tahir M, Simon CJ and Muehlbauer FJ 1993. Gene map of lentil: a review. *Lens Newsl.* 20: 3-10

Tanksley SD 1993. Mapping polygenes. *Ann. Rev. Genet.* 27: 205-233

Tay J 1989. Inheritance of resistance to ascochyta blight of lentil. M.Sc. Thesis, Department of Crop Science and Plant Ecology, University of Saskatchewan, Canada.

Tay J and Slinkard AE 1989. Transgressive segregation for Ascochyta resistance in lentil. *Can. J. Plant Sci.* 69: 547

Tourjee KR, Harding J and Byrne T 1994. Complex segregation analysis of Gerbera flower colour. *Heredity* 74: 303-310.

Tourjee KR, Harding J and Byrne T 1995. Interpretation of a selection plateau through complex segregation analysis: Euclidean distance index selection for flower colour in the Davis population of Gerbera. *Heredity* 75: 290-296.

Udupa SM and Weigand F 1997. DNA markers and breeding for resistance to ascochyta blight in chickpea. Proceedings of a symposium held at Aleppo, Syria, 11-12 April 1994. International Centre for Agricultural Research in the Dry Areas (ICARDA); Aleppo; Syria.

Udupa SM, Weigand F, Saxena MC and Kahl G 1998. Genotyping with RAPD and microsatellite markers resolves pathotype diversity in the ascochyta blight pathogen of chickpea. *Theor. Appl. Genet.* 97: 299-307

Upreti J and Dhar U 1996. Micorpropagation of Bauhinia vahlii Wright & Arnott- a leguminous liana. *Plant Cell. Rep.* 16: 250-254

Vaillancourt RE and Slinkard AE 1993. Linkage of morphological and isozyme loci in lentil, Lens culinaris L. *Can. J. Plant Sci.* 73: 917-926

Vakulabharanam VR, Slinard AE and Vandenberg A 1997. Inheritance of resistance to Ascochyta blight in lentil and linkage between isozyme. In Proc In Food Legume Res Conf III, Adelaide, Australia, p162

Verma DC, Litvay LD, Johnson MA and Einspahr DW 1983. Media development for cell suspensions of conifers. In Fujiwara, A. (ed.) 5th International Conference of Plant tissue and Cell Culture. Tokyo, Maruzen Co.

Visscher PM, Haley CS and Thompson R 1996. Marker-assisted introgression in backcross breeding programs. *Genetics.* 144: 1923-1932

Voisey CR, White DWR, Dudas B, Appleby RD, Ealing PM and Scott AG 1994. Agrobacterium-mediated transformation of white clover using direct shoot organogenesis. *Plant. Cell Rep.* 13: 309-314

Wang JK and Gai JY 1997. Identification of major gene and polygenes mixed inheritance and estimation of genetic parameters in F2 progeny. *Chinese J. Genet.* 24: 181-190

Warkentin TD and McHughen 1993. Regeneration from lentil cotyledonary nodes and potential of this explant for transformation by Agrobacterium tumefaciens. *Lens Newsl.* 20: 26-28

Weeden NF, Muehlbauer FJ and Ladizinsky G 1992. Extensive conservation of linkage relationships between pea and lentil genetic maps. *J. Hered.* 83: 123-129

Weising K, Kaemmer D, Epplen JT, Weigend F, Sexana M and Khal G 1991. DNA fingerprinting of Ascochyta rabiei with synthetic oligodeoxy-nucleotides. *Curr. Genet.* 19: 483-489

Weller JI 1986. Maximum likelihood techniques for the mapping and analysis of quantitative trait loci with the aid of genetic markers. Biometrics 42: 627-640

Whittaker JC, Curnow RN, Haley CS and Thompson R 1995. Using marker-maps in marker-assisted selection. *Genet. Res. Camb.* 66: 255-265

Wilcoxson RD 1981. Genetics of slow rusting in cereals. Phytopathology 71: 989-993

Williams DJ and McHughen A 1986. Plant regeneration of the legume Lens culinaris Medik (lentil) in vitro. *Plant Cell Tiss. Org. Cult.* 7:149-152

Williams DJ, McHughen A 1985. Plant regeneration of lentil in vitro. *Lens Newsl.* 12: 25

Witsenboer H, Kesseli RV, Fortin MG, Stanghellini M and Michelmore RW 1995. Sources and genetic structure of a cluster of genes for resistance to three pathogens in lettuces. *Theor. Appl. Genet.* 91: 178-188

Yamada Y 1962. Genotype by environment interaction and genetic correlation of the same trait under different environments. *Jap. J. Genet.* 37: 498-509

Ye G, McNeil DL and Hill GD 2000. Lentil Ascochyta blight and breeding for its resistance. *NZ Plant Protection* (in press).

Zamir D and Ladizinsky G 1984. Genetics of allozyme variants and linkage groups in lentil. *Euphytica* 33: 329-336

Zeng ZB 1993. Theoretical basis of precision mapping of quantitative trait loci. *Proc. Natl. acd. Sci. USA* 90: 10972-10976

Zeng ZB 1994. Precision mapping of quantitative trait loci. *Genetics.* 136: 1457-1468

APPENDICES

Based on the results obtained in this thesis and previous studies, a method for breeding for Ascochyta blight resistance in lentil was suggested in Chapter 10. This method is based on multi-location testing for selection of varieties with multiple resistance. Multilocation testing is required to determine the recommendation domains of any new cultivars from a breeding program. Multilocation testing is very costly, and careful design and data analysis are paramount. To develop a better analytical strategy for analysing multilocation testing data I have done an extensive literature review of the statistical methods for analysing plant variety trials. As a result, a review of two papers was prepared and presented in the 2000 New Zealand Agronomy Conference. Although, I did not conduct a multilocation test in my PhD study due to the time limitation, the methods I compiled should be very useful for any plant breeders. Therefore, this review is given here as appendices.

Some methods for analysing multisite plant variety trials

Estimating genotypic means at each site

Abstract

This paper is the first of two that discuss statistical methods for analysing multisite plant variety testing data sets. Methods for estimating genotypic means at each site are introduced. These methods include those that use data from each site including the classical and spatial methods as well as methods using data across all sites. The latter include the additive main effects and multiplicative interaction effect model (AMMI) and the multisite best linear unbiased prediction (BLUP). The implementation of these methods using SAS programs is outlined. The second paper deals with estimating and comparing genotypic means across sites and selection for stability and genotypic performance simultaneously.

Introduction

Multisite testing is commonly used in plant breeding programmes before new cultivars are recommended for release. The main purposes of multisite testing are to investigate genotypic performance over representative environments of the target production area, and to determine the areas where the tested genotypes are adapted (ie quantify genotype stability).

Genotypic means at each site and across sites provide the basic information for achieving these purposes. Surprisingly, practical breeders have paid little attention to this important aspect of their testing. Consequently arithmetic means are generally used regardless of the situations. Conversely, statisticians have long recognised that simple averages provide correct estimation of means only if some very stringent assumptions are satisfied (Cochran and Cox, 1957; Snedecor and Cochran, 1980; Pearce *et al.*, 1988). Many approaches have been developed to obtain more accurate estimates and/or comparison when the assumptions for using simple procedures cannot reasonably be made, but practical breeders frequently do not appreciate these approaches. One of the

reasons may be that the proposed methods are usually published in statistical journals that may not available to practical breeders. In this paper several procedures for estimating genotypic means at each site are given. A companion paper summarises the methods for estimating genotypic means across sites and comparisons among these means, and methods for simultaneous selection of performance and stability. Together these two papers form a discussion of some methods that practical breeders can use relatively easily to more effectively use data provided by multiple site testing.

Using observations at each site

Classical methods

A common practice in analysing multisite test data is to analyse the trial at each site separately. At each site, the trial is a typical one-factor experiment. Depending on the design used, standardised methods are available (Cochran and Cox, 1957; Pearce *et al.*, 1988). In the following sections, we assume the design at each site is a randomised complete block design (RCB) because it is the most commonly used design in multisite testing.

The arithmetic mean of a genotype across replicates is the easiest way to estimate a genotype's mean performance. This estimate is unbiased if observations are available for every replicate and the variance within replicates is the same (ie it is homogeneous). If there are missing values, the number of observations for each genotype is different and should be taken into consideration. The least-squares procedure has the property of providing estimates of means as if all the genotypes were included in all the replicates.

Spatial analysis

An important assumption for the above classical methods is that the residuals within each block are independently distributed with a constant variance. These methods are very inefficient if there is substantial heterogeneity within a block. This heterogeneity commonly arises if the number of genotypes tested is large. A group of statistical methods, collectively called spatial analysis, have been proposed to reduce the within-block heterogeneity. The following sections introduce several of these methods.

Papadakis's method and its modified versions

Papadakis (1937) first suggested that the performance of genotypes in a yield experiment should be adjusted for the local trend by an analysis of covariance with respect to the treatment (genotypic)-corrected yields of the adjacent plots. The working model for this method is

$$y_{ij} = \mu + g_i + \beta x_{ij} + \varepsilon_{ij}$$

where x_{ij} is the covariate calculated by taking the average of the genotype-corrected performance of its neighbouring plots, β is the regression coefficient associated with the covariate.

Note that the block effect is removed from this model because it is accounted for by the covariate. However, it may be better to keep the block effect in the model to ensure that the method never performed significantly worse than the randomised blocks model.

