PHYSIOLOGICAL AND AGRONOMIC EVALUATION OF RESPONSES OF FABA BEAN (*Vicia faba* L.) GENOTYPES TO WATER AND HIGH TEMPERATURE STRESSES : DEVELOPING SCREENING TECHNIQUES

By

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I dedicate this thesis to my loving parents

Mrs Girija Bhat

and

Mr Shankar Bhat

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ATT	Acquired thermal tolerance
BSA	Bovine serum albumin
CGR	Crop growth rate
CV	Coefficient of variation
DAS	Days after sowing
DPM	Disintegration per minute
EC	Electric conductivity
HMW	High molecular weight
HSP	Heat shock proteins
LMW	Low molecular weight
LSD	Least significant difference
NP water	Nanopure water (NANOpure Diamond^{TM}, Barnstead instruments)
PAGE	Polyacrylamide gel electrophoresis
r	Correlation coefficient
RI	Relative Injury
r_s	Spearman's rank correlation
RWC	Relative water content
SDS	Sodiumdodecylsulfate
SLA	Specific leaf area
TCA	Trichloroacetic acid
TDM	Total dry matter
TTC	2,3,5-triphenyltetrazoliumchloride
VDM	Vegetative dry matter
Rf value (pp156)	Is the ratio of the distance the protein band has moved with respect to the distance the dye front has moved (Ratio of the distance of the bottom of a protein band from the bottom of the well of the lane to the distance of the bottom of the dye front from the bottom of the well of the same lane).

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university, and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference is made in the text.

I consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

27th April 2001 Shambhu Bhat

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Summary

Faba bean (*Vicia faba* L.) is one of the important legumes of the Australian farming system (Siddique and Sykes, 1997, Pulse Australia, 2000). It is mainly grown in the southern and western cropping regions of the country where the climate is predominantly Mediterranean (Perry, 1994). This region is characterised by the occurrence of water shortage coupled with rising temperatures in spring, which coincides with the reproductive growth stage of faba bean (French, 1981, Richards, 1991). It is known that faba bean is susceptible to these two stresses, particularly during the reproductive stage. Although capable of producing high yield (5-6 t/ha) under favourable conditions, the average yield of faba bean is only 1.3 t/ha, and is highly variable across years. This has been mainly attributed to drought, high temperatures and diseases (Knight, 1994). Therefore, it is important to enhance the tolerance of the genotypes to drought and heat stresses to improve faba bean seed yield in the region.

This is the first study to evaluate a large number of genotypes representing the range in seed size and flowering times of faba bean for their response to drought and heat stresses. The aim of the study was to develop criteria that would allow selection for improved drought and heat stress resistance in faba bean. A number of physiological measurements relating to plant water relations under drought and well watered conditions, as well as measurements of dry matter production and yield, were made in a diverse range of genotypes. The work also investigated genotypic differences in acquired thermal tolerance (ATT) and heat shock protein (HSP) production in faba bean, which has not been studied previously.

The work showed that although the faba bean genotypes differ for physiological responses to drought stress, it is the whole plant response that is most likely to be useful in crop improvement programs. This study established that the response of total dry matter (TDM) of faba bean to water stress at 3-4 fully opened leaf stage could be used to screen the genotypes for differences in the seed yield sensitivity in the field. The technique of creating moisture stress described in this study is capable of generating data applicable to field conditions.

The study also established that genotypic differences exist for ATT of faba bean. The results indicated that cell membrane integrity of faba bean under heat stress is correlated with the stability of seed yield under the field conditions. A protocol was developed to incorporate radioactivity in the HSP of faba bean. The initial study showed there was little variation in production of HSP in faba bean. Also, HSP production was not correlated with the ATT. However, the protocol developed in this study to radiolabel the HSP provides a foundation to conduct further work to explore the role of HSP in developing thermal tolerance of faba bean and its relevance to yield improvement under heat stress.

Publications

The following conference proceeding was produced during the PhD candidature from present study:

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Chapter 1
Introduction

Sustainability is an important focus of Australian farming. As part of the efforts to maintain productivity in the cropping regions, pulses have been incorporated in the rotations. However, this is only a relatively recent phenomenon, with the major expansion of pulses occurring since the mid 1980s (Brinsmead et al., 1991, Nelson, 1995, Siddique and Sykes, 1997). Among the legume crops adapted to the cropping zone of southern and western Australia, faba bean has gained a place, particularly in the high rainfall regions (Krieg et al., 1996). It is well suited to the Mediterranean climate of the region and yields well in favourable conditions (Perry, 1994, Mwanamwenge, 1998). However, expansion of the area of cultivation has occurred in less favourable rainfall areas in the region (Loss and Siddique, 1997).

In spite of high its potential, the average yield of faba bean in Australia is only 1.3 t/ha (Pulse Australia, 2000). Its yields are highly variable from season to season, which is mainly attributed to low rainfall (Knight, 1994). Faba bean is known to be sensitive to drought stress (Kassam, 1973, Morgan et al., 1981, Turner et al., 1996). In southern Australia occurrence of water stress and increasing temperatures is common in spring (Richards, 1991). This coincides with the period when faba bean is in its reproductive stage, which is known to be very susceptible to these stresses (Baldwin, 1981, Katutar and Singh, 1990, Pilbeam et al., 1990b).

Water stress adversely affects leaf production and expansion, increases senescence and reduces the longevity of the faba bean canopy (Dantuma and Thompson, 1983,

Finch-Savage and Elston, 1982, Karamanos et al., 1982). Consequently it affects the dry matter accumulation of faba bean (Green et al., 1986). Water stress reduces the duration of the reproductive phase, increases abscission of reproductive parts and results in lower retention of pods and seeds (Peat, 1983, Lockerman et al., 1985, Grasshoff, 1990a). It also limits the assimilate availability to the reproductive parts and ultimately reduces the seed yield dramatically (Pilbeam et al., 1990a&b).

Susceptibility of faba bean to high temperature stress is also well documented. Saxena et al., (1988), reported that the crop experiences stress when temperature exceeds 20°C during the reproductive stage. High temperature hastens flowering in faba bean and also inhibits flower initiation (Evans, 1959, DeCosta, 1997). Agung, (1995) reported that heat stress reduced the duration of pod filling of faba bean. She observed that a rise in mean temperature by 1°C reduced the pod filling period of a large seeded genotype (Acc286) by 60 days and of a small seeded genotype (Fiord) by 19 days. This reduction resulted in lower individual seed weight and lower seed yield, highlighting the importance of the stress.

The optimum temperature for faba bean yield in southern Australian region has been estimated at 13°C to 14.5°C (Agung, 1995). This is little different to the optimum temperature for wheat in the region (McDonald et al., 1994). The large difference in the sensitivity to the stress between the two crops could be attributed to the water stress. Agung, (1995) reported that the effect of water stress on the faba bean seed yield was more important than that of the heat stress. However, because in this region faba bean is exposed to both drought and rising temperatures during the susceptible reproductive stage it is necessary to improve crop tolerance to both the stresses.

Currently little information is available on the usefulness of the physiological responses of the crop to water stress for identifying better yielding genotypes. There is virtually no information available on the genetic differences in the physiological response of faba bean to heat stress. It is valuable to have such information while trying to incorporate tolerance traits to improve the faba bean yield under drought and high temperature conditions. The present study was therefore designed to address these issues. This is the first study to examine the physiological responses of a large and diverse collection of faba bean genotypes to water and temperature stress, and to attempt to use the data to design a screening program for crop improvement. The following chapters describe in detail the field evaluations and glasshouse and laboratory experiments conducted to achieve this goal. It is hoped that the findings will provide a framework for future crop improvement efforts to increase the productivity of faba bean in southern Australian region.

Chapter 2

Literature review

2.1 Introduction

Researchers and farmers alike all over the world are becoming aware of the importance of sustainable agricultural practices. As a result, there has been a growing interest in the role of legumes in crop production. Farmers are realising the financial and other benefits of legumes in sustaining agricultural resources. Over the past fifteen years there has been a significant increase in the importance of grain legumes in crop rotations in Australia (Hamblin, 1987, Siddique and Sykes, 1997). Grain legumes used in Australian crop rotations have improved soil fertility and have also helped control weeds, diseases and insect pests (Hamblin, 1987).

Faba bean is one of the grain legumes that have become established in Australian agriculture. It is well suited to the Mediterranean climate of southern Australia and can produce relatively high yields (Perry, 1994, Mwanamwenge, 1998). Its market value as stock feed and human food, particularly in the Middle East, has made faba bean a commercially viable legume (O'Connell, 1991).

In southern Australia, there is a high probability of water stress along with rising temperatures during spring (French, 1981, Richards, 1991). Drought is one of the most important factors that adversely affects the performance of faba bean (Xia, 1990, Pilbeam *et al.,* 1990b). However, the crop is generally grown in higher rainfall regions of South Australia and hence there is a need for cultivars suited to low rainfall conditions of the state (McDonald *et al.,* 1994).

Improving the crop yield in the region has been a subject of the breeding program. Work has been carried out on varietal improvement, planting density and time of sowing (Marcellos and Knights, 1987, Adisarwanto, 1988). There is a lack of research on identifying and employing genetic variation as a tool in managing water stress and higher temperatures under the environmental conditions of southern Australia. The present study is aimed at improving the adaptation of the crop to the Mediterranean climatic conditions of this region. For this purpose, studies will be conducted to understand the genetic and physiological basis of the response of faba bean to water and higher temperature stresses. The information generated would be used to describe the crop performance under the climatic stresses and to develop strategies to improve the crop performance under the South Australian conditions.

2.2 Pulses and faba bean in Australia

2.2.1 Pulses in Australian agriculture

The extensive use of pulses in the Australian farming systems is a recent development but is considered as a significant aspect of current Australian crop production (Delane et al., 1989, Brinsmead et al., 1991, Nelson, 1995). The availability since the 1970s of suitable grain legume species, improved management practices and better marketing enhanced the whole farm profitability of including grain legumes in the crop production (Siddique and Sykes, 1997). It also helped extend legume cultivation to parts of the cereal belt considered too dry for pulses. The rotational benefits of the pulses, such as increased soil nitrogen and reduced disease incidence, have contributed to the sustainability of the farming system (Hamblin, 1987). The important pulses grown in Australia are lupin (*Lupinus angustifolius*), peas (*Pisum sativum*), chickpea (*Cicer arietinum*) lentil (*Lens culinaris*) and faba bean (*Vicia faba*) (Siddique and Sykes, 1997).

Currently, the area under pulse production in Australia is estimated at 2 million ha, comprising 10% of the cropped area (Pulse Australia, 2000). Western Australia is the major pulse producer in Australia with approximately 1 million ha, while South Australia has 258 000 ha. The production of pulses is 2 million tons every year, with a productivity of 1 t/ha. This is worth approximately $A400 millions at the farm gate. Of this, nearly 70% is exported. It is predicted that pulse production will increase mainly through increased productivity in the existing area. Until now lupin has dominated the development of the pulse industry. However, it is suggested that the majority of the expansion in pulse production in future will come from the chickpea and faba bean industry (Siddique and Sykes, 1997).

2.2.2 Faba bean in Australian farming

2.2.2.1 Cultivation

Faba bean yields are comparable to that of field peas in Australia (Walton and Trent, 1988, Siddique et al., 1993). Its cultivation in Australia began with the development of the variety Fiord in 1980 (Knight, 1994). Currently it is grown in an area of 154,000 ha with an average yield of 1.22 t/ha (Pulse Australia, 2000). Victoria and South Australia are the major producing states. In South Australia the crop is grown in high rainfall areas (Adisarwanto, 1988). Its expansion in Victoria has been mainly due to its ability to tolerate wet soil better than other pulses and the capacity to produce high yields in areas receiving more than 400mm rainfall (Siddique and Sykes, 1997). Recently faba bean has shown wide adaptability in Western Australia where its commercial production is rapidly increasing (Loss and Siddique, 1997).

Faba bean is a protein rich human food, and is also a recognised stock feed (Bond et al., 1994). Approximately half of the Australian faba bean crop is exported for

human consumption mainly to the Middle East and to also to Indonesia, Malayasia and Japan. While China dominates world production and supply of faba bean for human consumption, both quantity and quality of their produce is declining (Siddique and Sykes, 1997). This provides an opportunity for Australia to penetrate the human consumption market (Paull and Meyerlink, 1996).

2.2.2.2 Climatic constraints

The yield of faba bean is variable, which is attributed to diseases and susceptibility to moisture stress (Farah et al., 1988, Grzesiak et al., 1989, Grashoff, 1990a,b; Knight, 1994, Mwanamwenge, 1998). It is very sensitive to both high temperature and drought (Bond et al., 1985, Walton and Trent, 1988, Morgan et al., 1991; Turner et al., 1996). Moisture stress and high temperatures in the spring are known to limit faba bean growth (Loss et al., 1997a). In the field, reduced water supply and high temperatures often occur together affecting the productivity of many plant species (Ort and Boyer, 1985, Parry et al., 1989, Kpoghomou et al., 1990).

April to May is the optimum period for sowing faba bean crop in the southern Australia (Marcellos and Constable, 1986, Agung, 1995, Adisarwanto and Knight, 1997). Water shortage and increasing temperatures during the spring, generally of unpredictable severity, duration and timing, are the characteristic features of the Mediterranean climate of southern Australia (French, 1981, Richards, 1991, Loss and Siddique, 1994). This phenomenon coincides with stem elongation, flowering and pod filling stages (French and Shultz, 1984; Hamblin et al., 1987). Therefore, the probability of the crop experiencing water shortage and high temperatures during the reproductive stage in this climate is very high. This poses considerable risk to the production of faba bean.

Water stress accentuates the effect of heat, leading to closure of stomata and reduced transpirational cooling. This leads to an increase in the canopy temperature. Higher temperatures require higher transpirational cooling, which would be difficult when water supply is not adequate (Howarth. and Ougham, 1993). Therefore it is necessary to consider both drought and high temperature stresses while designing crop management under the Mediterranean climate of southern Australia.