Bartlett (1978) suggested that an iterated version of this method should be used. The first iteration is the same as the above. The analysis is then repeated iteratively based on the adjusted genotypic means from the previous iteration until the difference between the adjusted genotypic means in successive iterations is negligible.

Wilkinson *et al* (1983) suggested that the original nearest neighbour means instead of the treatment-corrected neighbour means should be used to adjust the target observation.

The advantage of this class of methods is its simplicity. However, by using different neighbour plots different results may be obtained. In practice various ways to form the covariate(s) can be tried and the best one used for final analysis. Usually the nearest four neighbours are used. Sometimes, two covariates are used. One is formed by using the two longitudinal neighbours and the other is formed by the two latitudinal neighbours.

The effectiveness of this method has been verified using real data sets (i.e. Mak *et al.*, 1978; Kempton and Howes, 1981; Bhatti *et al.*, 1991; Brownie *et al.*, 1993; Stroup *et al.*, 1994).

The necessary computations can be done as follows. Firstly, the unadjusted genotypic effect and the corresponding residuals are obtained by standard analysis of variance. Secondly, new variable(s) are formed by taking the average of the residuals of the neighbouring plots and finally an analysis of covariance is done by including the new variable(s) as covariate(s).

Schwarbach' weighted nearest-neighbour analysis

The weighted nearest-neighbour analysis proposed by Schwarbach (1985) is based on the following working model

$$y_{ij} = \mu + g_i + \frac{3}{4}NND_a + \frac{3}{4}NND_b + \varepsilon_{ij} = \tau_i + \frac{3}{4}NND_a + \frac{3}{4}NND_b + \varepsilon_{ij}$$

where NND_a is the first nearest neighbour difference and is calculated as follows: For each plot, the observation is adjusted by subtracting the mean of the two nearest neighbours ($NND_1 = y_m - \frac{1}{2}(y_{m-1} + y_{m+1})$, then the mean of NND_1 for each genotype is computed and denoted as NND_a; NND_b is the second nearest neighbour difference and is calculated as: the mean of the genotype at the m-th plot adjusted by subtracting the average of the two genotypes at the two nearest neighbour plots ($NND_2 = \bar{y}_m - \frac{1}{2}(\bar{y}_{m-1} + \bar{y}_{m+1})$), and then the mean of the adjusted values for each genotype is computed and denoted as NND_b.

By simulation, Schwarbach (1985) showed that this procedure is better than Wilkinson *et al.* 's (1983) method. The effectiveness of this method was also proven by Stroup *et al.* (1994).

Trend analysis based on specific function form

The local trend may be described by a function of plot position. The general model is

$$y_{ijk} = \mu + g_j + f(R,C) + \varepsilon_{ijk} = \tau_i + f(R_j, C_k) + \varepsilon_{ijk}$$

where y_{ijk} is the observed performance of the i-th genotype at the jk-th plot (j-th row and k-th column), $f(R_j, C_k)$ is a function of the position given by the row (R_j) and column (C_k) values.

The difficulty with this type of method is to determine the right function form. Polynomial regression based on the row and column positions has been used successfully (Kirk *et al.*, 1980; Tamura *et al.*, 1988; Brownie, 1993). However, it is difficult to determine the optimal degree for the polynomial regression. Tamura *et al.* (1988) developed a program based on SAS to determine the most suitable polynomial function. Once the most suitable function form is defined, the GLM including all the variables can be used to estimate the genotypic means.

Methods based on smoothing the trend

The spatial trend can also be modelled by using methods that do not rely on specific function forms. The least-square smoothing method proposed by Green *et al.* (1985) assumed that the second differences of the trend (ie, $t_i - (t_{i-1} + t_{i+1})/2$) are independent and discovered the underlying trend pattern by choosing a weighted coefficient that gives a smooth trend and minimal residual variance. This approach can be understood as calculating the local trend as a weighted average of plot performance adjusted for differences among genotypes. Weights are inversely proportional to the distance from the adjusted plot and depend upon the observed "fertility trend" (Clarke *et al.*, 1994). Similarly, Hackett *et al.* (1995) introduced the generalised additive model to represent the spatial trend. In their method, the residuals are smoothed against the explanatory variable 'X' (normally the position of the plot) by the use of the locally-weighted running line smoother. For each value of X, say x, and some given value k, the k nearest neighbours are identified and assigned a weight according to their distance from x. The weight decreases from one for the neighbour with the same value of X to zero for the most distant of the k neighbours. These k neighbours of the plot are used to fit a linear relationship between the observation and the explanatory variables by weighted least squares and the fitted value at point x estimates the smooth function at that point.

Clarke *et al* (1994) applied the least-squares smoothing to 12 experiments with hexaploid wheat and found that it was more efficient than the classical methods and the Papadakis method. They also developed a PASCAL program based on a procedure suggested by Green *et al.* (1985) to find the weight coefficient and the estimate of genotypic effect. For the generalised additive model, Hackett *et al.* (1995) showed how

to implement it by use of the S-plus software. SAS function PROC TPSPLINE can be used as well.

Correlated error model

The existence of local trends in a field trial usually implies that the neighbouring plots are more alike than those farther apart. By using a less restrictive assumption about the residuals, better estimates of the genotypic means can be obtained. To accommodate the spatial correlation structure of the residuals, the semivariogram concept used in geostatistics to model spatial correlation structure is used. Semivariogram is defined as one half the variance of the difference between two observations a given distance apart, and measures the spatial variability as a function of the distance between observations.

Zimmerman and Harville (1991) and Stroup *et al.* (1994) showed that the correlated error model approach was efficient, whereas Brownie *et al.* (1993) found that accounting for a trend with a correlated errors structure only was not effective.

SAS procedure PROC MIXED provides several covariance functions to model the correlated error structure and may be sufficient for analysing most field trials. However, PROC MIXED does not compute the semivariogram *per se*. External estimates of the parameters for the covariance function are required. SAS procedure VARIOGRAM can be used to determine the theoretical semivariogram model by computing the sample/ empirical semivariogram from the observed data set. The two companion functions 'Fvariogram' and 'Mvariogram' of Genstat were designed for finding suitable covariance functions from the observed data. Once a semivariogram model is selected, the unknown parameters describing variance and the spatial correlation can be estimated using the REML procedure and the genotypic means are estimated using generalised least squares.

Besag and Kempton's (1986) first difference model

Besag and Kempton's (1986) first difference method assumes that the first differences between adjacent plots (ie. $z_i = y_{i+1} - y_i$) are uncorrelated random variables with identical variance (σ_t^2) and other sources of variation are negligible. In other words, the systematic trend is assumed to be removed completely from the observations by first difference operation.

The advantages of this method are that the computation is simple and that the linear component of the trend can be removed completely and consequently the estimates of genotypic effects are better than those from the classical methods. The disadvantage is that the resulting estimates may be less accurate if the linear component of the trend only takes a small proportion of its overall variation. Baird and Mead (1991) applied this method to analyse data sets generated from a range of yield models and concluded that this method was more efficient than a RCB analysis and an incomplete block analysis when the yields were from models with trend components.

Generalised linear model using the differenced observations as the raw data provides the estimates of the genotypic means (see Appendix one).

Linear variance model

An extension of the above first difference model is the so-called linear variance model described by Williams (1986) and Besag and Kempton (1986). The linear variance model superimposes a white noise term with variance (σ_ε^2) on the plot observations of the first difference model. This model removes the assumption that the first difference operation eliminates the local trend completely. It can be understood as a two-step detrending process, the first step is to remove the linear component of the trend. The second step is to model the remaining trend as a random effect with mean zero and variance σ_t^2. Therefore, it increases the power of detrending as confirmed by Besage and Kempton (1986), Baird and Mead (1991) and Wu and Dutilleul (1999).

The procedure LVARMODEL of Genstat is specially developed for this method.

Autoregressive integrated moving average (ARIMA) analysis

Gleeson and Cullis (1987) proposed a method that assumes that the "experimental error" is white noise and the "spatial trend" can be regarded as random and represented by an autoregressive integrated moving average (ARIMA) model. The d-degree difference operation is used to simplify the ARIMA model. This model can be viewed as a two-step detrending process as well. The first step is to reduce the trend effect by differencing the original data (not necessarily first difference) and the second step is to model the remaining trend effect by regarding it as a random process with a covariance

function. Because the difference operation and the covariance function can be selected based on the actual data set, this model is more flexible.

Cullis and Gleeson (1991) extended this procedure to two-dimensional spatial analyses. The two-dimensional analysis was shown to provide more efficient estimates of the genotypic means.

Using more than 1,000 variety trials, Cullis and Gleeson (1989) demonstrated that the use of this method resulted in a reduction of 42% in variances of variety yield differences compared with complete block analysis, whereas incomplete block analysis resulted in a reduction of 33%. Studies showed that usually very simple ARIMA models worked very well in analysing field trials (Gleeson and Cullis, 1987; Kempton *et al.*, 1994, Grondona *et al.*, 1996).

The main disadvantage of this approach is the complicated computation involved in obtaining the estimates of the variance components. However, if the estimation of the trend effect is not of interest, we may assume that the trend effect and the local error can be modelled by the ARIMA model. In this case, the analysis can be easily carried out based on differenced yield using SAS procedure MIXED in the same manner as for the correlated error model. Standard Procedures are also available in S-Plus and Genstat software for one- or two-dimensional ARIMA analysis.

Random field models

Zimmerman and Harville (1991) proposed random field models to accommodate local trends. The local trend is modelled by including the "large-scale variation" and "small-scale" variation. The "large-scale variation" is normally modelled through the mean structure (difference, smoothing operation or using a specific function); and the "small-scale" variation is modelled through a spatially correlated structure (correlated error models). In this sense, many approaches mentioned above can be regarded as special forms of the random field models. For instance, the correlated error model takes account of only the 'small-scale' variation. However, the methods using specific function and the first difference approach model only the 'large-scale' variation. Clearly, it would be better if both the 'large-scale' and 'small-scale' variations could be taken into account. For instance, using a function or difference operation to account for the 'large-

scale' variation, and a correlated error to account for the 'small-scare' variation. The ARIMA analysis discussed in above section assumed a specific class of covariance function, other types of covariance functions may also be used.

Using observations from the whole test

All data in the multisite testing data set can be used to estimate (predict) the genotypic means at each site. The additive main effects and multiplicative interaction model (AMMI) and the multisite best linear unbiased prediction (BLUP) methods are introduced in the following sections.

AMMI Model

The AMMI model combines the usual additive analysis of variance (ANOVA) with principle component analysis (PCA). The additive part of the AMMI model is estimated first with ANOVA, and the multiplicative part is estimated using the PCA on the ANOVA's residuals. The direct estimation of the genotype-by-environment (GE) interaction is generated by the multiplication of a genotype interaction principle component axe (IPCA) score by an environment IPCA score (Gauch, 1988). The AMMI model can be written as

$$y_{ij} = \mu + g_i + e_j + \sum_{k=1}^{R} \lambda_k \alpha_{ik} \beta_{jk} + \theta_{ij}$$

Where y_{ij} is the mean of i-th genotype in j-th environment, μ is the overall mean, g_i is the effect of i-th genotype, e_j is the effect of the j-th environment, λ_k is the k-th singular value of the GE interaction residual matrix; α_{ik} and β_{kj} are corresponding principal component scores for genotypes and environments, respectively, and θ_{ij} is the residual which contains both the unexplained interaction and the pure experiment error.

The α_{ik} and β_{kj} are obtained by multiplying the square root of the k-th singular value with its corresponding eigenvectors of the genotypes and environments, respectively.

To accurately estimate genotypic means the optimum number of interaction principal component axes needs to be determined. Gauch (1988) suggested a postdictive and a predictive assessments for this. The postdictive assessment uses an F-test to identify the significance of each IPCA (root mean square difference between the observed and expected values, ie. the square root of error mean square). The predictive assessment splits the data set into a part for model construction and a part for model validation and uses the cross validation technique. The Root Mean Square of the Predictive Difference (RMSPD) and the mean square error (MSE) of the estimation [MSE(model)] are used to measure the success of the prediction. Smaller values of RMSPD and MSE (model) indicated good predictive success. RMSPD is calculated as follows: the difference between the prediction and validation observations are first squared and summed over all genotypes and environments and divided by the numbers of validation observations, and then its square root is taken. The MSE (model) can be computed approximately as

$$\sigma_M^2 = \sigma_{MV}^2 - \sigma_V^2 = (RMSPD)^2 - \sigma_\varepsilon^2 \cong \text{MSE(model)}$$

where σ_M^2 is the variance of the model; σ_V^2 is the variance of validation observations and can be estimated empirically by the error mean square σ_ε^2. σ_{MV}^2 is the variance of the difference between the model and the validation observations and can be empirically estimated by the MSE (model – validation).

Piepho (1994) suggested that when data-splitting procedures were applied to RCB designs, the complete block rather than single observation should be randomised. In the case that only one replicate is used for validation, the estimate of MSE (model) can be approximated as

$$\text{MSE (model)} = (RMSPD)^2 - \sigma_V^2 - b\hat{\phi}(b)/(b-1)$$

Where b is the number of blocks in the design, and v is the number of genotypes, $\hat{\phi}(b) = (MSB - \sigma_V^2)/v$ with MSREP being the mean square of block.

All the necessary computations for the AMMI model can be done by using SAS or other statistical software. Appendix two outlined a SAS-based program for this model.

Cornelius *et al.* (1996) proposed a method which adjusts the least square estimators of the main effects and multiplicative components above by multiplying them by their respective shrinkage factors. The shrinkage factors for the genotypic and environment main effects are $s_g = \max(1 - F_g^{-1}, 0)$, $s_e = \max(1 - F_e^{-1}, 0)$, where the F_g and F_e are the F-statistics for testing the genotypic and environment effects against the error mean square. The shrinkage factor for the k-th multiplicative component is $s_k = \max(1 - F_k^{-1}, 0)$ with $F_k = \frac{b\lambda_k^2}{df_k \sigma_\varepsilon^2}$, where df_k is the degrees of freedom associated with k-th multiplicative component, and can be computed as v + s –1 –2k. This approach uses all the multiplicative components, and consequently this difficult issue for the classical AMMI model is avoided. Simulation study showed that this approach is at least as good as the classical AMMI model (Cornelius and Crossa, 1999).

Multisite BLUP

The Best Linear Unbiased Prediction (BLUP) method was developed for predicting the random effect when the working model is a mixed linear model (Henderson, 1984). To apply the BLUP method in estimating the genotypic means, it is necessary to define at least one of the main effects as random. In the traditional sense, the genotypic effect is fixed because the experimenters are just interested in the particular set of genotypes. However, White and Hodge (1990) argued that the genotypic effect can be regarded as a random effect if the set of genotypes can be regraded as a random sample of a single population. More generally, an effect may be regarded as a random effect if the levels of the effect may reasonably be assumed to come from a probability distribution (Maclean *et al.*, 1991; Robinson, 1991). If we assume that genotype effect is random, and the environment effect is fixed, then the multisite linear model can be written as

$$y_{ij} = \tau_j + u_{ij} + \varepsilon_{ij}$$

where $\tau_j = \mu + e_j$ represents fixed effects and $u_{ij} = g_i + (ge)_{ij}$ represents random effects.

This model can be written in matrix notation as

$$y = X\beta + Zu + e$$

where X and Z are design matrices which link the observation in y with the fixed and random parts, respectively.

The BLUP of the random effect u is

$$CV^{-1}(y - X\beta^0)$$

The best linear unbiased estimation (BLUE) of the fixed part is

$$X\beta^0 = X(X'V^{-1}X)^{-}X'V^{-1}y$$

The BLUP of the genotypic means at each site is

$$\text{BLUP}(y) = X\beta^0 + CV^{-1}(y - X\beta^0)$$

where V is the covariance matrix between the observations in y and C is the matrix of covariances among the observations in y and the unobservable true genotypic effects.

The above equation is the general BLUP prediction equation in the sense that the C and V matrices can be of any type. It is clear that the BLUPs of genotypic means can be easily obtained once the matrixes V and C are determined. Therefore, the most important issue in the application of the BLUP method is to define V and C. In the following sections, several situations are considered.

Case 1 Homogenous mean variances

Assume that the first s rows of the Y vector are the means of the first genotype at '*s*' sites. If the test is balanced and the mean variances is homogenous, C and V matrices are block diagonals with s × s submatrices V^* and C^*, respectively. The non-diagonal elements of V^* and C^* are σ_g^2, and the diagonal elements are $\sigma_g^2 + \sigma_{ge}^2 + \sigma_\varepsilon^2$ and $\sigma_g^2 + \sigma_{ge}^2$, respectively, where σ_g^2 is the genotypic variance, σ_{ge}^2 is the genotype-by-environment variance and σ_ε^2 is pooled error.

Case 2 Heterogeneous GE variances and homogeneous within site error variances

In multisite testing, the mean variances are normally heterogeneous since genotypes contribute differently to the GE interaction and/or the within site error variance is different. The contribution of each genotype to the GE interaction variance is proposed as a stability parameter and termed the stability variance (Shukla, 1972). Therefore, a more realistic model should take the different stability variances into account. Assuming the within site error variance is homogeneous, genotypic effect is random and

environment effect is fixed, the V and C matrixes are block diagonal with s $\times$ s submatrices V^* and C^*, respectively. The non-diagonal elements of V^* and C^* are σ_g^2 and the diagonal elements are $\sigma_g^2 +_i \sigma_{ge}^2 + \sigma_\varepsilon^2$ and $\sigma_g^2 +_i \sigma_{ge}^2$, respectively.

Case 3 Both GE variances and within site error variances heterogeneous

If both the GE interaction variance and the within site error variance are heterogeneous, the V and C matrixes are the same as case 2 except that the within site error variance varies from site to site.

If the sole objective of the analysis is to predict genotypic means, SAS procedure MIXED can be used to carry out the analysis (for statements see Appendix 3) using the observed genotypic means at each site as the observational units.

Piepho (1997; 1998) extended this mixed model-based method to the linear model with multiplicative component. Again it assumes that genotypic effect is random and environmental effect is fixed, V and C are still block-diagonal matrixes with sub-matrixes V^* and C^*, respectively. The non-diagonal elements of V^* and C^* are $\sigma_g^2 + \sum_{k=1}^{R} \beta_{jk}\beta_{j'k}$, and the diagonal elements are $\sigma_g^2 + \sum_{k=1}^{R} \beta_{jk}^2 + \sigma_p^2$ and $\sigma_g^2 + \sum_{k=1}^{R} \beta_{jk}^2 + \sigma_p^2 - \sigma_\varepsilon^2$, respectively. Where σ_p^2 is the residual variance based on the cell mean model and contains the interaction variance and part of the error variance, $\sigma_\varepsilon^2 = \sigma_e^2 / r$, and β_{jk} is the k-th score of the j-th environment.