2.3 Faba bean plant

Faba bean (*Vicia faba*) is an annual plant, which requires cool conditions for optimum growth (Duc, 1997). Its stem is erect and hollow, with the height ranging from 50-200 cm depending on the environment and genotype. The stem growth of faba bean is indeterminate and the stems bear many nodes (5-25) (Chapman and Peat, 1978, Loss et al., 1997a). The indeterminate habit of growth causes vegetative growth, flowering and podding to occur simultaneously. Therefore vegetative parts may compete with reproductive parts for assimilates.

Faba bean has a tap root system with secondary roots. The roots bear nodules containing nitrogen-fixing bacteria (*Rhizobium leguminosarum* bv *viciae*) and also form endomycorrhizal associations (Duc, 1997). The root system is shallow, generally reaching depths of only 90cm or less (Day and Legg, 1983, Heeraman and Juma, 1993; Crawford et al., 1997).

Depending upon the genotype used and the prevailing environmental conditions, the time taken for the initiation and completion of the different growth stages of faba bean varies. The time from sowing to emergence in the field is 15-20 days (Agung, 1995; Loss et al., 1997a). The time from sowing to flowering varies with the

genotypes. Under the southern Australian conditions it ranges from 70-75 days after sowing (DAS) for very early flowering genotypes, to 95-100 DAS for very late flowering genotypes (Agung, 1995; SARDI Annual Reports 1994-98*)*. Similarly, the days to podding range from 110 DAS to 120 DAS depending on the genotype (Agung, 1995). The duration from first pod to last flower could be 20-30 days, depending on the sowing time, whereas the time taken from last flower to maturity could range from 25-35 days regardless of sowing time (Loss et al., 1997a).

The first flower appears on approximately the 10^{th} node, and the first pod at the 5^{th} to 9^{th} reproductive node depending on the environment and genotype (Adisarwanto, 1988). Faba bean produces 2-12 flowers on short racemes, axillary to the leaf (Agung, 1995, Duc, 1997). Because of the indeterminate growth, a large number of floral nodes could be produced, but the number of pods produced is very low because of the high abscission of flowers. The abscission may range from 36-94% (Kambal, 1969a, Clifford et al., 1990). The young pods may abscise, partly because of internal competition for assimilates with vegetative parts (Chapman et al., 1978, Peat, 1982). The number of ovules each pod carries may range from 3 to 12, depending upon the genotype (Duc, 1997). The small seeded genotypes have a 1000 seed weight of less than 500 g while that of the large seeded types exceeds 1 kg (Duc, 1997).

The dry matter production significantly affects the yield of faba bean (Marcellos, 1987, Siddique et al., 1993, Agung, 1995). A significant correlation between the dry matter at maturity and at flowering has been reported (Silim and Saxena 1992, Stutzel and Aufhammer, 1992). Harvest index (HI), which indicates the ability of the genotypes to partition dry matter to seed yield, was shown to be similar among a range of faba bean cultivars irrespective of seed size and maturity (Marcellos, 1987).

This may be because partitioning of dry matter to the seeds during seed growth does not differ among the genotypes belonging to different seed sizes and maturity groups (Agung and McDonald, 1998). The lack of significant genotypic differences for HI and partitioning of dry matter to seed during the seed growth indicate that dry matter partitioning does not have significant influence on the final seed yield. Therefore, seed yield is largely influenced by the total dry matter produced.

The main components of the faba bean seed yield are the number of pods per plant, number of seeds per pod and weight per seed (Kambal, 1969b). Seed numbers per pod and individual seed weight are considered most stable (Dantuma and Thompson, 1983). Therefore, the number of seeds per plant depends upon the number of pods produced. The seed number and individual seed weight can strongly compensate each other (Agung and McDonald, 1998). A positive association between the variation in grain yield and that in pod and seed number has been well demonstrated (Pilbeam et al., 1989, Katiyar and Singh, 1990). However, the possibility of seed yield not being correlated to any of its components has also been reported (Agung and McDonald, 1998). Therefore it could be inferred that the influence of individual components on the final seed yield depends upon the genotype and environment.

2.4 Response to water stress

2.4.1 Response of vegetative growth

Vegetative growth of faba bean comprising production, expansion and development processes of leaf, is mainly dependent upon availability of water (Dantuma and Thompson, 1983). It is reported to be more sensitive to drought than seed set and seed growth in faba bean (Plies-Balzer *et al.*, 1995). Similar responses have been recorded in other crops also (Hsiao and Acevedo, 1974, Hsiao et al., 1976). Water

deficit reduces leaf size of the faba bean by reducing its elongation and growth, and increases leaf senescence (Farah, 1981, Karamanos et al., 1982). Consequently longevity of the crop canopy can decline (Finch-Savage and Elston, 1982, Grashoff and Verkerke, 1991) which in turn, can reduce the efficiency of conversion of intercepted light (Green et al., 1985, Herz et al., 1992) and restrict the stem elongation and dry matter accumulation (Gej, 1992, Xia, 1994). Leaf area reductions of faba bean of up to 50% have been observed in the field due to the combined effects of reduced leaf growth and early senescence (Karamanos, 1978).

Faba bean leaves are succulent and need large amounts of water to maintain turgor (Bond et al., 1994). Generally in the field faba bean leaves do not maintain turgor for long under water deficit and wilt quickly. This appears to be associated with the lack of osmotic adjustment capabilities (Kassam, 1973, Finch-Savage and Elston, 1982, Grashoff and Verkeke, 1991). The loss of turgor could lead to the cessation of expansive growth like stem elongation (Hussian et al., 1990) and reduce the rate of stem dry matter accumulation (Grashoff, 1990a). Stomata close when plant loses water, adversely affecting photosynthesis, growth rate and the final yield (Green et al., 1986, Grzesiak et al., 1989, Xia, 1994). It can be summarized that water deficit restricts the ability of faba bean to produce dry matter by adversely affecting the leaf production and growth. This limits the photosynthetic capacity of the crop and inhibits its ability to produce critical biomass required for better yield performance.

2.4.2 Response of reproductive growth

In southern Australia flowering of faba bean genotypes generally starts in August and continues into September (Agung, 1995). Subsequently pod setting commences in early September. This is the time when soil moisture starts receding and

temperatures are increasing in the region. Hence, the later part of the reproductive growth is most likely to experience increasing water shortage and rising temperatures. Therefore it is important to understand the response of the reproductive growth to these stresses.

Flowering and pod setting stages of reproductive growth of faba bean are very sensitive to water stress (Dantuma and Thompson, 1983, Singh et al., 1987, Mohammad et al., 1988, Pilbeam et al., 1990b, Xia, 1990). The incidence of drought during the flowering period reduces leaf area, rate of photosynthesis and biomass accumulation of faba bean (Xia, 1994). Such a loss of leaf area during the pod filling reduces pod weight (Grzesiak et al., 1989). It was reported that when drought occurs soon after pollination, soybean embryos abort (Westgate and Peterson, 1993). This was attributed to the limited supply of carbohydrates due to reduced photosynthesis under water stress. In faba bean drought also limits the availability of assimilates to the reproductive sinks and reduces all the yield components and the final yield (Pilbeam et al., 1990 a,b). Reduced availability of assimilates to the growing seeds of faba bean results in lower grain yield (Tamaki and Naka, 1972, Pilbeam et al., 1990b). Drought induces abscission of flowers and pods (Peat, 1983), resulting in lower pod retention (Grashoff, 1990a). It reduces the duration of flowering (Xia, 1994) and number of days to maturity of faba bean (Lockerman et al., 1985). Water shortage was shown to shorten the seed filling period, and reduce the seed size and final yield of maize and soybean crops (Meckel et al., 1984, Westgate and Boyer, 1986, Quattar et al., 1987). Water deficit induced leaf senescence is linked to lower seed weight of faba bean (Dantuma and Thompson, 1983).

The seed growth of many crops depends upon the current photosynthesis (Yoshida,

1972). It was shown that vegetative growth and photosynthesis of faba bean are adversely affected by water stress (Pilbeam et al., 1990 a,b, Herz et al., 1992, Xia, 1994). Therefore, supply of current photosynthates to the reproductive parts of the crop would be limited under water stress. Drought during the pod development of faba bean was shown to affect the dry matter partitioning during that period and reduce assimilate availability to the reproductive parts, causing yield loss (Pilbeam et al., 1990 a,b). The ability to mobilise stem reserves of carbohydrates to maintain seed growth under limited water supply is an important mechanism of cereals (Westgate and Boyer, 1985, Phelong and Siddique, 1991, Palta et al., 1994). There is evidence that soybeans, dry beans and peas can also remobilise assimilates stored in stems to maintain a constant rate of seed growth under water stress when green leaf area is reduced (Meckel et al., 1984, Samper et al., 1984, Ney et al., 1994). This contribution in legumes can be as high as 53%, as in the case of dry beans (*Phaseolus vulgaris L.*; Samper et al., 1984). Not much information is available on the ability of the faba bean to mobilise its stem reserves of carbohydrates towards seed growth under stress. Agung, (1995) estimated that mainly stem reserves, and not pod walls, contribute to the seed weight. This contribution varied from 3% to 18% depending upon the seasonal conditions, time of sowing and the cultivar. In southern Australia where the possibility of occurrence of the terminal water stress is very high, significant retranslocation of dry matter to reproductive parts could be an advantage towards better yields.

Reproductive growth of faba bean is very sensitive to water stress. This is primarily because of the adverse impact on vegetative growth and photosynthesis. Consequently the assimilate supply to reproductive sinks becomes yield limiting factor. This is apart from the abscission of reproductive parts and reduction in the

length of pod filling period caused by water deficit.

2.4.3 Mechanisms for adaptation to drought

Plants develop the capacity to adapt to water stress. The mechanisms are mainly drought escape, drought avoidance with high tissue water potential (Levitt, 1982) and drought tolerance at low water potential (Turner, 1979). Drought escape is the mechanism whereby the plant completes its life cycle before the onset of water stress. This involves early germination and shorter lifespan or early maturity or both (Turner, 1979, French, 1990, Richards, 1991). Early sowing enables plants to grow under more favourable conditions of moisture supply and evaporative demand and have a longer period of pod filling (Richards, 1991). Baldwin, (1980), demonstrated the benefits of early sowing of faba bean in South Australia. There are also reports of early maturing accessions producing higher seed yields in South Australia (Adisarwanto, 1988). Soja *et al.,* (1988) observed that an early flowering genotype of faba bean showed the smallest yield reduction under drought compared to the late maturing genotypes. The authors suggest this may be due to its faster development enabling better utilisation of soil moisture and the occurrence of flowering and pod filling under less stressful conditions.

Drought avoidance is the ability of a plant to maintain high water status under drought and it involves maintaining water uptake and/or reducing water loss. This is possible through increased root size and increased hydraulic conductance of the roots (Turner, 1979). However, higher axial resistance for the flow of water in the roots was reported to be beneficial for wheat (Richards 1991). The utility of this character lies in the fact that water in the subsoil would be conserved for the uptake during the later stages of the crop when surface moisture is exhausted. In faba bean, the

information on genetic variation for rooting system and the water uptake capability is limited. However, Saxena *et al.,* (1981) reported that one selection extracted water from deeper layers than another selection. Selection of a deeper rooting system was suggested to be included in the breeding program of faba bean (Day and Legg, 1983).

Drought tolerance is the ability of the plant to maintain metabolic activities at low tissue water potential and mainly involves osmotic adjustment among other traits (Boyer, 1983). Osmotic adjustment is defined as an accumulation of solutes that decreases osmotic potential resulting in maintaining turgor at low water potential (Radin, 1983). This helps maintain active growth and photosynthesis at lower leaf water potential (Morgan, 1984). Therefore, assimilate supply to roots could be continued, resulting in maintaining water extraction and thereby, physiological activities (Turner, 1986). Although it was suggested that osmotic potential could be useful in improving its performance under drought conditions (Day and Legg, 1983), faba bean was shown to lack osmoregulation (Grashoff and Verkerke, 1991).

Genetic variation exists for flowering time and maturity length of faba bean. It is possible to use early sowing and short duration varieties to avoid stress. However, genetic variation in the ability of faba bean to avoid or tolerate drought is not widely reported. Although it reportedly lacks osmotic adjustment, the genetic differences for drought tolerance traits should be explored because of the implications for its production in rainfed systems.

2.5 Assessing faba bean response to water stress

Several traits have been used to evaluate the response of crop plants to water stress.

In general, leaf water characters, stomatal conductance, growth and yield characters are some of the main traits being employed. A considerable amount of evidence is available on the usefulness, limitations and difficulties in the adaptation of such traits to breeding programs. The selection of a particular trait or combination of traits would also depend upon the requirements of the research program.

Physiological traits such as transpiration efficiency, carbon isotope discrimination, and osmoregulation have been used to assess the plant responses in breeding programs. There is a considerable body of work available describing the benefits and problems of breeding on the basis of physiological traits. In general, for a trait to be employed in a breeding program it should have a proven role in stress tolerance, be related to the yield stability under stress and should be highly heritable (Rascio et al., 1987, Singh et al., 1992). The trait's measurement should be quick, easy, inexpensive and appropriate for screening large number of accessions (Singh et al., 1992). Also, there should be a suitable stage of expression of the trait for the evaluation.

Some of the traits have proven to be useful for screening but by and large, outcomes of improving yield on the basis of physiological traits are variable and few have been adopted in routine breeding programs. Advantages associated with a single trait are small because each trait improves a plant function rather than the yield itself. To be able to influence the crop performance substantially, a trait should integrate several aspects of the growth. This is unlikely and therefore a combination of multiple physiological criteria appropriate to the target environment should be used in selection process (Blum, 1983, Shiferaw and Baker, 1996). The responses to stress may vary among the growth stages. Therefore, breeding for drought and heat tolerance should involve selection of parents possessing high degree of tolerance at

various critical stages of growth (Shiferaw and Baker, 1996). Often the techniques of measurement are time consuming and expensive. Transpiration efficiency, carbon isotope discrimination and osmoregulation are some of the examples of such traits. For these reasons major criteria in many breeding programs still are grain yield and yield stability under environmental stress (Brockner and Frohberg, 1987). However, it is appropriate to examine leaf stomatal conductance and plant water characters to understand the plant responses to water stress.