Conclusion

The classical methods for estimating the genotypic means at each site of a multisite variety trial are simpler but do not take the possible spatial heterogeneity among plots into consideration and do not use all the information contained in multisite test data. Spatial analysis using the information of other plots, particularly the neighbouring plots can therefore significantly improve estimates of true means. However, there is no clear

rule to guide the selection of the appropriate spatial model. In practice several of the models could be used and the best one determined. Most of the methods can be easily implemented using SAS and/or Genstat, which are commonly accessible. Moreover, specialised software such as ASREML (Gilmour *et al.*, 1996) has been developed. Therefore, it is now time for practical breeders to become familiar with these methods and for those familiar with the methods to make simple "plug in the data" versions of computer software available so that full use can be made of their benefits.

The AMMI model is more flexible in the sense that there is no requirement for a large number of genotypes/sites. However, to determine the number of interaction principle component axes to use for the classical AMMI model is not a trivial problem. The shrinkage estimators seem to be better than the classical AMMI model. The multisite BLUP is better than the classical AMMI when variance components can be estimated accurately. In addition, the missing GE combinations do not create serious problems. However, to obtain accurate estimates of the variance components, the number of tested genotypes and/or the number of sites must be large. As with the classical methods, neither AMMI nor multisite BLUP takes possible systematic heterogeneity among plots into account. Using the spatially adjusted values as the raw data for the AMMI or multisite BLUP analysis the advantages of both types of methods could be explored. Patterson and Nabugoomu (1990) outlined such a two-step procedure. Cullis *et al.* (1998) developed a method that combines spatial analysis and multisite BLUP into a single step, and showed that it is more efficient than the two-step procedure. However, special software is required to carry out the analysis and as stated above simplified 'plug in the data' software and recognition of the advantages of the methods are needed, if this approach is to be widely used..

References

Baird, D. and Mead, R. 1991. The empirical efficiency and validity for two neighbour models. *Biometrics* ***47***, 1473-1487

Bartlett, M.S. 1978. Nearest neighbour models in the analysis of field experiments. *Journal of Royal Statistics Society, B* ***40***, 147-174

Besag, J.and Kempton, R. 1986. Statistical analysis of field experiments using neighbouring plots. *Biometrics* ***42***, 231-251

Bhatti, A.U., Mulla, D.J., Koehler, F.E., and Gurmani, A.H. 1991. Identifying and removing spatial correlation from yield experiments. *Soil Science Society of American Journal 55*,1523-1528

Brownie, C., Bowman, D.T., and Burton, J.W. 1993. Estimating spatial variation in analysis of data from field trials: A comparison of methods. *Agronomy Journal* ***85***, 1244-1253

Clarke, F.R., Baker, R.J., and DePauw, R.M. 1994. Moving mean and least squares smoothing for analysis of grain yield data. *Crop Science* ***34***, 1479-1483

Cochran, W.G. and Cox, G.M. 1957. *Experimental design.* 2nd Ed, John Wiley & son Inc. New York

Cornelius, P.L., Crossa, J., and Seyedsadr, M.S. 1996. Statistical tests and estimators of multiplicative models for genotype by environment data. *In* Genotype by environment interaction (eds., M.S. Kang and H.G. Jr. Gauch HG. Boca. Raton). pp.199-234. CRC Press.

Cornelius, P.L, and Crossa J. 1999. Prediction assessment of shrinkage estimators of mutiplicative models for multienvironment cultivar trials. *Crop Science* ***39***, 998-1009

Cullis, B.R. and Gleeson, A.C. 1989. The efficiency of neighbour analysis for replicated variety trials in Australia. *Journal of Agriculture Science, Cambridge* ***113***, 233-239

Cullis, B.R.and Gleeson, A.C. 1991. Spatial analysis of field experiments- an extension to two dimensions. *Biometrics* ***47***, 1449-1460

Cullis, B.R., Gogel, B., Verbyla, A., and Thompson, R. 1998. Spatial analysis of multi-environment early generation variety trials. *Biometrics* ***54***, 1-18

Gauch, H.G. 1988. Model selection and validation for yield trials with interaction. *Biometrics* ***44***, 705-715

Genstat 5 Committee 1993 *Genstat 5, Release 3, reference manual.* Clarendon Press, Oxford

Gilmour, A.R., Thompson, R. and Cullis, B.R. 1996. ASREML. Biometric Bulletin 3. NSW Agriculture, Organge, NSW, Australia

Gleeson, A.C. and Cullis, B.R. 1987. Residual maximum likelihood (REML) estimation of a neighbour model for field experiments. *Biometrics* ***43***, 277-288

Grondona, M.O., Crossa, J., Fox, P.N., and Pfeiffer, W.H. 1996. Analysis of variety trials using two-dimensional separable ARIMA processes. *Biometrics* ***52***, 763-770

Green, P., Jenison, J., and Secheult, A.H. 1985. Analysis of field experiments by least squares smoothing. *Journal of Royal Statistics Society, B* ***47***, 299-315

Hackett, C.A., Reglinski, T., and Newton, A.C. 1995. Use of additive models to represent trends in barley field trial. *Annual Applied Biology* ***127***, 391-403

Henderson, C.R. 1984. *Application of linear models in animal breeding*. University of Guelph. Guelph, Ontario

Kempton, R.A. and Howes, C.W. 1980. The use of neighbouring plot values in the analysis of variety trials. *Applied Statistics* ***30***, 59-70

Kempton, R.A., Seraphin, J.C. and Sword, A.M. 1994. Statistical analysis of two-dimensional variation in variety yield trials. *Journal of Agriculture Science, Cambridge* ***122***, 335-342

Kirk, H.J., Hayes, F.L., and Monroe, R.J. 1980. Application of trend analysis to horticultural field trials. *Journal of American Society of Horticultural Science* ***105***, 189-193

Maclean, R.A., Sanders, W.L., and Stroup, W.W. 1991 A unified approach to mixed model theory. *American Statistician* ***45***, 54-64

Mak, C., Harvey, B.L., and Berdahl, J.D. 1978. An evaluation of control plots and moving means for error control in barley nurseries. *Crop Science* ***18***, 870-873.

Papadakis, J.S. 1937. Methode statistique pour des experiences sur champ. Bulletin de I'Institute d' Amelioration des Plantes a Salonique No. 23.

Patterson, H.D. and Nabugoomu, F. 1992. REML and the analysis of series of crop variety trials. In Proceedings from the 25-th international biometric conference, 77-93. Statistics Section AgResearch: Hamilton, New Zealand.

Pearce, S.G., Clarke, R.F., and Kempton, R.E. 1988. *Manual of crop experimentation.* London: Charles Griffin and Company Ltd, New York: Oxford University Press.

Piepho, H.P. 1994. Best linear unbiased prediction (BLUP) for regional yield trials: a comparison to additive main effects multiplicative interaction (AMMI) analysis. *Theoretical and Applied Genetics* ***89***, 647-654

Piepho, H.P. 1997. Analysing genotype-environment data by mixed models with multiplicative effects. *Biometrics* ***53***, 761-766

Piepho, H.P. 1998. Empirical best linear unbiased prediction in cultivar trials using factor-analytic variance-covariance structures. *Theoretical and Applied Genetics* ***97,*** 195-201

Qian CG, Basford KE, IH DeLacy, and Cooper M 2000. Evaluation of experimental designs and spatial analysis in wheat breeding trials. *Theoretical and Applied Genetics* ***100***, 9-16

Robinson, G.K. 1991. That BLUP is a good thing: the estimation of random effects. *Statistical Science* ***6***, 15-51

SAS Institute 1997. SAS/STAT software: changes and enhancements through release 6.12. SAS Institute Inc., Cary, North Carolina, USA

Schwarzbach, E. 1985. Weighted nearest-neighbour analysis. *Biometrics* ***41***, 1088

Shukla, G.K. 1972. Some statistical aspects of partitioning genotype-environment components of variability. *Heredity* ***29***, 237- 245

Snedecor, G.W.and Cochran, W.G. 1980. *Statistical methods.* 7th edn. Iowa State University Press, Ames, Iowa, USA

Stroup, W.W., Baenziger, S.P., and Mulitze DK. 1994. Removing spatial variation from wheat yield trials: A comparison of methods. *Crop Science* ***86***, 62-66

Tamura, R.N., Nelson, L.A., and Naderman, G.C. 1988. An investigation of the validity and usefulness of trend analysis for field plot data. *Agronomy Journal* ***80***, 712-718

White, T. and Hodge, G. 1989. *Predicting breeding values with applications in forest tree improvement.* Pp367, Kluwer Academic Publishers; Dordrecht; Netherlands

Wilkinson, G.N., Eckert, S.R., Hanock, T.W., and Mayo, O. 1983. Nearest neighbour (NN) analysis of field experiments (with discussion). *Journal of Royal Statistics Society B* ***45***, 151-211

Williams, E.R. 1986. A neighbour model for yield experiments. *Biometrika* ***73***, 279-287

Wu T., and Dutilleul P. 1999. Validity and efficiency of neighbour analysis in comparison with classical complete and incomplete block analysis of field experiments. *Agronomy Journal* ***91***, 721-731

Zimmerman, D.L. and Harville, D.A. 1991. A random field approach to the analysis of field-plot experiments and other experiments. *Biometrics* ***47***, 223-239

Appendix 1: SAS programs for implementing some spatial methods

In the following programs, A is a data set consisted of differenced observations, ROW and COL are variables to index the row and column positions of the plots, respectively.

1.1 Simple RCB design analysis

Using GLM	Using MIXED
PROC GLM; CLASS GEN BLOCK; MODEL YIELD = GEN BLOCK; LSMEANS GEN; RUN;	PROC MIXED; CLASS GEN BLOCK; MODEL YIELD=GEN; RANDOM BLOCK LSMEANS GEN; RUN;

1.2. Correlated error model

Assume that the error covariance structure can be modelled by a spherical covariance function.

Keep block structure	Ignore block structure
PROC MIXED NOPROFILE; CLASS GEN BLOCK; MODEL YIELD = GEN BLOCK; PARMS () () ()/NOITER; REPEATED /SUB = BLOCK TYPE = SP(SPH) (ROW COL); LSMEANS GEN; RUN;	PROC MIXED NOPROFILE; CLASS GEN; MODEL YIELD = GEN; PARMS () () ()/NOITER; REPEATED /SUB = INTERCEPT TYPE = SP(SPH) (ROW COL); LSMEANS GEN; RUN;

(Note: PARMS statement defines the parameters of covariance function estimated externally)

Other available covariance function are: EXP: exponential, GAU: Gaussian, and POW: Power.

1.3. First difference

The difference operation is done within block	Block structure is ignored
PROC MIXED DATA = A METHOD = REML;	PROC GLM DATA = A;
CLASS GEN BLOCK;	CLASS GEN ;
MODEL YIELD = GEN;	MODEL YIELD = GEN;
RANDOM BLOCK;	LSMEANS GEN;
LSMEANS GEN;	RUN;
RUN;	

1.4. Trend analysis: see Brownie *et al.*,(1993)
1.5. Nearest neighbour analysis: see Brownie *et al.*,(1993)

Appendix 2 SAS program for AMMI model.

```
/* Obtain GE means*/
Proc Glm;
Class site gen;
Model yield = site block(site) gen gen* site;
Lsmeans gen*site;
Out = mset lsmean = my;
/* Obtain GE residual */
 Proc Glm set = mset;
   Class site gen;
    Model my = site gen;
   Output out = setR Residual = ry;
/* Get multivariate form of GE means*/
Proc sort data = mset;
 By gen;
Data mset (keep= y1-ys gen);
 Array yy(s) y1-ys;
 Do site = 1 to s;
 Set mset;
 By gen;
 yy(site) = myield;
 If last.gen then return;
Data mset (keep= y1-ys);
  Set mset;
/* Get multivariate form of GE residual*/
Proc sort data = setR;
 By gen;
Proc transpose;
 Out = RGE (rename (_1=yr1 _2=yr2 s=yrs[1]));
 By gen; Id = site;
Data RGE (keep= y1-ys);
 Set RGE;
/*Singular value decomposition*/
Proc iml; s = ?[1];
v = ?[2];
s = ?[2];
Use RGE;
Read all into D;
E = D'*D;
V= D*D';
Call eigen (E1, E2, E);
Call eigen (V1, V2, V);
C = E1[1,1];
E = E2[,1]; V= V2[,1];
;/* first multiplicative component/
Y1=C'*V*E';
C= E1[2,1]; E= E2[,2]; V = V2[,2];
/* second multiplicative component*/
Y2=C'*V*E';
/* multiplicative effect*[3]/
GE = Y1+ Y2;
/*overall, genotypic & site means*/
Use mset;
Read all into M;
OM = M[:];/* overall mean*/
OM = [v,s,OM];
/*Genotypic mean*/
VM = M[,+];
Vm = VM/s;
Do i = 1 to s;
 VM = VM|| VM; End;
VM = VM [1:v, 1:s];
/*site means*/
SM = M[+,];
SM = SM/v;
Do i = 1 to v;

SM= SM//SM;
End;
SM = SM [1:v, 1:s]
Reset print;
Pre = Gm + SM +GE – OM;
/*Compute RMSPD*/
Y = Pre – Val[4];
RMSPD = Y[##];RMSPD = RMSPD/(v*s);
RMSPD = RMSPD**0.5;
```

[1] the 's' should be replaced by the number of sites.

[2]: the question marks are to be replaced by the number of genotypes and the number of sites.

[3] assume two multiplicative components are required.

[4]Val is v×s matrix consists of GE means obtained using validation part of data.

Appendix 3 Multisite BLUP

Assume that environment effect is RANDOM and genotype effect is FIXED. In following programs, the BLUE of fixed (GEN) effect and the BLUP of the random (SITE) are obtained; the BLUPs of genotypic means at each site can be computed in terms of the mixed linear model. If plot means are used, BLUP (y_{ij}) = BLUE (GEN_i) + BLUP ($SITE_j$) + BLUP [($BLOCK(SITE_j)$)]+ BLUP [$(GEN*SITE)_{ij}$]. If genotypic means at each site are used, BLUP (y_{ij}) = BLUE (GEN_i) + BLUP ($SITE_j$). Alternatively, the ESTIMATE statement in PROC MIXED can be used to obtain the BLUPs of genotypic means at each site.

3.1 Using plot means

Homogeneous within site error variances	Heterogeneous within site error variances
PROC MIXED; CLASS GEN SITE BLOCK; MODEL YIELD = GEN/DDFM = SATTERTH SOLUTION; RANDOM SITE BLOCK(SITE) GEN*SITE; RUN;	PROC MIXED; CLASS GEN SITE BLOCK(SITE); MODEL YIELD = GEN/DDFM = SATTERTH SOLUTION; RANDOM SITE BLOCK(SITE) GEN*SITE/SOLUTION; REPEATED /SUB = SITE TYPE = UN SOLUTION; RUN;

3.2 Using genotypic means at each site

Homogenous mean variances	Heterogenous mean variances
PROC MIXED METHOD = REML; CLASS GEN SITE; MODEL YIELD = SITE/SOLUTION; RANDOM INT GEN/SOLUTION; RUN;	PROC MIXED METHOD = REML; CLASS GEN SITE; MODEL YIELD = SITE/SOLUTION; RANDOM INT /SUB = SITE /SOLUTION; REPEATED / GROUP = GEN TYPE = UN(1); RUN;

Methods for analysing multisite plant variety trials

II Selection for yield and stability

Abstract

This is the second of two manuscripts describing statistical methods for analysing multisite plant variety trials. The first paper reviewed the methods for estimating genotypic means at each site. In this paper, we summarised methods for estimating and comparing genotypic means across sites including analysis of variance, nonparametric methods for testing genotypic effect, and some methods for selecting high-yield and stable genotypes including joint regression, segmented regression, principle coördinate analysis, general superiority measure, yield-stability statistic, safety-first, expected utility maximisation and desirability index.

Additional key words: *ANOVA, nonparametric method, mixed model*

Introduction

The existence of Genotype by Environment (GE) interactions is well documented in the plant breeding literature. Although selecting specific genotypes for specific environments is the best way to utilise GE interactions, it may not be practicable. GE interactions usually cannot be related to a single or even a few environmental factors thus it may be not possible to group cultivation environments into groups which give the same GE response. If the cultivation environments can be grouped then limited resources for plant breeding may dictate stable genotypes with wide applicability are the best option. Because good performance is the main objective of plant breeding, selection of stable genotypes means that simultaneous selection for good performance and stability is needed. Therefore, breeders must weigh the importance of a genotype's stability relative to its mean performance across sites. Methods have been suggested to assist in the simultaneous selection of yield and stability and some of these are summarised in this paper.

Estimating and comparing genotypic means across sites

The genotypic mean across sites is an important indicator of the potential of the genotype to be successful in the whole production region. Therefore, an accurate assessment of yield performance of new genotypes across environments is crucial for plant breeding programmes (Cullis *et al.*, 1996).

Estimating genotypic means across sites

The simplest way to estimate the across-site mean is to take the arithmetic average. However, the arithmetic average is an unbiased estimate only if all genotypes are tested at all the sites and the genotypic mean variance is homogenous.

When not all the sites contain all the genotypes, but the genotypic mean variance is homogenous, the least square means can be used to account for the different numbers of test sites for different genotypes. This is the "fitting constants" method (Searle, 1971). When genotypes are absent in environments with generally high responses, their means are corrected upwards. Means for genotypes absent in unfavourable environments will be corrected downward (van Eeuwijk 1995). The general linear model (GLM) for analysis of variance provided by statistical software such as SAS and Genstat includes the fitting constants method as one option. However, the assumption underlying this method is that there is no genotype-by-site interaction, which is clearly unsatisfied by most of the multisite tests.

Gauch and Zoble (1990) proposed a method based on an expectation maximisation algorithm to input the missing values by use of the additive main effects and multiplicative interaction effect (AMMI) model. This model was discussed in the first paper of this review (Ye *et al.*, 2000). The Gauch and Zoble (1990) method involves the following steps. (1) Compute cell means for every cell with data, then initialise the additive parameters by computing the unweighted genotype means, environment means and grand mean. (2) Initialise the interaction residuals as usual for cells with data, but input an interaction residual of zero for missing cells. (3) Solve the multiplicative parameters. (4) Re-estimate and revise each missing cell with the current AMMI model.