2.5.1 Stomatal conductance

Stomatal resistance for water movement is considered a useful trait in evaluating plant responses to water stress. This is because stomata close under water stress to limit transpirational loss of water (Boyer, 1970, Hsiao, 1982). In the study conducted by Kassam, (1973), the conductance of faba bean did not decrease until the leaf water potential and turgor dropped to a threshold low value. Similar non-linear relationships between the conductance and plant water status have been described for other crops aslo (Boyer, 1970, Hsiao et al., 1976). Stomatal closure is considered to help conserve plant water and improve its use efficiency under stress. This was demonstrated by Nerkar *et al.*, (1981), who found that the faba bean genotypes having lowest conductance also showed greatest water use efficiency.

The existence of genotypic variation in faba bean for stomatal conductance under water stress is inconclusive. Nerkar et al., (1981), reported that significant genotypic differences exist among the faba bean genotypes for conductance. Ricciardi and Steduto, (1988), found that conductance at different depths of the canopy did not show any significant differences among the genotypes. It should be noted that Nerkar et al., (1981), conducted their experiments under controlled conditions while the

latter's was a field study. A later study on response to water stress also concluded that faba bean genotypes did not differ for conductance (Grashoff and Verkerke, 1991). In this study they found the expansive vegetative growth of faba bean to be more sensitive than conductance to water stress. Because the faba bean yield is correlated to dry matter accumulation, conductance assumes importance in screening the genotypic response. Therefore, although faba bean yield is not directly related to conductance (Ricciardi and Steduto, 1988), it may have a useful role in screening the genotypes for their response to water stress. The sensitivity of conductance as a screening index has also been reported for other crops (Shiferaw and Baker, 1996).

2.5.2 Plant water characters

In the following review, references to plant water characters/status are made with respect to leaves. The plant water characteristics namely, relative water content, osmotic potential and water potential, quantitatively reflect plant water status. Hence, they have been studied in characterising the plant responses to water stress. Midday water potential of the faba bean was shown to be one of the most sensitive indicators of plant water status (Karamanos et al., 1982). Similar sensitivities were reported for wheat also (Rascio et al., 1987). Plant water status of faba bean was reported to affect the seed yield from the early growth stages, and cell turgor throughout the growth period was found to influence the seed yield (Karamanos, 1984). Karamanos, (1978), observed a linear relationship between the mean plant water potential and total leaf area of faba bean. In a later study (Karamanos, 1984) it was demonstrated that a high correlation exists between the total dry matter and water potential of faba bean, mainly because of the strong dependence of leaf area on the water potential. Karamanos et al., (1982), obtained a close correlation between the plant turgor at noon and final leaf size of the faba bean in field. It was observed that the vegetative

expansive growth of faba bean linearly declined with decreasing plant turgor and water potential (Grashoff and Verkerke, 1991). Therefore it is obvious that leaf area and the total dry matter are strongly influenced by the plant water status.

The evidence for the existence of genotypic variation for plant water characters in faba bean is very weak. Ricciardi and Steduto, (1988), and Grashoff and Verkerke, (1991), have reported a lack of genotypic variation for the plant water characters of faba bean grown in the field. The first of these two studies found that the significant genotypic differences for seed yield and its components were not related to the leaf water characters. The lack of an ability of faba bean to osmotically adjust under the water stress has been demonstrated (Grashoff and Verkerke, 1991). This study also found that the cultivars were unable to maintain turgor during the water shortage. At the same time it shows that the vegetative growth and dry matter accumulation are heavily influenced by plant water status. Because of the dependence of the faba bean seed yield on dry matter accumulation, plant water status will indirectly affect the crop performance under water stress. It has to be noted that most of these studies have used limited number of genotypes. The information on the response of plant water status of diverse genotypes to water stress has been lacking in Australian conditions. It is pertinent to conclude that water relations of a range of genotypes possessing diverse morphological and ecological/adaptive characters need to be studied under water stress. This will provide valuable information on the crop response to water stress and help identify ways to screen genotypes.

2.5.3 Limitations of plant water relations

Having stated the importance of quantifying the plant water status, it would be worthwhile to remember the difficulties also. Measurement of commonly studied

plant water traits in the field is tedious, time consuming and labour intensive (Shiferaw and Baker, 1996). There is a lack of consistency in the ability of these techniques to evaluate drought response. It is also necessary to measure them within a short period, normally during mid-day. Consistency in timing of sampling and age of the plant part sampled is therefore of great importance in such measurements. All the measurements are destructive and hence need considerable amount of plant material for sampling. The difficulties in replicating the stress conditions from experiment to experiment increase the variability of the measurements.

2.6 Response to high temperature stress

Temperature is an important environmental factor influencing the growth and development of plants. Occurrences of temperatures above the optimum for crop production in the field are common. Hot weather can be detrimental for survival, reproductive development and productivity of several crops (Boyer, 1982, Fischer, 1986, Hall, 1992). Temperature stress reduces crop yield by affecting different aspects of plant physiology such as photosynthesis rate, growth rate, plant water relations, seed set and assimilate partitioning (Howarth et al., 1997, Paulsen, 1994).

Generally two types of high temperature stresses are recognised, heat stress and heat shock. Wardlaw and Wrigley, (1994), defined temperatures between 25°C-32°C as heat stress temperatures, those above 32°C as heat shock temperatures and those above 40°C as severe shock temperatures for temperate cereals. Heat shock temperatures occur for only small duration (Wardlaw and Wrigley, 1994). Heat stress causes alteration in the rate and duration of metabolic processes in the cells whereas heat shock results in new physiological processes being initiated. Thus, heat shock effects are more severe than of the heat stress (Howarth and Ougham, 1993).

Heat shock affects cells structurally by disruption of the cell membrane and functionally by enzyme deactivation, protein denaturation and disruption of metabolic pathways (Blum, 1988; Porter et al., 1994). The structural impact involves specific phase changes in the lipid bilayer of cell membrane, leading to its disruption (Suss and Yordanov, 1986) and loss of semi permeability (Berry & Bjorkman, 1980). Consequently, the ability of the plasmalemma to retain solutes is affected (Christiansen, 1978; Lin et al., 1985). The stress effect on the functional aspects of cells injures photosynthesis and causes reduction in growth (Levitt, 1980a, Al-Khatib & Paulsen, 1999). It also causes respiratory depletion of substrates and reduction of chloroplast photochemical activity (Blum, 1988).

2.6.1 General crop responses

High temperatures affect both legumes and cereals by many and complex mechanisms. It is pertinent to mention a few important mechanisms operating under heat stress during the reproductive phase, which are relevant under terminal stress conditions. It was reported that temperatures above 30°C limit growth and adaptation of several legumes (Wery et al., 1994). Hot weather adversely affects the ability of legumes to retain reproductive organs and achieve satisfactory pod set. Excessive abscission of flowers and pods under hot conditions was found to contribute to yield reduction of common bean (Li et al., 1991). In cowpea, hot weather caused male sterility and lack of fertilization and the resultant reduction in pod-set was the major cause of the yield loss (Warrag & Hall, 1984; Ahmed et al., 1992). Hot weather during the reproductive growth of cereals is known to reduce the duration of grain fill and individual grain weight. In wheat this is because heat retards conversion of sucrose to starch in the developing grains (Bhuller & Jenner, 1986). Consequently post anthesis heat stress reduces the weight of individual wheat kernels rather than

the number of grains produced (Wardlaw et al., 1989). A shortened grain growth period contributes more to this reduction than the reduced rate of grain filling (Weigand & Cuellar, 1981). Therefore, heat stress during the grain fill hastens grain maturity and result in smaller, shrivelled grains (Saadalla et al., 1990b). These responses of cereals and legumes are important for faba bean also, because the growing season of faba bean in the southern Australia is characterized by terminal drought and accompanying rising temperatures. As pointed out earlier, the probability of later stages of reproductive stage of the crop experiencing this stress is very high.

The exposure of most organisms to temperatures 8°C-10°C above the normal growth condition activates a number of genes normally inactive, producing a group of proteins called heat-shock proteins (HSPs) (Vierling, 1991). HSPs are arbitrarily grouped into different classes based on their molecular weight (O'Connell, 1994, Ristic et al., 1996). HSPs with more than 60 kiloDaltons (kD) molecular weight are classed as high molecular weight (HMW), and they are constitutively expressed in plants (O'Connell, 1994). The HSPs between 15 to 30 kD are classified as low molecular weight (LMW), and are the most abundantly synthesised in plants under heat stress (Waters et al., 1996). Some HSPs are produced under normal conditions during the different developmental stages (Coca et al., 1994, DeRocher and Vierling, 1994). Production of stress proteins has also been reported under water stress (Almoguera et al., 1993) and other abiotic stresses (Vierling, 1991).

In the field, high temperatures and water stress occur together, resulting in plants being unable to cool through transpiration (Howarth and Ougham, 1993). As a result, the canopy temperature increases under water stress (Burke, 1990) rendering it

difficult to separate effects of the two stresses When the heat shock occurs, HSP synthesis takes primacy over the plant's response to other stresses (Howarth and Ougham, 1993), affecting the ability of the plant to respond to other stresses that are also operating. Therefore, it is very difficult to study the effects of high temperatures in isolation in the field and will have to be necessarily conducted under the controlled environment.

2.6.2 Response of faba bean

Relatively little information is available on the response of faba bean to high temperatures. Faba bean is considered to be sensitive to high temperature and water stress (Bond et al., 1985, Walton and Trent, 1988, Morgan et al., 1991, Turner et al., 1996). When the conditions are hot and dry, it looses turgor quickly, which leads to early closure of stomata. As a result, there would be a reduction in CO_2 fixation (Chaves, 1991). It was observed that exposure to high temperature (30°C/25°C day-night regime) for 6 days lowered the water potential of young faba bean plants, causes injury to photosynthesis and affects growth (McDonald and Paulsen, 1997). The rates of leaf production, expansion and of leaf area development of faba bean are all adversely affected by high temperature and drought (Dantuma and Thompson, 1983).

Faba bean is susceptible to high temperature, particularly during the reproductive stage (Loss et al., 1997a,b, McDonald and Paulsen, 1997) when it experiences stress if mean temperatures exceed 20°C (Saxena et al., 1988). Higher temperature is known to hasten its flowering (DeCosta, et al., 1997). At temperatures particularly above 23°C, flower initiation may be inhibited (Evans, 1959) and the number of open flowers produced would be low (Abdalla and Fischbeck, 1978). Heat induced

abscission of reproductive parts has been reported for several other legumes also (Richards, 1991; Agtunong et al., 1992).

High temperatures are associated with reduced pod filling period and increased seed growth rate of faba bean (Dekhuijzen and Verkeke, 1986). However, this increase is unlikely to compensate the effect of shortened pod filling period, and the individual seed weight is adversely affected (Agung, 1995). A reduction in the duration of the reproductive stage was observed in soybeans also (Sapra and Anaele, 1991). The reduction of seed weight under high temperature could be partly attributed to the earlier senescence of the pod wall and the consequent early termination of sucrose transport to the seeds from it (Sjodin, 1971, Chapman and Peat, 1978, Pilbeam et al., 1990a,b). Heat stress during the seed filling may limit the seed size partly by affecting the cell division in the cotyledons (McDonald & Paulsen, 1997). Similar results have also been reported for cereals (Bhuller and Jenner, 1986).

2.6.3 Thermal tolerance

Although heat stress damages all the crops, plants do have some level of tolerance to elevated temperatures. Such a tolerance inherently present in the plants is variously known as intrinsic thermal tolerance, thermal tolerance (TT) and heat tolerance (HT) (Li et al., 1991). When the plants are subjected to above optimum but not lethal, temperatures they develop an ability to tolerate much higher temperatures (Chen et al., 1982, Krishnan et al.). Such a thermal protection against lethal temperatures developed by a plant following exposure to a higher but non-lethal temperature has been defined as acquired thermal tolerance (ATT) (Chen et al., 1982). There exists a wide range of genetic variation among several crops for the ability to acquire thermal tolerance (Blum, 1988). The HT and ATT are two different phenomena and have

different roles in the crop adaptation to temperature stress (Li et al. 1991).

For plant performance in the field, ATT is more relevant than HT because sub-lethal chronic heat stress is more common in field crops than direct heat killing and is also because metabolic changes occurring during such acclimation process contribute more to crop adaptation to heat stress than the HT (Chen et al., 1982, Howarth et al., 1997). For example, it was reported that heat acclimation enables common bean genotypes to reduce heat injury. Their ATT, but not the HT, was positively and significantly related to post stress performance for dry weight, pod set, pod weight and pod yield (Li et al., 1991). Similar reports for sorghum and pearl millet are also available (Howarth et al., 1997). Chaisompongpan et al., (1990) concluded that genotypes with higher acclimation potential experience less injury to photosynthesis under heat stress. Because of the differences in their ability to acclimate, genotypes with higher heat acclimation potential may have received less injury, leading to a better post-stress growth and development.

2.7 Assessing faba bean response to temperature stress

Increasingly faba bean production in southern Australia is moving to low rainfall regions considered unfavourable because of the high probability of occurrence of terminal drought and high temperature stresses (Loss and Siddique, 1997). Therefore an improvement in heat tolerance of faba bean is necessary to sustain its production in the region. Central to this is availability of techniques to determine the thermal sensitivity of crop varieties to identify appropriate breeding materials (Howarth et al., 1997). This involves identification and quantification of traits providing thermal tolerance, and a knowledge of their genetic regulation (Porter et al., 1995). Such traits can be used to screen and identify the best parents for a crossing program

(Srinivasan et al., 1996, Gavuzzi et al., 1997). Genetic variation among and within crop species for thermal tolerance and for post stress performance exists (Blum, 1988, Srinivasan et al., 1996, Howarth, et al., 1997). The trait, controlled mainly by additive genetic effects, is amenable for improvement through breeding (Porter et al., 1994). However, a lack of information on the genetic diversity for heat tolerance and of screening techniques to elucidate them is an important barrier in improving heat tolerance of many crops (Wery et al., 1994).

Desirable characters of a screening technique

Estimating heat tolerance involves exposing a plant or a plant part to a regulated heat stress, followed by estimating the degree of resultant tissue injury (Onwueme, 1979). Heat injury is a function of temperature level, duration of exposure, rapidity of stress and genotypic sensitivity (Li et al., 1991). Therefore, standardisation of a screening technique is required to ensure its repeatability across seasons and locations (Blum & Ebercon, 1981, Clarke, 1983, Gavuzzi et al., 1997). For this, screening conditions providing the greatest genetic differences must be employed (Sullivan, 1972).