(5) Fit the AMMI model to these revised data. (6) Iterate this process until convergence, ie. the imputed missing values show acceptably small changes.

A more efficient method to deal with the unbalanced GE data is to use a mixed model by assuming either genotype or environment effects to be random. Using this method the predicted means of the unobserved cells (genotype and environment combination) can be obtained by replacing the necessary terms in the model with their expected value. In other words, all the random effects are set to zero and the fixed effect to their generalised least squares estimate. Indeed, the predicted mean for any cell corresponds to the hypothetical means that would have been obtained were the data orthogonal and equally replicated (van Eeuwijk, 1995). Whether the genotype or environment effect is assumed to be random depends on the situation. In general, an effect can be regarded as a random effect if the levels of the effect may reasonably be assumed to come from a probability distribution (Maclean *et al.*, 1991; Stroup and Multize, 1991; Piepho, 1994). In practice the random effect should have sufficient degrees of freedom (say 10) to allow proper checking of the distributional assumptions about this effect.

When the genotypic mean variances are not homogeneous, the estimation of genotypic means becomes more complicated. Unfortunately, genotypic mean variances are usually not homozygous across sites due to the heterogeneity of GE interaction which is an indicator of different stabilities of tested genotypes and /or the heterogeneity of the within site experimental errors. In this case, the across-site genotypic means (arithmetic) are mostly affected by the mean from the sites with larger variances, and the generalised or weighted least squares method is a method to get more precise estimates. Using this method observations in different sites are weighted by the reciprocal of the error mean squares of the site. Thus, the genotypic means from the sites with higher precision have a greater influence on the estimation. By defining a weight factor in the GLM, the weighted least squares means can be obtained easily if all effects are regarded as fixed. Another method is to use a mixed model. The heterogeneous variances can be taken into account in mixed model analysis by correctly defining the two variance –covariance matrixes (see Ye *et al.*, 2000). SAS procedure MIXED is very useful in analysing multisite data sets. Using the 'REPEATED /SUB = SITE TYPE = UN' statement, a site-

specific error variance will be generated, and the estimate for fixed effect and the prediction for random effect will be obtained.

Using within environment error variances estimated directly from the observed data as weights has been criticised by many authors. For instance, more weight is usually given to the less productive sites since the error variance is usually positively correlated to the environmental yield response (Crossa, 1990; Gauch, 1988). Some authors have argued that more weight should be put on the more productive sites because they are of more interest to the experimenters (Crossa, 1990; Gauch, 1988). To overcome this problem Cullis *et al.* (1996) and Frensham *et al.* (1997) first modelled the error variances as a function of the log of site mean yield and other environmental variables. The predicted error variances from the prediction equation were then used as weights for estimating genotypic means across sites. The advantages of this procedure are: 1) It provides insight into those factors affecting error variance. 2) The influence of data recording and transcription errors is reduced. 3) It does not require the additivity or homogeneity assumption is to be true for the other random effects.

Using error variances as weights does not take the heterogeneity of the GE interaction mean square into account. This can be overcome by weighting the genotypic means by the reciprocals of the residual mean squares which include both the interaction and the error variances (Bernardo, 1992). The residual mean square for the i-th genotype can be estimated as

The heterogeneous interaction variance can also be dealt with using the MIXED procedure of SAS. Using the 'REPEATED / GROUP = GEN TYPE = UN' statement, a genotype-specific variance can be generated. As for the error variance, Frensham *et al.* (1997) proposed a method which models the GE interaction variances by a log-linear function of the explanatory variables. This approach produces a GE interaction variance for each genotype. Alternatively, Denis and Dhorne (1989) modelled the GE interaction variance using genotypic and/or environmental variables directly in the mixed model analysis. This method is known as the "mixed factorial regression".

When the weighted least square method is used, the following points apply. Firstly, unless the variances are quite different, the simple arithmetic means are still valid, although the significance tests using a pooled error are no longer valid. Secondly, the

weighted means can lose their expected superiority over the arithmetic mean if the estimated variances lack sufficient precision (Yates and Cochran, 1938). This was further confirmed by Bernardo (1992) using a corn yield trial containing 34 varieties and 53 environments. Thirdly, multisite testing data usually exhibits large ranges in site mean yields so that much of the heterogeneity may be related to scale. Therefore, data transformation may be necessary to remove scale-dependent heterogeneity, otherwise, misleading interpretation of the heterogeneity may be obtained (Frensham *et al.*, 1997).

Comparing genotypic means across sites

Generally the purpose of multisite testing is the statistical estimation of genotypic performance. However, breeders are sometimes interested in comparing the genotype means across environments. Analysis of variance (ANOVA) combined with multiple comparisons is normally used by breeders to achieve this objective. Nonparametric methods have also been suggested by different authors to be used when the assumptions underlying ANOVA cannot be satisfied.

ANOVA

For convenience, assume there are '*v*' genotypes tested in '*s*' environments with '*b*' replications in randomised block design, and '*n*' individuals are planted within each plot. The general model for analysing a multisite test based on cell (plot) means can be written as;

$$y_{ijk} = \mu + g_i + e_j + b_{k(j)} + (ge)_{ij} + \varepsilon_{ijk}$$

Where y_{ij} is the mean of the i-th genotype in the j-th environment, μ is the overall mean, g_i is the effect of the i-th genotype, e_j is the effect of the j-th environment, $b_{k(j)}$ is the effect of k-th block in j-th environment, $(ge)_{ij}$ is the interaction of the i-th genotype with the j-th environment, and ε_{ijk} is the error associated with the mean of the i-th genotype in the k-th block in the j-th environment.

When all genotypes are tested at all the sites and the within-site error variances are homogeneous, ANOVA based on the cell means is the simplest method to compare the genotypes. The ANOVA table is given in Table 1. If both genotypic and

environmental effects are assumed to be fixed, the significance of genotypic differences can be tested by F = MSG/MSE with v-1 and $(v\text{-}1)s(b\text{-}1)$ degrees of freedom. Assuming that genotypic effect is fixed and the environmental effect is random or if both effects are random, the significance of genotypic difference can be tested by F = MSG/MSGE with $(v\text{-}1)$ and $(v\text{-}1)(s\text{-}1)$ degrees of freedom.

When the within-site error variances are not homogeneous, most practitioners use some forms of transformation to remove the heterogeneity and then ANOVA is done based on the transformed data. However, the interpretation of the results from analysing transformed data may become difficult or biologically meaningless. Another alternative is to combine sites into groups that have homozygous within group variance, and the combined analysis is done for each group separately. However, the results from different groups cannot be combined to give a recommendation over all the sites.

Table 1. Combined analysis of variance for multisite data.

source	df	MS
Genotype	v-1	MSG
Site	s-1	MSE
Block(Site)	$(b\text{-}1)s$	MSB
Genotype*site	$(v\text{-}1)(s\text{-}1)$	MSGE
Error	$(v\text{-}1)s(b\text{-}1)$	MSE

Sometimes, instead of the plot means the genotypic means at each site have to be used to perform an ANOVA. In this case, the interaction and the experimental error can not be separated. The ANOVA table is given in Table 2. The usual assumptions for an ANOVA are more difficult to satisfy because the interaction mean squares for genotypes are unlikely to be the same, and the covariances between a pair of genotypes are unlikely to be the same as well.

However, an ANOVA can be valid under a less restrictive assumption about the variance-covariance structure. The sufficient and necessary condition for a valid ANOVA is the circularity structure of the variance-covariance matrix (Winer *et al.*, 1992). In the context of a GE two-way table, this condition requires that for each pair of

genotypes 'i' and 'k', the quantity $\sigma_{ii} + \sigma_{kk} - 2\sigma_{ik}$ is a constant, where σ_{ii} and σ_{kk} are the variances of the i-th and k-th genotypes respectively, and σ_{ik} is the covariance between the i-th and k-th genotypes. In other words, the variance of the difference between the observations of genotype 'i' and 'k' in the same environment is the same.

When circularity is violated, the F-test for the significance of the differences between genotypes F = MSG/MSGE can be approximated by the F-distribution with $\varepsilon(v-1)$ and $\varepsilon(v-1)(s-1)$ degrees of freedom, ε is a measure of departure from circularity. If the ε value is less than 0.8, the departure from the circularity is serious. The ε can be estimated by using the sample variances and covariances as suggested by Geisser and Greenhouse (1958).

Table 2. ANOVA of multisite testing data based on genotypic means at each site.

Source	df	MS	F
Genotype	v-1	MSG	MSG/MSGE
site	s-1	MSE	
residual	(v-1)(s-1)	MSGE	

$$\varepsilon = \frac{v^2(\bar{s}_{ii} - \bar{s}_{..})^2}{(v-1)\sum\sum s_{ik}^2 - 2v\sum s_{i.}^2 + v^2 s_{..}^2}$$

where s_{ik} is sample covariance of the i-th and k-th genotypes, $\bar{s}_{..} = \frac{\sum_i s_{ii}}{v}$ is the average of all genotype variances, $\bar{s}_{ik} = \frac{\sum_i \sum_k s_{ik}}{v^2}$ is the pooled covariance between all pairs of genotypes, and $\bar{s}_{i.} = \frac{\sum_k s_{ik}}{v}$ is the average covariance between the i-th genotype and all other genotypes.