A valid heat tolerance test should relate to plant responses to the stress such as tissue growth, or with critical physiological processes and field performances (Blum and Ebercon, 1981, Howarth et al., 1997). The traits used should be quantitative, heritable, and associated with the yield (Blum, 1988). Rapid, simple and inexpensive screening techniques are required to evaluate large populations (Winter et al., 1988).

2.7.1 Acquired thermal tolerance (ATT)

An improvement in the ability of crops to tolerate heat stress is required to sustain and enhance crop production. It is also evident that screening for thermal tolerance is

necessary for germplasm enhancement through plant breeding. The literature shows that the ATT of crops, rather than the HT, is more relevant in the field condition. Therefore, quantifying ATT would form the basis of an improvement in crop production in hot weather conditions.

To quantify ATT several questions have to be answered. Among them, choosing how to evaluate the heat injury, which criterion to evaluate, at what growth stage the evaluation should be done, which plant part has to be used are the most important ones. After deciding on these, the screening techniques will have to be standardised for the crop. The following brief review of heat tolerance research elucidates some of these aspects and affords to plan out the current study.

ATT of crops can be evaluated in the field or under controlled conditions. While field evaluation is very relevant, it has some associated difficulties. Heritability under hot and dry environments of grain yield, which is the primary criterion used in field evaluation, is very low (Blum, 1988). Cell membranes tend to acclimate in response to the prevailing ambient temperatures providing variable levels of heat tolerance (Martineau et al., 1979). Therefore to validly compare the results of different screening tests, environmental conditions prior to and during the screening should be identical. However, high temperature events in the field are inherently variable and non-reproducible. Such an inconsistency causes variation in the outcome of the field screening across the years (Martineau et al., 1979, Gavuzzi et al., 1997, Marcum, 1998). It is also known that a number of environmental factors such as water stress, soil nitrogen and air relative humidity interact with heat stress in the field (Levitt, 1980b, Wehner and Watschke, 1981, Perdomo et al., 1996). These interactions compound the effect of heat stress on plant growth and yield (Marcum, 1998). Apart

from low heritability and inconsistent nature of heat stress events, field evaluations occupy an entire season. Locations and facilities may not always be conveniently available for this. These difficulties render field evaluation and whole plant screening inconsistent and inefficient. On the other hand, laboratory based techniques have control over the screening conditions and are reproducible. They are less affected by environmental conditions and can be performed any time of the year and therefore are very convenient (Gavuzzi et al., 1997, Howarth et al., 1997).

2.7.1.1 Criteria to evaluate ATT

It is desirable that criteria chosen to evaluate ATT be involved in heat reactivity, be heritable and be associated with the yield. A criterion that can judge cell injury under stress would meet such requirements (Onwueme, 1979, Levitt, 1980a,b). The earlier discussion on the mechanism of heat injury indicates the validity of studying the structural and functional responses to quantify ATT. The literature shows that measurement of electrolyte leakage from the tissue and reduction of 2,3,5-triphenyl-tetrazolium-chloride (TTC) are two commonly used approaches that have shown promising results (Blum, 1988). Therefore it is pertinent to review them here.

Electrolyte leakage assay

The electrical conductivity (EC) test is based on the electrolyte leakage from the tissue in response to high temperature. The ease with which electricity passes through a solution containing such electrolytes leaked from the leaf tissue is defined as EC for the purpose of this project. As mentioned earlier, high temperatures disrupt cell membrane and reduce its ability to retain solutes. Consequently membrane permeability increases, leading to cell electrolyte diffusion (Berry & Bjorkman, 1980, Lin et al., 1985, 1986, Collins et al., 1995). Therefore, the electrolyte leakage

is assumed to be proportional to the injury sustained by the cell membrane and thus capable of quantifying heat injury (Srinivasan et al., 1996). Functional cell membrane systems and their integrity during the heat stress are widely reported as central to drought and heat resistance in plants (Sullivan, 1972, Raison et al., 1980, Ristic et al., 1992). ATT is also associated with plasmalemma and organelles remaining intact (Collins et al., 1995). Therefore, electrolyte leakage from plant tissue under heat stress could be used to quantify heat injury and assess ATT. Such an estimation of ATT has been reported in wheat (Blum & Ebercon, 1981, Shanahan et al., 1990, Fokar et al., 1998), barley (Gavuzzi et al., 1997), tomato, potato, beans, soybean (Martineau et al., 1979, Chen et al., 1982, Sapra & Anaele, 1991), common bean (Li et al., 1991, Schaff et al., 1987), chickpea, pigeon pea, groundnut (Srinivasan et al., 1996), and turf grass (Marcum, 1998).

Technique of EC test

Measurement of EC includes treating plant or its tissue in distilled water at specified temperatures, allowing for electrolyte leakage into the surrounding media and measuring initial EC. Total EC of all the cell contents, obtained by killing the tissue in the same bathing media, is measured after this. Heat injury is calculated on the basis of the two measurements. Either whole seedlings (Howarth et al., 1997) or leaf tissue (Martineau et al., 1979, Chen et al., 1982, Shanahan et al., 1990) can be used to obtain electrolyte leakage. When leaf tissue is used, leaf discs (Martineau et al., 1979, Chen et al., 1982, Shanahan et al., 1990, Srinivasan et al., 1996), or pieces of a specified length (Saadalla et al., 1990a,b, Fokar et al., 1998) are heat shocked.

The electrolyte leakage from plant tissue into the surrounding media after the treatment is not immediate. This is a gradual and slow process, necessitating a long

incubation. Although shaking for a specified time can speed it up (Onwume, 1979, Blum & Ebercon, 1981, Chen et al., 1982), incubation at 10°C for up to 24 hours allows proper diffusion of electrolytes (Srinivasan et al., 1996, Howarth et al., 1997, Fokar et al., 1998). Total electrolyte leakage that represents entire cell contents, is obtained by disrupting the membranes of all the cells in the tissue. This is commonly achieved by autoclaving (Martineau et al., 1979, Saadalla et al., 1990a, Marcum, 1998) or by boiling (Shanahan et al., 1990, Howarth et al., 1997).

TTC reduction assay

Reduction of TTC salt to red coloured formazan by the electron transport enzymes in mitochondria forms the basis of TTC reduction assay (Towill & Mazur, 1975). TTC reduction has been associated with cytochrome a-a_3 in plant mitochondria where it may substitute oxygen as final electron acceptor from the electron transport chain (Kalina & Palmer, 1968). Therefore it evaluates mitochondrial enzymatic activity involved in the electron transport chain and represents the functional response of plant tissue to heat stress. While electrolyte leakage is related to membrane integrity, it does not evaluate the functional ability of the cells under high temperatures. Therefore only TTC reduction is capable of identifying genotypes equipped with better functional response to heat stress (Porter et al., 1994). Examples of this trait being used to quantify the thermal tolerance of crops include spring wheat (Fokar et al., 1998), winter wheat (Krishna et al., 1988, Porter et al., 1994), diploid wheat (Vierling & Nguyen, 1992), tomato, potato, beans and soybean (Chen et al., 1982).

Technique of TTC reduction assay

This assay includes incubating treated plant parts, commonly leaf tissue, in a TTC solution in the dark, extracting the formazan produced and reading the intensity of

the colour formation. The TTC solution is prepared in 50mM phosphate buffer (pH 7.4). Although Chen et al., (1982) reported 0.08% of TTC solution to be adequate, the more common concentration is 0.8% (Krishnan et al., 1989, Fokar et al., 1998). The purpose is to provide TTC in excess. Because of the need to get TTC into the cells to enable its reduction by live cells and the associated difficulties, vacuuming has been employed (Chen et al., 1982). Infiltration is enhanced by adding surfactants such as Tween-20 to the TTC solution (Porter et al., 1994). The live cells reduce TTC and form red coloured formazan, which is extracted by 95% ethanol (Towill & Mazur, 1975). Formazan can be extracted by boiling or by incubation. In the first method, TTC treated leaf tissue is boiled to dryness in ethanol, followed by adding ethanol again, and vortexing to get formazan into solution (Chen et al., 1982, Krishna et al., 1989). Alternatively, the TTC treated tissue is incubated in ethanol for up to 24 hours in the dark at room temperature (Porter et al., 1994, Foker et al., 1998). Reading the intensity of colour development, a measure of the extent of TTC reduction, follows extraction of formazan. The colour absorbance is read either at 485 nm (Towill & Mazur, 1975, Chen et al., 1982, Krishna et al., 1989) or at 530 nm (Steponkus & Lanphear, 1967, Porter et al., 1994, Fokar et al., 1998).

Relevance of electrolyte leakage assay

Although EC and TTC reduction provide different perspectives of the thermal tolerance of the plant, they should be compared to assess their value in a breeding program. It has been shown that the EC test is a valid measure of ATT and is related to whole plant and field performances of several crops. Positive correlation between the heat injuries measured by the EC test conducted in the growth chamber and in the field has been reported in sorghum (Howarth et al., 1997). These results were also correlated with the seedling growth under heat stress. Significant correlation between

cell membrane thermal stability (CMT) estimated by the EC test and whole plant heat tolerance has been reported in soybean (Martineau et al., 1979), wheat (Saadalla et al., 1990b, Shanahan et al, 1990) and turf grass (Marcum, 1998). ATT quantified by measuring EC was positively and significantly related to the grain yield and quality of wheat in various regions with the hot climate (Saadalla, et al., 1990b, Fokar et al., 1998). Wide variation among the wheat genotypes for the EC trait was also reported (Saadalla et al., 1990b). The researchers suggested that CMT was associated with the ability of wheat to maintain productivity and grain quality under heat stress. There is evidence that the EC test is consistent across the years in wheat and soybean (Shanahan et al., 1990, Martineau et al., 1979).

There is also evidence about the significant correlation between the TTC reduction and grain yield, its quantitative heritability and genetic variation (Porter et al., 1995). However, in a recent study of spring wheat, the trait was not related to the grain yield in different regions around the world (Fokar et al., 1998). The conflict in the outcomes of the two reports may be primarily due to the nature of field evaluation. In the first study, the genotypes were examined at a single location for grain yield while the second was a multilocation study spread around the globe. This brings into play local environmental variability and the existence of influence of stresses other than high temperatures. The researchers concluded that although TTC reduction was highly heritable, it was less predictive of plant performance in the field than the EC test. Currently the evidence for the suitability of the TTC reduction as a selection criterion is not as strong as it is for EC measurement, but it has to be remembered that TTC reduction represents cell functionality under heat stress. Since EC and TTC reduction measure different aspects of cellular response, together the may provide complete information of the heat response of the plants.

It can be concluded that heat tolerance quantified by measuring EC can predict whole plant performance and grain yield of several crops. With a good standardisation of the procedure, and provided it is relevant for the expected field stress during the growth stage, it can possibly be employed to screen for heat tolerance in breeding programs. Although the reports of suitability of TTC reduction are conflicting, it is worthwhile testing because it measures the cell functionality under heat stress.

2.7.1.2 Suitability of seedling stage for screening

For a breeding program, seedling evaluation is ideal as it is quick and convenient to handle. However, the growth stage at which plants are screened should be carefully chosen because it directly affects the ability of the test to predict field performances. An ability to significantly predict thermal tolerance of the fully developed plants in growth chamber and in field based on seedling screening has been reported in wheat, sorghum, pearl millet, and soybean. For example, Martineau et al., (1979) reported consistent genotypic differences across sampling dates for membrane integrity of soybean during the vegetative stage, suggesting that any phase of vegetative growth could be used for the assay. Significant correlation between the ability of the sorghum seedlings to acquire thermal tolerance and their survival in the field at extremely high temperatures of semiarid tropics has been reported (Howarth et al., 1997). A significant correlation for thermal tolerance, measured using electrolyte leakage as well as TTC-reduction assay on leaf samples, between green seedling stage (10 day old) and flowering stage of wheat has been established in the field and glasshouse experiments (Reynolds et al., 1994, Fokar et al., 1998).) Membrane integrity of hardened wheat seedlings measured in the laboratory was found to be associated with tolerance of grain yield to post anthesis heat stress in the field (Saadalla et al., 1990b). It should be noted here that high temperature affects many

different aspects of physiology such as photosynthetic rate, assimilate partitioning and growth rate. Only at extreme temperatures will membrane thermal integrity affect them (Howarth et al., 1997) and it could be useful only if such extreme temperatures occur. Thermal tolerance of leaves at the seedling stage may be only a vegetative response. However, heat stress during the reproductive phase of an indeterminate plant like faba bean elicits both vegetative and reproductive responses. It suggests a possibility that the seedling stage may not be appropriate to screen for grain yield response. However, the evidence in the literature shows screening seedlings for thermal tolerance can be correlated with the grain yield response of plants to higher temperatures in the field. Although information on the mechanism underlying this relation is lacking, it can be concluded that evaluation of growth chamber grown seedlings for thermal tolerance can validly represent the thermal tolerance of fully developed field grown plants.

2.7.1.3 Suitability of leaves for screening

Thermal tolerance has been measured at whole plant level and by bioassays. At the whole plant level yield components are the main characters studied (Shonnard & Gepts, 1994). Bioassays include cell membrane integrity measured by electrolyte leakage (Shanahan et al., 1990, Fokar et al, 1998) and TTC reduction (Krishnan et al., 1989, Porter et al., 1995) using leaf samples. The difficulties associated with field screening of thermal tolerance and low heritability of yield suggests a necessity to adopt a bioassay for the purpose. Youngest fully open leaves are commonly used in bioassays (Martineau et a., 1979; Fokra et al., 1998; Marcum, 1998).

Immature leaves of pepper (*Capsicum annum L.*) reportedly had lower CMT values than the mature leaves obtained by measuring EC (Anderson et al., 1990). On the

other hand, greatest genotypic differences were observed only in the youngest fully expanded leaves of several crops including soybean (Martineau et al., 1979) and wheat (Kokar et al., 1998). Therefore, using only fully expanded youngest mature leaf appears to be the best practice to ensure the repeatability of the results.