Huynh and Feldt (1976) gave a modified estimate of ε, and this is preferable when ε is not much smaller than unity.

$$\varepsilon_{adj} = \frac{s(\nu - 1)\varepsilon - 2}{(\nu - 1)[(s-1) - (\nu - 1)\varepsilon]}$$

When the number of sites is larger than the number of genotypes, the circularity of the covariance structure can be tested by Mauchley's procedure and the Hotelling multivariate T^2 test can be used. Because this is rarely the case in plant variety tests, the detail of these methods are not given, but interested readers can refer to Winer *et al.* (1992) and (Piepho, 1996).

When multiple comparisons between means are made under the condition that circularity is violated, one may use paired t-tests. That is to carry out two-way ANOVA for each pair of genotypes separately (the 'lsmeans' statement associated with MIXED of SAS provides this pair-wise comparison) and the experiment-wise error rate can be controlled using the Bonferroni procedure: the paired comparisons are performed at the $2\alpha/(\nu-1)\nu$ significant level, where α is the predetermined significance level.

Nonparametric methods

In the previous section a method is introduced to deal with the situation when the underlying assumptions of the ANOVA are violated. There are some nonparametric methods, which do not rely on any restrictive assumption, and may be used as alternatives. All nonparametric methods transform the original observations into ranks, and further analyses are done by using the ranks. Because we are only interested in testing the genotypic effect, in the following sections, only methods for testing the genotypic effect are introduced.

1. Hildebrand (1980) method: The original observations are expressed as the derivations from the replication mean, then the derivations are transformed into a single rank order. The test statistic

$$\frac{12}{\nu(\nu sb+1)}\sum_{i=1}^{\nu}(\bar{R}_{i..} - \bar{R}_{...})^2$$

is approximately χ^2-distributed with ν -1 degrees of freedom

2. Kubinger (1986) method: The original observations are ranked into a single rank order (R_{ijk}), then the ranks are transformed by subtracting the average rank within the particular replicate ($\overline{R}_{ij.}$) and adding the overall rank of the genotype ($\overline{R}_{i..}$), that is, $R^*_{ijk} = R_{ijk} - \overline{R}_{ij.} - \overline{R}_{i..}$. The test statistic

$$\frac{12}{v(vsb+1)}\sum_{i=1}^{v}(\overline{R}^*_{i..} - \overline{R}_{...})^2$$

is approximately χ^2-distributed with v -1 degrees of freedom.

3. Van der Lann-de Kroon (1981) method: The original observations are ranked for each site separately into the ranks (R_{ijk}). The test statistic

$$\frac{12}{vsb^2(sb+1)}\sum_{i=1}^{v}\overline{R}^2_{.j.} - 3v(sb+1)$$

is approximately χ^2-distributed with s -1 degrees of freedom.

When comparisons among genotypes are made the sign test or the signed Wilcoxon test can be used (they are available in most commercial statistical software).. Again, the significance level needs to be modified using the Bonferroni procedure. In addition, the Spearman and the Kendall rank coefficient can also be used for each pair of genotypes.

Selection for yield and stability

Joint regression analysis

Finlay and Wilkinson (1963) developed a method to study the genotypic stabilities using multisite testing data. This method is known as joint regression analysis nowadays. It consists of regressing genotypic means at each site onto the environmental indexes defined as the environmental means. The stable genotypes are those with regression coefficients less than one.

Westcott (1987) method.

Westcott (1987) proposed a method based on principle coordinate analysis. His definition of the dissimilarity between two genotypes in a given environment is

$$S_j(i,k) = \frac{y_{mj} - (y_{ij} - y_{kj})/2}{y_{mj} - y_{lj}}$$

where y_{mj} and y_{lj} represent the genotypes with the highest and lowest mean performance in the j-th environment, respectively; y_{ij} and y_{kj} are the mean performance of genotypes i and k in the j-th environment, respectively. When more than one environment is considered the similarity between the i-th and k-th genotypes is the mean of $S_j(i,k)$ across environments. The measure of similarity between any pair of genotypes compares their average performance with the best genotype (y_{mj}) in a given environment. Genotypes with smaller $S_j(i,k)$ values are closer to y_{mj}.

This method can be used for selecting stable genotypes. The testing environments are ranked in descending order according to their means (ie. environmental index); the sites outside the lower and upper quartile are the poor and good sites. Genotype performance is first analysed for the poorest site, next the two poorest sites, and so on. The same procedure is applied to the good sites. For each cycle of analysis, a two-dimensional diagram is developed that represents the first two principle coordinates. Genotypes that have consistently shown an above average performance throughout the cycles are the most stable genotypes.

General superiority measures

Lin and Binns (1988) defined the measure of general superiority P_i as the mean square of the distance between a genotype's response and the maximum response at each site averaged over all sites. They demonstrated that P_i may be regarded as the mean square (MS) of the joint effect of the genotypic (G) and GE interaction.

$$P_i = \sum_{j=1}^{s} \left(y_{ij} - y_{mj}\right)^2 / 2v$$

Where y_{mj} is the maximum response among all genotypes in the j-th site.

The smaller the value the better the genotype.

Yield-stability statistic

Kang (1991) proposed the "rank sum method". Ranks are assigned to mean yield with the highest yield receiving the rank of 1 and another rank is assigned to the stability parameter (stability variances) with the lowest value having a value of one. Then the yield rank and stability rank are summed for each genotype. The genotypes with smaller rank sums are preferred. Kang (1993) modified this method and gave another name called the yield-stability statistic (YS_i), the necessary calculations are as follows:

1. Rank genotypes according to yield: The genotype with lowest yield receiving a rank of 1.
2. Adjustment of yield rank: +1 if the genotype mean yield is higher than the overall mean yield for a test (OMY), +2 and +3 if the genotype mean yield is higher than OMY by one least significance difference (LSD) or two LSDs or more respectively; -1 if the genotype mean yield is lower than OMY, -2 and -3 if the genotype mean yield is lower than OMY by one LSD or more and lower than OMY by two LSD's or more. The adjusted rank was labelled (Y_i).
3. Assignment of stability rating (S_i): $S_i = 0$ if stability variance is not significant; and –2, -4, and -8 if it is significant at 10%, 5% and 1% probability levels, respectively.
4. Compute and select genotypes: $YS_i = Y_i + S_i$.

   The genotypes that had YS_i values larger than the average are selected.

Segmented regression analysis

When selecting for wide adaptation for variable environmental conditions, the selected genotypes should ideally posses relatively high yield and stable performance in high stress environments. At the same time the genotypes should posses the capability to respond positively to favourable environments. Therefore, the environments are grouped into high-yielding and low-yielding groups first, and then the response pattern for each group is fitted to a linear model by joint regression (Finlay and Wilkinson, 1963). The ideal genotypes are the genotypes with regression coefficients in low-yielding environments less than one and regression coefficients in high-yielding environments larger than one.

Safety-First

Eskridge (1990) introduced a decision-making concept known as safety-first to the selection of stable genotypes. The model he used was the Kataoka (1963) model. Based on this model, the general safety-first index is

$$\text{SFI}= \bar{y}_{i.} - Z_{(1-\alpha)} S_i^2$$

Where α is an acceptable probability of having a very low performance, $\bar{y}_{i.}$ is the sample mean yield across sites for the i-th genotype, S_i^2 is a measure of stability for the i-th genotype, and $Z_{(1-\alpha)}$ is the (1-α) percentile from a standard normal distribution.

The genotypes with larger SFI values are the desirable ones. Eskridge (1990) developed the safety-first index for several commonly used stability parameters.

Expected utility maximisation

Eskridge and Johnson (1991) introduced the expected utility maximisation (EUM) to select stable plant cultivars. It can be separated into four major steps:

1. Enumeration of all possible choices: If the single 'best' genotype from a set of genotypes is selected, then the list of all possible choices is simply the set of genotypes being evaluated.
2. Define utility function: To evaluate the genotypes by the utility function the utility function should have the following characteristics. First, if a breeder prefers A to B, then the utility of A is larger than B. Second, the scale on which the utility is defined is arbitrary. This means that the ordering of genotypes must not change under a positive linear transformation. Finally, it is likely to be a concave function of performance, where the curvature of the utility function defines the breeder's attitude toward stability. The more curved the utility function, the greater the importance placed on stability. Eskridge and Johnson (1991) used the negative exponential utility function as the functional form. Therefore, $U(Y)=1- e^{-aY}$, where 'a' is defined as the stability preference coefficient, $a \geq 0$.
3. Specify a probability distribution of genotype response, $f(y_{ij})$: The performances of each genotype in all environments are rarely fully tested and need to be predicted.

Application of EUM to selection requires these 'predictions' be made in terms of a probability distribution for each genotype. Eskridge and Johnson (1991) assumed that genotypic performance is normally distributed, and sample estimates of mean and variance from trials were used to replace the unknown true parameters.

4. Calculate the indices for selection of stable genotypes based on EUM: The 'value' the breeder may expect to obtain from the i-th genotype is simply the expected value of the utility of genotype performance.

$$E[U(y_{ij})] = \int U(y_{ij}) f(y_{ij}) dy_{ij}$$

where integration is over all possible yields.