2.7.1.4 The test conditions for ATT

The previous review on heat tolerance and its screening highlighted the importance of the treatment conditions. For a screening method to be applicable to the field performance and be repeatable, the test conditions should be precisely standardised. This involves deciding the temperature regimes to be used, the duration and method of exposure to those temperatures, the stage and plant part to be evaluated, the criterion to measure heat injury and the methods of those measurements. A review of the literature shows wide variations in the conditions used by researchers, making it necessary to develop specific methods relevant for the crop and problems at hand.

Pre-treatment

The procedure of measuring ATT should allow thermal tolerance to develop among the genotypes under study. Chen et al., (1982) reported that plants needed to be pre-treated at a non-lethal higher temperature before testing for ATT to elicit genotypic differences. Subsequent reports, both from the controlled environment studies and the field experiments, have substantiated this requirement (Gavuzzi et al., 1997, Howarth et al., 1997). Shanahan et al., (1990) suggested that evaluations in the field might not elicit the differences in the genotypic ability of wheat for ATT because of the absence of pre-treatment. This was proven by the inability of the wheat leaf to acquire thermal tolerance when it was not acclimated through prior heat treatment (Porter et al., 1994). Therefore, screening for heat injury must involve pre-treatment

that can induce genotypic differences for heat tolerance.

Researchers have used different temperatures to pre-treat various crops. For example, 35°C was used to pre-treat tomato, potato, chickpea, pigeon pea, groundnut and soybean (Chen et al., 1982, Srinivasan et al, 1996), 37.5°C for beans (Chen et al., 1982), 37°C for common bean (Li et al., 1991) and 34°C for spring wheat (Fokar et al., 1998). There are reports of pre-treating the same crop at different temperatures in different experiments. While Saadalla et al., (1990a) used 34°C to pre-treat winter wheat, Porter et al., (1994, 1995) used 37°C. The first study used 10-14 day old seedlings while the latter used 7-day old seedlings. In another study on pearl millet, a very high temperature of 43°C was used to pre-treat 2-days old seedlings. Here the evaluation was targeted to seedling emergence in the semi-arid tropics where soil temperature could reach about 50°C at the time of emergence (Howarth et al., 1997). Therefore, the pre-treatment temperature should be standardised to the crop, to the stage of evaluation and for the problem being addressed.

The duration and method of pre-treatment also need standardisation. Howarth et al., (1997) pre-treated 2-day old pearl millet seedlings in a thermal-gradient water bath for 2 hours. Similarly Saadalla et al., (1990a) used a constant temperature water bath to treat 10-14-days old wheat seedlings. On the other hand, pot-grown seedlings were pre-treated in a growth chamber for up to 24 hours (Onwueme, 1979, Chen et al., 1982, Porter et al., 1994, Fokar et al., 1998). The earlier work focussed on pre-treating pot grown seedlings under lights (Onwueme, 1979, Chen et al., 1982). However, recent research has favoured dark conditions and high humidity (>90%) to minimise transpirational cooling of leaves (Porter et al., 1994, Fokar et al., 1998). Hence the growth stage, the plant material used and transpirational cooling influence

the duration and method of pre-treatment.

Temperatures for pre-treatment can be standardised by different methods. Saadalla et al, (1990a) pre-treated wheat seedlings at 34°C for 0, 6, 12, 24 and 48 hours. They achieved greatest sensitivity in detecting genetic differences with 48 hours. This method was used to standardise the pre-treatment temperatures of soybean and pearl millet also (Martineau et al., 1979, Howarth et al., 1997). On the other hand, Chen et al., (1982) pre-treated seedlings for 24 hours at temperatures from 20°C to 40°C. Srinivasan et al., (1996) used a similar approach for groundnut, pigeon pea, chickpea and soybean. After identifying a critical range in temperature, they fine-tuned it by varying temperature by only 2°C and chose 35°C. The approach of varying only the duration at constant temperature ensures that where genotypes differ only slightly, instrument error in maintaining temperatures does not affect the result (Onwueme, 1979, Chen et al., 1982, Li et al., 1991). In general, standardisation of temperature level involves identifying the temperature providing greatest genotypic differences. This can be done by keeping the temperature constant while varying the duration of exposure, or *vice versa*. However, testing for different durations at a particular temperature appears to be more practical.

Heat shock treatment

In the heat shock (HS) treatment, pre-treated plant tissue is subjected to a higher temperature, above that of the pre-treatment, to obtain maximum genetic differences for heat injury (Martineau et al., 1979, Li et al., 1991). Chen et al., (1982) used 50°C for heat shocking tomato, potato, soybean and beans. A HS treatment at 48.5°C to 50.5°C for 15 minutes provided maximum differences among the soybean genotypes (Martineau et al., 1979). For wheat, the HS temperature was reported to be 44°C

(Blum & Ebercon, 1981, Shanahan et al., 1990). Typically, a range of temperatures and durations are tested to obtain a level of heat injury at which the genotypic differences are maximum (Sullivan, 1972, Onwueme, 1979, Chen et al., 1982). The objective of HS treatment, therefore, is to injure the cell membrane of the pre-treated tissue to a level at which the genotypes will show maximum differences.

In summary, the screening techniques to evaluate heat injury need to be standardised. They should be capable of inducing the genotypic differences for ATT and measuring those differences. For this, pre-treatment of plants at a high temperature and a subsequent heat shock at a still higher temperature are necessary. The temperature regimes and durations can be standardised either by testing different temperature levels at a constant duration or vice versa. The conditions providing maximum differences between the genotypes for heat injury should be chosen.

Methodology of measuring heat injury

Heat injury can be quantified by measuring EC or TTC reduction. Although there are other criteria such as photosynthesis, chlorophyll content and dry weight (Vierling & Nguyen, 1992, Srinivasan et al., 1996, Howarth et al., 1997) and yield components (Li et al., 1991) apart from yield itself (Saadalla et al., 1990b, Fokar et al., 1998), EC and TTC reduction are the methods most frequently adopted for bioassays (Chen et al., 1982, Shanahan et al., 1990; Porter et al., 1995; Fokar et al., 1998). A survey of the methodology of the two measurements has been provided earlier in the sections on electrolyte leakage assay and TTC-reduction assay.

2.7.2 Heat shock proteins

HSPs are localised in cytoplasma, chloroplast and endomembrane (Hernandez &

Vierling, 1993). Although their precise functions are not known yet, it is suggested that the HSPs are involved in heat tolerance (Ristic et al., 1996). A number of HSPs function as molecular chaperones (Boston et al., 1996). This involves binding to proteins in non-native structural state, thereby facilitating protein folding, targeting and degradation. During high temperature stress molecular chaperones reportedly prevent irreversible protein denaturation (Parsell & Lindquist, 1993). It is suggested that this protective role may also be associated with stabilising cell structures, which keeps plasmalemma and organelle intact and thereby functional (Collins et al., 1995). Related to this, it was shown that HSPs were associated with various cell membranes (Cooper & Ho., 1987). Also, Ristic et al., (1991), reported that the pattern of HSP synthesis in maize was similar to the results of membrane injury.

Major advances in unravelling the functions of HSPs have occurred only recently. For example, Lee and co-workers demonstrated the chaperone function of LMW-HSPs *in vitro* in 1995. They showed that in pea, HSPs 18.1 kD and 17.7 kD prevent thermal aggregation by binding to the proteins in non-native state (Lee et al., 1995). In further *in vitro* studies it was suggested that LMW-HSPs not only prevent protein aggregation under heat stress but may also facilitate the refolding of those bound proteins in association with other chaperones (Lee & Vierling, 2000). Involvement of HSP 70 kD with LMW-HSP in renaturation of such a denatured, bound protein has been demonstrated *in vivo* in *Arabidopsis thaliana* (Forreitar et al., 1997). However, *in vivo* evidence of HSP functions in plants has not been reported so far. A strong correlation between the productions of LMW-HSP localised to chloroplast and plant thermal tolerance was observed in plants of six divergent *Anthophyta* species including peas and maize (Downs et al., 1998). It was suggested that these HSP are involved in thermal protection rather than repair while the reverse may be true for

HSP 70 kD. Lee et al (1995) also reported similar conclusions on the LMW-HSPs of pea. In 1996, Hartl reported the chaperonic role of HMW-HSP in protein folding and importing into organelle. In further clarification on the function of LMW-HSPs localised in chloroplast, it has been demonstrated that they protect sensitive photosystem II and consequently the whole chain of electron transport during heat stress (Heckathorn et al., 1998). In summary, LMW-HSP are involved in protective role wherein they bind to denatured proteins during heat stress while HMW-HSP repair these damaged proteins by refolding them during the recovery process.

The majority of the work on HSPs have been confined to *in vitro* conditions. However, there are a few reports of *in vivo* studies also. Production of HSPs in wheat under a simulated field environment has been documented, where HSP synthesis was positively correlated with heat tolerance (Nguyen et al., 1994). Kimpel & Key (1985) observed that the profiles of HSP messenger RNA accumulation were similar between field grown and laboratory planted soybeans when subjected to heat shock. They concluded that HSPs accumulate in response to a gradual, as well as a sudden increase in temperature. Burke et al., (1985), have reported accumulation of HSPs in field grown cotton that was heat stressed. As mentioned earlier, *in vivo* studies with Arabidopsis have demonstrated the chaperonic activity of HSP 70 and LMW-HSP (Forreiter et al., 1997). It is expected that as the knowledge of the HSPs widens through *in vitro* study, more work will focus on the field experimentations.

It is suggested that the synthesis of HSPs is involved in the development of thermal tolerance (Vierling, 1991). Correlations between the ATT due to heat treatment and synthesis of HSPs were reported in wheat (Joshi et al., 1997), sorghum (Howarth & Kirsten, 1994), soybeans (Kimpel & Key, 1985), corn (Jorgensen et al., 1992) and

various other crops. Besides, the requirement of specific HSPs for the establishment of heat tolerance has been reported (Lee et al., 1994, Schirmer et al., 1994). In wheat, a positive correlation exists between synthesis of specific LMW-HSPs and the degree of tolerance acquired following exposure to elevated temperatures (Krishnan et al., 1989, Vierling & Nguyen, 1992). The researchers found that the seedling survival at HSP inducing temperature was positively correlated with the ATT. The ATT was measured using TTC-assay. In maize, synthesis of LMW-HSPs was related to ATT measured using EC (Jorgensen et al., 1992). However, it is not known if ATT and HSP are related causally or if HSPs are just a consequence of the high temperatures. In this regard it is appropriate to note that while ATT is only transient, HSPs persist much longer, even for a number of days (Howarth, 1991). Hence the presence of HSPs in a cell need not imply its thermal tolerance at that time. In a related work in sorghum it was observed that HSP synthesis and development of thermal tolerance were correlated but the decay of the tolerance and degradation of HSP were not linked (Howarth & Kirsten, 1994). Also the nature of the temperature fluctuations in the field make the plant's ability to survive continued heat shocks and to synthesise HSPs each time heat shock is encountered more critical than the ability to survive a single heat shock (Howarth and Ougham, 1993). Therefore it is possible that *de novo* synthesis of HSP is required for the development of thermal tolerance.

Apart from the work on their expression and their relationship with the development of thermal tolerance, researchers have also been trying to unravel the genetics of the HSP synthesis. Qualitative and quantitative diversity of HSP synthesis has been established in diploid wheat (Vierling & Nguyen, 1990). Similar genetic variation was reported from maize (Jorgensen & Nguyen, 1995) and sorghum (Jorgensen et al., 1993). But there is a lack of study of inheritance of differences in HSP expression

and their relationship with the development of TT (O'Connel, 1994). This anomaly is being addressed now. Joshi et al., (1997) have demonstrated genetic linkage between ATT and the differential expression of a member of HSP 26 gene family in wheat using the TTC-assay. Genetic linkage between heat tolerance of creeping bent grass (*Agrostis palustris* Huds) and the presence of HSP 25 polypeptide has also been reported (Park et al., 1996). Genetic inheritance of HSP synthesis in maize has also been reported (Jorgensen & Nguyen, 1995). The study showed that the synthesis of all parental HSP in F-1 generation conformed to dominant inheritance pattern.

In summary, HSP are associated with thermal protection. They are considered to be molecular chaperones involved in preventing protein degradation under heat stress and refolding of damaged proteins during the recovery. These functions of HSP have been demonstrated *in vitro*. Although currently there are only few *in vivo* studies on the HSP synthesis and functions, they support the findings of *in vitro* experiments. It is expected that future research will concentrate more on *in vivo* studies. Apart from chaperone activity, HSP have been correlated with the development of thermal tolerance in response to heat stress, both in the field grown and laboratory grown plants. However, the exact mechanism underlying this relation is not known yet. In this regard, present efforts are concentrating on studying the genetic linkage between differences in HSP synthesis and their relationship with thermal tolerance. It is hoped that more relevant information on the HSP synthesis and thermal tolerance will be forthcoming in future which can be incorporated in crop improvement programs.

2.7.2.1 The need to screen for HSP

Plants produce unique HSP under heat stress, which may be involved in a protective role. At least some HSP may be involved in adaptation, tolerance or recovery

processes under water stress (Almoguera, et al. 1993). Protein structure being an important site of perception of temperature inside the cells, a study of HSP should provide insight into the importance of regulation of heat shock response at the site of perception itself (Levitt, 1980a, Ho, 1987). Therefore, it might be useful to screen the faba bean genotypes for their ability to produce HSP at higher temperatures.

The objective of screening genotypes for HSP production is to study the possibility of using them as markers to improve germplasm for hot environments (Krishnan et al., 1989). This requires information on diversity for inheritance of HSP genes and the role of HSPs in acquiring thermal tolerance (Blum, 1988). Therefore, the first step should be to study genetic diversity in HSP synthesis and its relationship with the genetic diversity in thermal tolerance. This should be followed by a comparative analysis of HSP synthesis in cultivars possessing different thermal tolerances. Investigations of the inheritance of the differences in HSP synthesis, in the ability to acquire thermal tolerance and of their relationship should provide a sound basis for crop improvement under temperature stress (Blum, 1988, Krishnan et al., 1989).

2.7.2.2 The test conditions

Although plants produce HSP in response to heat stress, the temperature at which maximal HSP synthesis occurs is dependent upon the optimum growth temperature of a crop (Howarth and Ougham, 1993). Also, within a species there are differences for this temperature among the genotypes and between the growth stages (Howarth, 1989). Hence, it is more appropriate to study the qualitative differences in the HSP production at an appropriate temperature than the quantitative differences.

Screening for HSP becomes meaningful for crop improvement when it is combined

with tests of thermal tolerance. Standardising test conditions for thermal tolerance has already been reviewed. As a result, temperature regime to study HSP synthesis can be adapted from that for thermal tolerance work, depending on the experimental requirements. Even though HSP can be expressed both by sudden and gradual increases in temperature (Kimpel and Key, 1985), use of single high temperature to elicit HSP synthesis is common. In general, the heat shock temperatures used for measuring thermal tolerance are adopted to induce HSP synthesis (Krishnan et al., 1989, Vierling, 1991, Howarth & Kirsten, 1994, Joshi et al., 1997).

The HSP are produced in vegetative and reproductive parts of plants (Blumenthal et al., 1990). Accordingly researchers have used leaf (Ristic et al., 1991, Jorgensen and Nguyen, 1995), hypocotyl (Collins et al., 1995), coleoptile (Blumenthal et al., 1994), roots or whole seedlings (Howarth et al., 1997). It would only be appropriate to use the same plant part to study both HSP synthesis and thermal tolerance. At the end of heat shock, total protein content is extracted from the plant tissue, commonly using sodiumdodecylsulphate (SDS) buffer (Laemmlli, 1970). This crude protein extract is electrophoresed and separated into bands. Polyacrylamide gels are commonly used to electrophorese crude protein extract (Laemmli, 1970, Krishnan et al., 1989).

Separation of the HSP by one dimension gel electrophoresis is difficult because of the minute quantity in which they are produced (Vierling & Nguyen, 1990). There are alternatives such as the use of two-dimensional gel electrophoresis (Jorgensen and Nguen, 1995) and chromatographic separations. These are specialised techniques and more difficult compared to the single dimensional gel electrophoresis. However, labelling of the HSP with radioactivity makes it possible to use the single dimension gel electrophoresis to separate stress proteins (Martino-Catt et al., 1993, Howarth and

Skøt, 1994). After the electrophoresis of labelled proteins, the gels can be subjected to fluorography and autoradiography to visualise the different protein bands on the X-Ray film (Howarth and Skøt, 1994, Jorgensen and Nguyen, 1995).

Radio-labelling is a common method of studying the stress proteins. Generally the plants are labelled during the final hour of the heat treatment (Jorgensen et al., 1992) wherein a radioactive amino acid is introduced into the plant system. The most common label used is the amino acid S^{35}Methionine (Joshi et al., 1997, Vierling & Nguyen, 1990). Other labels such as Trans S^{35}label ($_{L}MetS^{35}$; $_{L}CysS^{35}$) (Park et al., 1996), and $_{L}(3,4,5\text{-}^{3}H(N))$ leucine (Kimpel et al., 1990) have also been used. The plants can be labelled *in vivo* or *in vitro*. The *in vivo* labelling involves directly introducing the radioactive material into the plant tissue, of an intact plant (Martino-Catt et al., 1993, Howarth and Skøt, 1994). Theoretically *in vivo* labelling allows experimentations on field or pot grown intact plants. However, the huge logistical requirements of space for plants of large architecture and the safety requirements render the method unpractical in certain circumstances. The *in vitro* labelling is done on the harvested tissue, by introducing the label into an incubating buffer during the heat stress (Krishnan et al., 1989, Jorgensen and Nguen, 1995). This procedure is easy to handle irrespective of plant architecture because only the harvested tissue is labelled. Therefore, depending upon the situation and the facilities available, both the methods have applicability in analysing the HSP production.

HSPs may have a protective role under heat stress. They are possibly involved in adaptation to drought and other stresses that act through cell membrane disruption. Therefore it is appropriate to use HSP markers to improve plant performance under heat stress. However, screening for HSP becomes relevant when combined with tests

for thermal tolerance. While HSP can be induced by gradual increase in temperature also, frequently a single high temperature is used. Although HSP are produced in different parts of the plant, leaves are commonly used in experimentations. Because of the minute quantity in which they are produced, the HSP need to be labelled with radioactivity to be effectively resolved on gels. Plants can be labelled both *in vitro* and *in vivo*, but the latter procedure is of limited use due to logistical requirements.

2.8 General conclusions

Legumes are relatively new in Australian agriculture. Faba bean is an important legume grown on large areas of southern and western parts of Australia. Its value in the crop rotations is recognised. The industry caters to human consumption, feed market requirements and is export driven. However, the highly unpredictable yield is the main constraint for the expansion of area and production of the crop. The yield variations are attributed to the susceptibility to drought, heat and diseases. Water stress results in stomatal closure and hinders transpiration. As a result the plants cannot attain transpirational cooling and the canopy temperature increases. Therefore it is difficult to separate the water and temperature stresses, which accentuate the effect of each other. The Mediterranean climate of South Australia is characterised by the terminal water deficit coupled with rising temperature. Hence the reproductive stage of the faba bean is likely to experience the combined effect of the two stresses.

Water deficit and high temperatures adversely affect many components of growth and yield of faba bean. Water stress restricts dry matter production and reduces the duration of flowering and maturity. Faba bean needs large amounts of water to maintain turgor and lacks osmotic adjustment under water stress. Abscission of the reproductive parts occurs under the stress. Finally, the seed yield and all its

components are reduced. High temperature causes injury to the photosynthetic apparatues and lowers the water potential of faba bean. Flowering is reported to be very susceptible to temperature stress, with flower initiation delayed and fewer flowers being produced. Duration of pod filling gets shortened under higher temperature. Thus, susceptibility of the reproductive stage adversely affects the final yield.

Crop improvement under water and temperature stress depends on the ability to identify genotypes differing for traits relevant to the yield. This requires identifying traits affecting the growth and yield, and understanding their contribution to the stress tolerance. Because a single trait is unlikely to substantially alter the crop response, a number of them will have to be considered. The ability of a trait to combine several important processes of growth and yield formation and the ease with which it can be quantified are the major factors to be considered while deciding on a trait. Selection of such traits should be targeted to the specific locations.

Studies on faba bean improvement under water stress have mainly concentrated on the field experiments. The usefulness of the traits associated with water relations has yet to be studied and established in improving the stress response of the crop. Genetic variation exists for the flowering time, maturity and seed yield of faba bean. Cell membrane integrity and stress protein productions have not been studied in faba bean, nor has there been any effort in using them for crop improvement. Very little information is available on the effect of high temperature on faba bean production. Since rising temperatures in the southern Australia cropping zone accompany terminal water stress, it is necessary that faba bean improvement should include better responses to temperature stress as well. Therefore the present study will

evaluate the response of faba bean to water and temperature stresses. The usefulness of the physiological characters in assessing the genotypic response will be studied. The ability of the cell membrane integrity, estimated by EC and TTC methods, in evaluating the faba bean response will be examined. Attempts will be made to study the HSP synthesis and their usefulness in evaluating the genotypic variations.

Chapter 3

General Materials and Methods

3.1 Introduction

The current study involved agronomic and physiological evaluation of selected faba bean genotypes for the purpose of developing techniques to screen for response to drought and high temperature stress. During the present study two field experiments were conducted, one in 1995 and one in 1998, both at Roseworthy, South Australia. Apart from the field trials, several glasshouse experiments were also conducted. The materials and methods specifically employed in each study are described in detail in Chapters 4, 5 and 6. This chapter provides information on some common materials and methods used in various experiments. Generally these methods are based on the results of preliminary trials conducted specifically for the purpose.

3.2 Measurement of leaf water status

Leaf water status was measured using relative water content, leaf water potential, osmotic potential and leaf stomatal conductance. All these parameters were recorded on the youngest fully opened leaf between 12 noon and 2 pm.

3.2.1 Relative water content (RWC)

Sets of 10 leaf discs, each of 0.72 cm^2, were punched from leaves in each replicate in both the control and water stressed treatments using a core borer. The leaf discs were collected in an airtight container and transported to the laboratory on ice. The fresh

weights of these discs were determined and they were floated at room temperature on distilled water in a petri dish for 8 hours under a light intensity approximating the compensation point without any increase in the temperature of the leaf tissue. The surface of these discs was gently blotted with tissue paper and their turgid weight was recorded. The discs were dried in an oven at 85°C for 48 hours to obtain dry weight. The RWC was calculated as follows (Turner et al., 1978):

$$\text{RWC (\%)} = \frac{(\text{fresh weight} - \text{oven dry weight})}{(\text{turgid weight} - \text{oven dryweight})} \text{X100}$$

3.2.2 Leaf water potential

Leaf water potential was measured by the pressure chamber method (Scholander et al., 1964), using the youngest fully emerged leaf. Immediately after harvesting, within seconds the leaf was covered in an airtight plastic bag and sealed to reduce evaporation loss (Turner, 1981). The leaf was inserted in the pressure chamber with the cut end of the petiole protruding from the chamber through a rubber seal. The chamber was immediately pressurised with compressed air slowly (0.005 MPa/s) until the sap was expressed at the cut end. A low powered lens was used to detect the expression of sap from the cut end.

3.2.3 Osmotic potential

Osmotic potential was measured using a vapour pressure osmometer (Wescor, Logan, Utah, USA). A leaflet from the leaf on which water potential was measured (youngest fully opened leaf) was immediately frozen in liquid nitrogen and brought to the laboratory. There it was thawed to the room temperature and sap expressed using a 1ml plastic syringe. This sap was used to determine osmotic potential.

3.2.4 Stomatal conductance

Stomatal conductance was measured using a porometer (Mk3 Delta-T Devices, Cambridge, England). The porometer does not take into account boundary layer component. Conductance values recorded from both sides of the leaves were added. Only the fully lit leaves were used to record the conductance.

3.3 Growth measurements

Growth parameters recorded in the experiments included leaf area, crop growth rate (CGR), specific leaf area (SLA) and dry weight. The leaf area was measured using a Patten Electronic Planimeter, (Patten Industries, South Australia). Dry weight of the leaves was obtained and specific leaf area (cm^2/g) was calculated as follows:

$$SLA = \frac{\text{Leaf area}\,(cm^2)}{\text{Leaf weight}\,(g)}$$

Dry weight of plants or plant parts was obtained by drying them in an oven at 85°C for 48 hours. Dry weight was used to obtain crop growth rate. (CGR, g/plant.day) was calculated as follows:

$$CGR = \frac{TDM_2 - TDM_1}{t_2 - t_1}$$

Where, TDM_1 and TDM_2 are the total dry weights (g/plant) recorded at t_1 and t_2 respectively, where t_1 and t_2 are the number of days after sowing of these consecutive harvests respectively. The stage t_2 is later than the stage t_1.

3.4 Roseworthy site details

The field experiments of 1995 and 1998 were conducted in paddock E7 at Roseworthy Agriculture College farm. The farm is situated at latitude 34.53° S and longitude 138.69° E, at an elevation of 68.0 m above mean sea level. The paddock

has mallee brown soil (US Soil Taxonomy - Xerochrept), with surface pH of about 7.5 and sub-soil pH of 8.5. The subsoil contains free limestone.

Details of the long-term average rainfall and temperature and values for years 1995 and 1998 are provided in Appendix 3.1.

The land preparation for sowing consisted of ploughing and harrowing. The design used for the field experiments was randomised complete block design (RCBD). Six row cone seeder was used for sowing. During sowing fertiliser was also ploughed in. Weed control was done manually. The crop was sprayed with fungicide to protect against chocolate spot.

3.5 Seed yield harvest

In the field trials ten plants were harvested from the two central rows of each plot. They were used to record seed yield, seed number, pod weight and pod number. Only the pods that contained seeds were counted and weighed. These parameters were converted to per m^2 by multiplying with the number of plants per m^2.

Seed to pod ratio (%) was calculated as follows:

$$\text{Seed:Pod} = \frac{\text{Seed yield (g/plant)}}{\text{Pod yield (g/plant)}} \text{X100}$$

3.6 Statistical analysis

The data obtained were analysed using analysis of variance (ANOVA) technique, using the Genstat statistical package (Lawes Agricultural Trust, IACR, Rothamsted),. Least significant difference (LSD) was calculated at 5% probability, wherever applicable (Gomez & Gomez, 1983). Correlation coefficient (r) and Spearman's rank

correlation (r_s) were obtained where applicable (Gomez and Gomez, 1983). The data were checked for normality to see whether transformation was required, but no transformation was needed. The coefficient of variation (CV) was also calculated for many of the data sets to allow variability between different experiments to be compared.

Chapter 4

Effect of water regimes on the seed yield of faba bean genotypes under the field conditions

4.1 Introduction

Faba bean is an important grain legume that, under favourable conditions, produces high yields (Perry, 1994). The Mediterranean climate of southern Australia is well suited to the crop (Perry, 1994, Mwanamwenge, 1998) and its performance in the region has been comparable to, and in some instances better than, that of peas, the major pulse crop grown in the region (Walton and Trent, 1988, Siddique et al., 1993). While its cultivation has mostly been concentrated in southern Australia (Krieg et al., 1996) recently there have been large increases in the area sown in the Western Australia (Loss and Siddique, 1997).

Currently the average yield of faba bean in Australia is only 1.3 t/ha (PulseAustralia, 2000). The growth and yield of faba bean is affected by several factors, among which rainfall is important (Grashoff, 1990a, b, Knight, 1994). The Mediterranean climate of southern Australia is characterised by a high incidence of drought during the spring (French, 1981). As a result, the chances of crops experiencing drought stress during the reproductive stage are very high (Richards, 1991). This shortage of moisture generally coincides with flowering and pod filling stages in faba bean.

By adversely affecting both the vegetative and reproductive growth, moisture stress considerably reduces faba bean yield. Drought reduces leaf size, increases leaf senescence and therefore, longevity of the leaf canopy declines (Farah, 1981, Finch-

Savage and Elston, 1982, Karamanos et al., 1982). Moisture stress adversely affects the rate of photosynthesis, stem dry matter accumulation and growth rate thereby affecting the yield potential of the crop (Green et al., 1986, Grzesiak et al., 1989, Grashoff et al., 1990a). Duration of flowering, number of days to maturity and rate of biomass accumulation during the reproductive growth of faba bean are all reduced under drought (Lockerman et al., 1985, Xia, 1994). As a result it limits the availability of assimilates to the reproductive sinks, reducing the seed yield and yield components of the crop (Pilbeam et al., 1990b).