If the performance of the i-th genotype in the j-th environment y_{ij} is normally distributed with the mean $E(y_i)$ and variance $V(y_i)$, then the general form of an expected utility index is;

$$E(y_i)-(a/2)\ V(y_i)$$

The genotype with largest index value is considered to be the 'best'. In practice, a stability model needs to be chosen so as to estimate $E(y_i)$ and $V(y_i)$.

Hernandez *et al.* (1993)'s desirability index

Hernandez *et al.* (1993) proposed a desirability index which is expressed as the area under the regression function. It can be written as;

$$D_i = \bar{y}_{i.} + b_i C$$

where $\bar{y}_{i.}$ is the mean yield of the i-th genotype, b_i is the linear regression coefficient of the i-th genotype on the environmental index (I) which is defined as the mean of an environment minus the grand mean, and $C= \frac{I_a + I_b}{2}$ is the mean of the environmental indices at two extreme environments.

Conclusion

The heterogeneous variances of the genotypic means, within-site error variances and/or the heterogeneous GE interaction variances complicate the estimation of genotypic means across sites. The across-site genotypic mean of a genotype is a weighted mean of its means at every site. Therefore, the method for determining the weights is very important. The method introduced in textbooks and applied by plant breeders currently is to use the within-site error variances as weights (Cochran and Cox, 1957). Modelling error variance using a function of other variables has been used to replace the sample error variances as weights. A mean which is more meaningful in the context of breeding may be more appreciated. With a multitrait selection index, appropriate weights need to be determined by the relative importance of each trait in determining the economic value of the genotypes. Similarly the genotypic means at different sites may also be weighted by the relative importance of the production conditions represented by each site.

For comparing the genotypic means across sites, the ANOVA plus multiple comparison method is often difficult to justify. Since the stability of a genotype should also be taken into consideration when selection is made, the possibility of two genotypes with very similar performance and stability may be rare. Though breeders may be more interested in ranking genotypes rather than detecting statistical significance, Therefore, it is still required to test for differences among genotypes by statistical methods. Geisser and Greenhouse (1958)'s method for F test, and nonparametric methods may be more appropriate.

The presence of significant GE interactions in multisite testing is the rule rather than the exception. Selection for genotypes with good stability has always been the objective of a plant breeder. Many methods for simultaneous selection of performance and stability have been developed recently. The relative performance of these methods under different situations is unknown. The general superiority measure and Westcott method use the relative performances at each site ,and do not require an explicit stability parameter in developing the selection criteria, and are simple. The main disadvantage of these two methods is that breeders cannot make a subjective evaluation of the importance of the stability. The desirability index requires that the genotypic response can be

explained by a linear model. Because a linear model rarely models the genotypic response satisfactorily, this index may be of limited use in practice. Similarly, the segmented regression method requires that linear models in each environmental group can model the genotypic responses. The rank sum method, yield-stability statistic, the safety-first method and the expected utility maximisation procedures all use stability parameters explicitly. Therefore, they all face the problem of selecting a form of stability parameter since different types of stability parameters may result in the selection of different genotypes. The safety-first and the expected utility maximisation procedures need to define a probability distribution of the genotypic performance across sites and the parameters of the distribution need to be estimated accurately. Thus they can only be used when the number of sites is large. Another obvious difficulty of the expected utility maximisation procedure is the specification of a utility function.

References

Bernardo, R. 1992. Weighted vs. unweighted mean performance of varieties across environments. *Crop Science* ***32***, 490-492

Cochran, W.G. 1954. The combination of estimates from different experiments. *Biometrics* ***10***, 101-119

Cochran, W.G., and Cox, G.M. 1957. Experimental design. 2nd Ed, John Wiley & son Inc. New York

Crossa, J. 1990. Statistical analyses of multilocation trials. Advances in Agronomy 44, 55-85

Cullis, B.R., Gleeson, A.C., and Thomson, F.M. 1982. The response to selection of different procedures for the analysis of early generation variety trials. *Journal of Agriculture Science* ***118***, 141-148

Cullis B.R., Thomson, F.M., Fisher, J.A., Gilmour, A.R., and Thompson, R. 1996. The analysis of the NSW wheat variety database. I Modelling trial error variance. *Theoretical and Applied Genetics* ***92***, 21-27

Denis J.B., and Dhorne, T. 1989. Modelling interaction by regression with random coefficients. *Biuletyn Oceny Odmian* ***21-22***, 65-73

Eskridge, K. M. 1990. Selection of stable cultivars using a safety-first rule. *Crop Science* ***30***, 369-374

Eskridge, K.M., and Johnson B.E. 1991. Expected utility maximisation and selection of stable plant cultivars. *Theoretical and Applied Genetics* ***81***, 825-832

Frensham, A., Cullis, B.R., and Verbyla, A.P. 1997. Genotype by environment variance heterogeneity in a two-stage analysis. *Biometrics* ***53***, 1373-1383

Gauch HG 1988. Model selection and validation for yield trials with interaction. *Biometrics* ***44***, 705-715

Gauch, H.G., and Zobel, R.W. 1990. Imputing missing yield trial data. *Theoretical and Applied Genetics* ***79***, 753-761

Geisser, S., and Greenhouse, S.W. 1958. An extension of Box's results on the use of F-distribution in multivariate analysis. *Annals of Mathematical Statistics* ***29***, 885-891

Gilmour, A.R., Cullis, B.R., and Verbyla, A.P. 1997. Accounting for natural and extraneous variation in the analysis of field experiments. *Journal of Agricultural, Biological and Environmental Statistics* ***2***, 269-293

Hernandez, C.M., Crossa, J., and Castillo, A. 1993. The area under the function: an index for selecting desirable genotypes. Theoretical and Applied Genetics 87, 409-415

Hildebran, H. 1980. Asymptoisch verteilungsfreie Rangtests in linearen Modellen. *Medicinal Information Statistics* ***17***, 344-349

Huynh, H., and Feldt, L.S. 1976. Estimation of the Box correction for degree of freedom from sample data in randomised block and split plot designs. *Journal of Educational Statistics* ***1***, 69-82

Kang, M.S.1991. Modified rank-sum method for selecting high yielding and stable crop genotypes. *Cereal Research Communication* ***19***, 361-364

Kang, M.S. 1993. Simultaneous selection for yield and stability in crop performance trials: Consequences for grower. *Agronomy Journal* ***85***, 754-757

Kataoka, S. 1963. A stochastic programming model. *Econometrica* ***31***, 181-196

Kempton, R.A., and Fox, P.N. (eds) 1997. Statistical methods for plant variety evaluation. Chapman & Hall.

Kubinger, K.D. 1986. A note on nonparametric tests for the interaction in two-way layouts. *Biometrics Journal* ***28***, 67-72

Lin, C.S. Binns, C.S., and Lefkovitch, L.P. 1986. Stability analysis: Where do we stand? *Crop Science* ***26***, 894-900

Lin, C.S. 1988. A superiority measure of cultivar performance for cultivar×location data. *Canada Journal of Plant Science* ***68***, 193-198

Little ,T.M. and Hills, F.S. 1978. Agricultural experimentation: Designs and analysis. John Wiley & Sons Inc. New York

Lowe, W.J., Stonecypher, Hatcher, A.V. 1983. Progeny test data handling and analysis. In Progeny testing of forest trees. South Coop Series bull No 275. Texas A & M Univ. College Station, TX pp51-68

Maclean, R.A., Sanders, W.L., and Stroup, W.W. 1991. A unified approach to mixed model theory. *American Statistician* ***45***, 54-64

Pearce, S.G., Clarke, R.F., and Kempton, R.E. 1988. Manual of crop experimentation. London: Charles Griffin and Company Ltd, New York: Oxford University Press

Piepho, H.P. 1994. Best linear unbiased prediction (BLUP) for regional yield trials: a comparison to additive main effects multiplicative interaction (AMMI) analysis. *Theoretical and Applied Genetics* ***89***, 647-654

Piepho HP. 1996. Comparing cultivar means in multilocation trials when the covariance structure is not circular. *Heredity* ***76***, 198-203

SAS Institute 1997. SAS/STAT software: changes and enhancements through release 6.12. SAS Institute Inc., Cary, North Carolina, USA

Searle, S. R. 1971. Linear models. John Wiley & Sons. New York

Snedecor, G.W. and Cochran, W.G. 1980. Statistical methods. 7th edn. Iowa State University Press, Ames/IA

Stroup WW, Mulitze DK. 1991. Nearest neighbour adjusted best linear unbiased prediction. *American Statistician* ***45***, 194-200

Van Eeuwijk F. A., Keizer, L. C. P., and Baker, J. J. 1995. Linear and bilinear models for the analysis of multi-environment trials: II. An application to data from the Dutch Maize variety trials. *Euphytica* ***84***, 9-22

Van der Lann-de Kroon 1981. Distribution-free test procedures in two-way layouts: A concept for rank-interaction. *Statistics* ***35***, 189-213

Westcott, B. 1986. Some methods of analysing genotype-environment interaction. *Heredity* ***56***, 243-253

Westcott, B. 1987. A method of assessing the yield stability of crop genotypes. *Journal of Agriculture Science* ***108***, 267-274

Winer, B.J., Brown, D.R., and Michelis, K.M. 1992. Statistical principles in experimental design. 3rd edn. McGraw-Hill, New York

Yates, F. and Cochran, W.G. 1938. The analysis of groups of experiments. *Journal of Agriculture Science* ***28***, 556-580