In southern Australia, faba bean is generally grown in areas where annual rainfall exceeds 400mm (McDonald et al., 1994, Siddique and Sykes, 1997). Even in these higher rainfall areas the crop is exposed to periods of water stress. Therefore, to increase the production and extend the area of cultivation of faba bean it is necessary to have cultivars that are tolerant to drought and can perform well in the low rainfall conditions (McDonald, et al., 1994). This requires assessing the extent of genetic variation for yield performance under such situations. A field study was conducted in 1995 at Roseworthy, South Australia, for this purpose, to study and compare the genotypic variation among the faba bean genotypes for growth and seed yield under rainfed and irrigated conditions. The aim was to measure the total dry matter accumulation during the growth of various genotypes of faba bean and the final seed yield and its components to accomplish the objectives.

4.2 Materials and methods

Eight faba bean genotypes were chosen for the field study to represent the various seed sizes and flowering times found within the breeding program of South Australia. They included Fiord, the first released and the most widely adapted

genotype in the country. The selected genotypes and their characteristics were as follows:

Genotype	Seed size	Seed weight (g/seed)	Maturity group	Flowering time (Days after sowing)
Fiord	Small	0.48	Early	66-70
Icarus	Medium	0.83	Late	96-101
Ascot	Small	0.45	Medium	69-76
Acc286	Large	1.35	Early	52-56
Ac527	Medium	0.79	Medium	73-77
Acc617	Large	1.15	Medium	71-77
Acc820	Large	1.98	Medium	68-73
Acc973	Small	0.37	Late	94-100

The treatments consisted of eight genotypes grown with or without irrigation, and in three replications. The experiment was conducted in a split-plot design with irrigation in the main plots and genotypes in the sub-plots (Appendix 4.1). The trial was sown on the 4 July 1995, which was later than the optimum period. The dimensions of individual plots were 10m x 1.5m with six rows of plants. The row spacing was 25 cm and the sowing density was 40 plants/m^2. Plots were later thinned to 25-30 plants/m^2. Irrigation commenced 87 days after sowing (DAS) (on 30 September) after most of the genotypes had flowered (Appendix 4.2). Early flowering genotypes were well into podding when irrigation commenced whereas the other genotypes had just reached the early flowering stage. Water was supplied weekly through a drip irrigation system to the intended main plots. The other set of main plots (rainfed) received only the incident rainfall. Drip irrigation and an isolation distance of 2 m between the main plots within replications were used to avoid movement of water between the main plots. Irrigation was continued until physiological maturity of all the genotypes commenced (last irrigation supplied on 9 November). Total water supplied for the experimental area was equivalent to 22 cm of rainfall (80 m^3 of water for 360 m^2 area).

Growth during the season was estimated from three dry matter harvests, at 58, 87 and 116 DAS, broadly corresponding with the early flowering, early podding and cessation of podding stages of the genotypes being studied. Five plants were harvested at each observation and dried at 85°C for 48 h. and total dry weight was recorded (g/plant). At the harvest on 116 DAS, the number of viable pods produced and their dry weight (g/plant) were recorded. Pods more than 1 cm long were defined as viable pod at this harvest. At maturity, ten plants were harvested from the two central rows of each plot for yield (g/plant). They were used to record seed yield, seed number, pod yield and pod number. At maturity, only the pods that contained seeds were counted and weighed. No attempt was made to record the total dry weight at maturity because of the complete senescence of leaves. Whole plot seed yields were estimated from a machine harvest.

Data from August and September were analysed as a randomised complete block design with six replicates as there was no irrigation imposed at that time. Least significant difference (LSD at 5% probability), crop growth rate (CGR) (g/plant/day) and seed to pod ratio (seed:pod) were calculated as described in Chapter 3.

4.3 Results

4.3.1 Total dry matter production and crop growth rate

The faba bean genotypes differed significantly for dry matter production (TDM) (Table 4.1). At 58 DAS Acc820 and Acc286, the large seeded genotypes, produced significantly more TDM than the small seeded genotypes. At 87 and 116 DAS, Acc527 recorded the highest TDM but was similar to Acc286, Acc820, Acc617 and Fiord. These genotypes included a range of flowering times from very early to medium duration. Acc973 consistently produced significantly less TDM at all the

harvests. Although Acc973 and Icarus were very late flowering genotypes, their TDM was equivalent to the earlier flowering genotypes like Ascot and Fiord at all the harvests. Hence, it appears that the dry matter production in the early stages of growth was affected more by seed size than by the flowering time. TDM under rainfed condition was significantly lower than under irrigation at 116 DAS (46% reduction), however, the genotype and water regime interaction was not significant (Table 4.1). This could be attributed to the relatively high variability in the data (CV=26%). TDM of the genotypes under the two water regimes were not correlated (r=+0.25; n=8) suggesting that rankings of genotypes differed under the two water regimes.

The genotypes differed significantly for CGR from sowing to 58 DAS and between 59 and 87 DAS. Water stress significantly reduced CGR at 116 DAS (54% relative reduction), but its interaction with the genotypes was not significant (Table 4.2). This could be attributed to the high variability in the data (CV=35%). CGR of the genotypes under the two water regimes were not correlated (r=-0.13; n=8), indicating that they maintained different relative rankings under the two water regimes.

Generally large seeded genotypes recorded higher CGRs during the early stages of growth, than the small and medium seeded genotypes. Acc820 and Acc286, both large seeded genotypes, recorded significantly the highest CGR from sowing to 58 DAS. Acc527 recorded the highest CGR from 59 to 87 DAS, but this was not significantly different to Acc617 and Acc286. Acc973 and Ascot, small seeded genotypes, consistently recorded the lowest CGR at each of these harvests. Under irrigation, the seed size did not confer any advantage to the genotypes for CGR (87 DAS-116 DAS). This trend is similar to that of the dry matter production (Table 4.1).

Table 4.1: Dry matter production of eight faba bean genotypes under rainfed and irrigated conditions at Roseworthy, 1995

Genotypes		Total dry weight (g/plant) at Days after sowing				
		58	87	116		
				Rainfed	Irrigated	Average
Small seed size						
Early maturing	Fiord	1.9	8.1	25.6	45.6	35.6
Medium maturing	Ascot	1.6	7.0	20.4	36.6	28.5
Late maturing	Acc 973	1.1	5.6	21.4	30.9	26.2
Medium seed size						
Medium maturing	Acc 527	2.4	10.2	28.1	49.3	38.7
Late maturing	Icarus	2.0	7.6	18.2	42.3	30.3
Large seed size						
Early maturing	Acc 286	3.2	10.1	24.8	36.3	30.6
Medium maturing	Acc 820	3.4	9.9	28.9	49.1	39.0
Medium maturing	Acc 617	2.3	9.7	20.5	58.8	39.6
				23.5	**43.6**	
Least significant difference (p=0.05)		**0.5**	**1.9**			
Genotype				**10.2**		
Water level				**5.1**		
*Genotype*Water level*				**non-significant**		

Table 4.2: Crop growth rate of eight faba bean genotypes under rainfed and irrigated conditions at Roseworthy, 1995

Genotypes		Crop growth rate (g/plant/day) during the intervals (Days after sowing)				
		0-58	59-87	88-116		
				Rainfed	Irrigated	Average
Small seed size						
Early maturing	Fiord	0.03	0.22	0.61	1.29	0.95
Medium maturing	Ascot	0.03	0.18	0.47	1.02	0.74
Late maturing	Acc 973	0.02	0.15	0.55	0.86	0.71
Medium seed size						
Medium maturing	Acc 527	0.04	0.27	0.64	1.32	0.98
Late maturing	Icarus	0.03	0.20	0.37	1.18	0.78
Large seed size						
Early maturing	Acc 286	0.06	0.24	0.57	0.84	0.71
Medium maturing	Acc 820	0.06	0.23	0.71	1.29	1.00
Medium maturing	Acc 617	0.04	0.26	0.40	1.66	1.03
				0.54	**1.18**	
Least significant difference (p=0.05)						
Genotype		**0.01**	**0.03**	**non-significant**		
Water level				**0.18**		
*Genotype*Water level*				**non-significant**		

4.3.2 Pod production

Overall, there was a 52% reduction in pod number under rainfed condition at 116 DAS (Table 4.3). However, for Acc973, Acc286 and Acc820, pod number was not affected by irrigation. Relative reduction in the pod numbers of the genotypes at 116 DAS ranged from 24% (Acc973) to 75% (Acc617). At maturity, pod numbers were reduced by 65% under rainfed condition and significant reductions occurred in all the genotypes, but the differences between the genotypes was less (from 58% for Fiord to 88% for Acc820). The relative reductions of pod numbers under the rainfed condition at the two harvests were not correlated ($r=+0.04$; $n=8$), suggesting that water stress continued to reduce the pod numbers beyond 116 DAS. Because the interaction of water regimes and genotypes was significant for pod numbers at 116 DAS but not at maturity, it can be argued that the extent of relative reduction of pod numbers beyond 116 DAS varied among the genotypes.

Genotypes differed significantly for the number of pods produced per plant at 116 DAS and at maturity. In general, large seeded genotypes produced fewer pods than the small seeded genotypes under the two water regimes at both the harvests. Among the small seeded genotypes Fiord recorded higher reduction in pod number under water stress at 116 DAS than the late maturing Acc973. This was an effect of the difference in flowering time because, while Fiord did not experience a further reduction, Acc973 recorded a much higher reduction in pod numbers at maturity than at 116 DAS. Among the large seeded genotypes, early flowering Acc286 recorded a lower reduction in pod number than Acc617 at 116 DAS. However, the final reduction in the pod numbers of the genotypes indicates that Acc617 did not record further pod loss while Acc286 experienced a high reduction. This suggests that

Table 4.3: Pod production of the faba bean genotypes as influenced by the water regimes at Roseworthy, 1995

Genotype		Number of pods produced per plant						Relative reduction of pod number (%) under rainfed condition	
		At 116 Days after sowing (DAS)			At maturity				
		Rainfed	Irrigated	Average	Rainfed	Irrigated	Average	116 DAS	Maturity
Small seed size									
Early maturing	Fiord	16	40	28	11	25	18	60	58
Medium maturing	Ascot	16	31	23	9	23	16	48	62
Late maturing	Acc 973	22	29	26	9	22	15	24	60
Medium seed size									
Medium maturing	Acc 527	16	37	26	8	22	15	57	63
Late maturing	Icarus	7	21	14	4	14	9	67	71
Large seed size									
Early maturing	Acc 286	10	16	13	4	12	8	38	68
Medium maturing	Acc 820	5	8	6	1	8	5	38	88
Medium maturing	Acc617	5	20	13	2	9	6	75	74
		12	**25**		**6**	**17**			
Least significant difference (p=0.05)									
Genotype			**7**			**6**			
Water level			**3**			**2**			
*Genotype*Water level*			**9**			**non-significant**			

although early flowering enabled Acc286 to produce more pods by 116 DAS under rainfed condition, the genotype did not retain most of them until maturity. Therefore, while early flowering time enabled small seeded genotypes not to experience further pod loss under rainfed condition beyond 116 DAS, late flowering time did so for large seeded genotypes.

Water stress significantly affected the pod dry weight at maturity but not at 116 DAS (Table 4.4). At maturity, the relative reduction in pod dry weight under rainfed condition was 64% compared to 17% at116 DAS. Reduction in the pod weight of the genotypes under water stress has been reported earlier (Grzesiak et al., 1989). This response was attributed to the adverse effect of water stress on photosynthesis and the resultant reduction in the availability of assimilates to the reproductive sinks (Pilbeam et al., 1990a,b, Xia, 1994).

The interaction between water stress and genotypes was not significant for pod weight at both 116 DAS and maturity. This could be because of the high variability (CV=47%) of data of pod weight at 116 DAS. The relative reduction of pod weight of the genotypes was variable. At 116 DAS, it ranged from no change (Icarus and Acc617) to a high of 36% (Fiord) (Table 4.4) while at maturity, all the genotypes recorded similar and very high reductions of pod weight (57% for Acc527 to 66% for Acc286 and Acc973). Also, the relative reductions of the pod weights at the two harvests (116 DAS and maturity) were not correlated($r=+0.12$; $n=8$). Therefore, the genotypes differed for the length of the period of increasing pod dry weight between rainfed and irrigated regimes. This is supported by the increase in the pod weights of all genotypes under the irrigated condition beyond 116 DAS, which, under rainfed condition, was limited to the late and medium flowering genotypes Icarus, Acc973,

Table 4.4: Pod weight accumulation of the faba bean genotypes as affected by the water regimes at Roseworthy, 1995

Genotype		Pod dry weight (g/plant)						Relative reduction of pod weight (%) under rainfed condition	
		At 116 Days after sowing (DAS)			At maturity				
		Rainfed	Irrigated	Average	Rainfed	Irrigated	Average	116 DAS	Maturity
Small seed size									
Early maturing	Fiord	15.1	23.5	19.3	13.7	36.0	24.9	36	62
Medium maturing	Ascot	12.3	13.4	12.9	12.2	34.4	23.3	8	65
Late maturing	Acc 973	3.9	5.9	4.9	10.5	30.6	20.6	34	66
Medium seed size									
Medium maturing	Acc 527	8.8	9.2	9	15.4	35.7	25.6	4	57
Late maturing	Icarus	5.2	5.2	5.2	10.9	31.2	21.1	0	65
Large seed size									
Early maturing	Acc 286	16.4	18.7	17.6	13.8	40.9	27.4	12	66
Medium maturing	Acc 820	9.9	12.9	11.4	15.0	43.4	29.2	23	65
Medium maturing	Acc617	8.7	8.7	8.7	13.4	38.1	25.8	0	65
		10.0	**12.0**		**13.0**	**36.0**			
Least significant difference (p=0.05)									
Genotype			**6.2**			**5.0**			
Water level			**non-significant**			**2.5**			
*Genotype*Water level*			**non-significant**			**non-significant**			

Acc527, Acc617 and Acc820 (Table 4.4). However, the pod weights of the genotypes under the two water regimes at 116 DAS and at maturity were highly correlated to each other ($r=+0.92$, $p<0.01$, and 0.79, $p<0.05$ respectively; $n=8$). This suggests that the genotypes maintained similar relative rankings under the two water regimes at each of the two harvests (116 DAS and maturity). Therefore, although the duration of the period of pod filling of the genotypes was longer under irrigation, that difference between the genotypes within each of the water regimes was similar at maturity as at 116 DAS.

The genotypes differed significantly for pod weight accumulation at 116 DAS and at maturity (Table 4.4). At 116 DAS, among the small and medium seeded genotypes the early flowering genotypes recorded significantly higher pod dry weights than the later flowering genotypes. For example Fiord and Acc286 had the highest pod weights and Icarus and Acc973 the lowest. However, at maturity, this trend was confined to the small seeded genotypes, where it was not significant. Therefore, in contrast to 116 DAS, flowering time and the seed size did not influence the pod dry weights of the genotypes at maturity.

4.3.3 Final seed number, seed yield, 100 seed weight and pod dry matter partitioning to seeds

Interaction between water stress and genotype was significant for the number of seeds produced at maturity (Tables 4.5 and 4.6). The loss of seeds under water stress has been attributed to the loss of flowers and pods (Peat, 1982, Grashoff, 1990a). The seed numbers of the genotypes under the two water regimes were significantly correlated ($r=+0.97$, $n=8$, $p<0.01$). This suggests they had similar rankings under the water regimes. The relative reduction of the seed number was very high and varied from 44% for Acc820 to 66% for Acc973 (Table 4.6). Generally small seeded

genotypes produced more seeds than the large seeded genotypes under both the water regimes. Among the small and medium seeded genotypes, those that flowered early produced more seeds and had a lower relative reduction of seed numbers than the late flowering genotypes. However, the very high relative reductions of these genotypes indicate that early flowering time may not contribute substantially to lowering the sensitivity to water stress. On the other hand, early flowering large seeded genotype Acc286 produced more seeds than the late flowering Acc820 and Acc617 only under irrigated condition. The higher relative reduction of the seed numbers of Acc286 under rainfed condition also confirms the observation that early flowering may not contribute to better seed retention (Tables 4.5 and 4.6).

The interaction between the water regimes and genotypes was significant for seed yield (Tables 4.5 and 4.6). The genotypes did not differ significantly for seed yield under rainfed condition and seed size did not contribute to the differences in the seed yield of the genotypes under either water regime. The relative reductions of seed yield per plot and per plant were very high and similar. It varied from 44% for Acc617 to 71% for Acc286 (Table 4.5). Irrespective of the flowering time, small seeded genotypes recorded similar reductions of per plant seed yield. However, among the large seeded genotypes early flowering Acc286 recorded a higher loss of seed yield per plant (71%) than that of the medium flowering Acc617 (44%)(Table 4.5). This could be because of the higher relative loss of the seed numbers of Acc286 compared to the other two large seeded genotypes that flower relatively later. Therefore, early flowering time did not provide for better seed retention or better seed yield of the genotypes. Per plot seed yield of the genotypes, was higher than the average yield for the region and previous reported results (Agung, 1995). This could be mainly attributed to the high rainfall of 1995 (Appendix 3.1).

Table 4. 5: The effect of water regimes on the per plant seed production and seed yield of the faba bean genotypes at Roseworthy, 1995

Genotype		Seed number per plant			Seed yield (g/plant)			Relative reduction (%) under rainfed condition of	
		Rainfed	Irrigated	Average	Rainfed	Irrigated	Average	Seed number	Seed yield
Small seed size									
Early maturing	Fiord	27	69	48	12.0	31.2	21.7	61	62
Medium maturing	Ascot	23	63	43	10.1	29.0	19.6	63	65
Late maturing	Acc 973	16	47	31	8.4	24.2	16.3	66	65
Medium seed size									
Medium maturing	Acc 527	19	39	29	13.9	27.0	20.5	51	49
Late maturing	Icarus	11	27	19	8.0	22.9	16.0	59	61
Large seed size									
Early maturing	Acc 286	9	24	16	10.4	35.5	23.0	63	71
Medium maturing	Acc 820	7	12	10	12.5	25.2	19.0	42	50
Medium maturing	Acc617	10	18	14	11.4	20.4	16.0	44	44
		15	**37**		**11.0**	**27.0**			
Least significant difference (p=0.05)									
Genotype			**8**			**4.1**			
Water level			**4**			**2.1**			
*Genotype*Water level*			**11**			**5.8**			

Table 4. 6: The effect of water regimes on the net plot seed production and seed yield of the faba bean genotypes at Roseworthy, 1995

Genotype		Seed number per plot			Seed yield (g/plot)			Relative reduction (%) under rainfed condition of -	
		Rainfed	Irrigated	Average	Rainfed	Irrigated	Average	Seed number	Seed yield
Small seed size									
Early maturing	Fiord	11070	30375	20723	5685	15795	10740	64	64
Medium	Ascot	9300	24825	17063	4845	13605	9225	63	64
maturing	Acc 973	7230	21315	14273	4680	14115	9398	66	67
Medium seed size									
Medium	Acc 527	7485	15465	11475	5985	14085	10035	52	58
maturing	Icarus	4335	10890	7613	4380	12480	8430	60	65
Large seed size									
Early maturing	Acc 286	3660	10305	6983	5790	17565	11678	64	67
Medium	Acc 820	2715	4875	3795	5805	17190	11498	44	66
maturing	Acc617	4080	7320	5700	5310	15780	10545	44	66
		6234	**15671**		**5310**	**150077**			
Least significant difference (p=0.05)									
Genotype			**3195**			**1425**			
Water level			**1590**			**720**			
*Genotype*Water level*			**4515**			**2025**			

The TDM at 87 DAS and CGR from 59-87 DAS were highly correlated with the (per plot) seed yield of the genotypes under rainfed condition (r=+0.81 and +0.76 respectively, n=8, p=0.05). Also, TDM of the genotypes under rainfed condition at 116 DAS and CGR from 88-116 DAS were highly correlated to the (per plot) seed yield of the genotypes under rainfed condition (r=+0.89, p<0.01 and +0.76 respectively, p<0.05 n=8). There was no significant correlation between the TDM and seed yield, or between CGR and seed yield under the irrigated condition.

The reduction in the per plant pod weight of the genotypes under water stress at maturity and the per plant seed yield under rainfed condition were highly negatively correlated (Figure 4.1). This correlation indicates the importance of maintaining pod dry weight under water stress for the seed yield of the faba bean genotypes. The per plant relative reductions of the mean seed yields and of mean seed numbers of the genotypes under the rainfed condition were highly positively correlated to each other (Figure 4.2). This illustrates the influence of seed retention on the ability of a faba bean genotype to maintain seed yield under water stress.

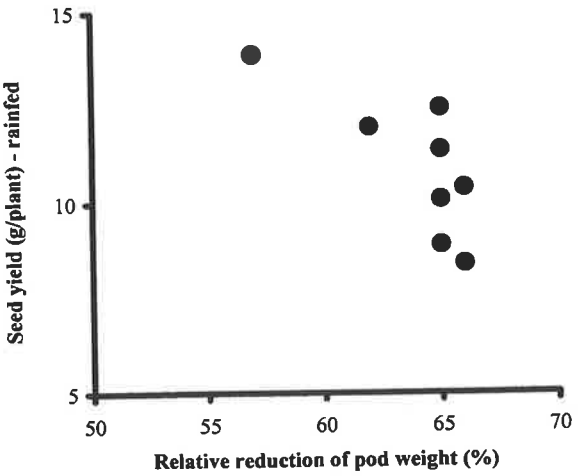

Figure 4.1: Effect of relative reduction of pod weight on the seed yield of the faba bean genotypes under rainfed condition (r=-0.75, n=8, p<0.05)

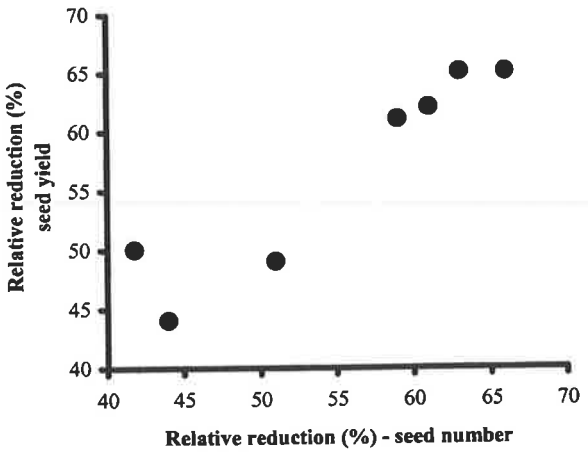

Figure 4.2: Effect of relative reduction of seed number per plant on the relative reduction of seed yield per plant of the faba bean genotypes (r=-0.93, n=8, p<0.01)

The interaction between water stress and the genotypes was significant for the 100 seed weight (Table 4.7), because only the large seeded genotypes Acc286 and Acc820 recorded significant relative reduction (20%, and 13% respectively). The relative reductions (per plant) of seed number and of seed yield of all the genotypes were positively correlated (r=+0.92; n=8, p<0.01).

Interaction between water regimes and the genotypes for the seed:pod ratio was small (Table 4.7). Generally only the large seeded genotypes showed change in seed:pod ratio under rainfed condition. Among them, early maturing Acc286 recorded a 13% reduction of the seed:pod ratio while the late maturing Acc820 (30%) and Acc617 (38%) recorded a significant increase. It indicates that early flowering time renders large seeded genotypes incapable of maintaining seed:pod ratio whereas the late maturing large seeded genotypes had an opportunity to maintain sink capacity by losing pods by virtue of their late flowering time. This is supported by the higher relative reduction of pod number of Acc617 and Acc820 than that of Acc286 under rainfed condition (Tables 4.3 and 4.5). As a result, the

Table 4.7: Pod partitioning and 100 seed weight of the faba bean genotypes under different water regimes at Roseworthy, 1995

Genotype		Seed : Pod ratio (%)			100 seed weight (g)		
		Rainfed	Irrigated	Average	Rainfed	Irrigated	Average
Small seed size							
Early maturing	Fiord	87	87	87	45	46	45
Medium maturing	Ascot	83	84	84	43	47	45
Late maturing	Acc 973	79	78	79	52	52	52
Medium seed size							
Medium maturing	Acc 527	93	76	85	72	70	71
Late maturing	Icarus	82	73	78	83	84	84
Large seed size							
Early maturing	Acc 286	76	87	82	119	148	133
Medium maturing	Acc 820	83	58	71	178	205	191
Medium maturing	Acc617	86	53	70	112	115	113
		84	**75**		**88**	**96**	
Least significant difference (p=0.05)							
Genotype				**8**			**5**
Water level				**3**			**4**
*Genotype*Water level*				**12**			**8**

relative reduction of seed yield of Acc286 was higher than those of Acc617 and Acc820.

4.4 Discussion

4.4.1 Dry matter accumulation of the genotypes

The genotypes differed significantly for dry matter production and CGR at 58, 87 and 116 DAS. They recorded large reductions in dry matter accumulation and CGR under rainfed conditions at 116 DAS. This is consistent with the earlier reports that limited water supply results in the reduction of stem dry matter accumulation (Grashoff et al., 1990a) and of biomass accumulation (Xia, 1994). Moisture stress also reduces the longevity of the faba bean canopies (Finch-Savage and Elston, 1982). The net effect of water stress, therefore, is a reduction in the total biomass accumulation. The genotypes with the bigger seed size accumulated higher dry matter and recorded higher CGR in the early stages of crop growth. The early flowering trait did not provide a similar advantage (Tables 4.1 & 4.2).

The positive correlation between TDM of the genotypes at 116 DAS and the seed yield under rainfed condition suggests that dry matter accumulation and an ability to sustain it under water stress during podding was an important determinant of yield. This interpretation points to the importance of the stage of development on the yield formation under water stress as well as to the TDM itself. With either possibility, the TDM could be used to differentiate the genotypes for their yield performance under limited water supply.

4.4.2 Pod production of the genotypes

Water stress resulted in high reduction of pod numbers at 116 DAS and maturity (Table 4.3). This is consistent with earlier reports (Korte et al., 1983). The reduction in the pod numbers could be attributed to the abscission of flowers and pods (Peat, 1982, 1983). The pod numbers produced by the genotypes under the two water regimes were highly positively correlated at 116 DAS and at maturity, but the lack of correlation between the relative reductions in pod number at these two stages indicates that water stress continued to affect pod retention beyond 116 DAS.

Seed size of the genotypes appeared to have affected pod numbers while flowering time did not. Generally the large seeded genotypes recorded fewer pods than the small seeded genotypes. Because the large seed size restricts the number of pods an individual plant can support, the ability of such genotypes to retain pods is very important for yield under rainfed condition. The flowering time affected the relative reduction in pod numbers of the genotypes under water stress depending on their seed size. For small seeded genotypes, early flowering time resulted in lower reduction of pod numbers under water stress during the later stages of reproductive growth and for large seeded genotypes late flowering time provided that benefit.

The interaction effect between water treatment and genotype was high for the pod weight at maturity, with the relative reduction of pod weight under rainfed condition ranging from 57% to 66%. Although for the late maturing genotypes podding occurred mainly after 116 DAS, genotypes of all flowering times recorded similar pod weights under rainfed condition at maturity. Therefore the late flowering time did not disadvantage the genotypes for pod weight. The seed size did not influence the final pod weight despite affecting the number of pods produced by a genotype.

By considering these arguments together, it can be said that an ability to maintain pod weight under rainfed condition was important for better seed yield of the genotypes.

The relative reduction of pod weight under rainfed condition at 116 DAS was generally lower than that for pod number but at maturity both the reductions were very high (Tables 4.3 and 4.4). This indicates existence of a compensatory increase of pod weight in response to the loss of pods during early podding, but it was not reflected at the final harvest. Therefore, the genotypes could not sustain such compensation under continued water stress during the later stages of pod filling.

4.4.3 Yield of the genotypes

Interaction between water stress and the genotypes was significant for number of seeds produced and seed yield. Genotypes recorded very high reductions of both the seed numbers and seed yield under rainfed condition. Small seeded genotypes generally produced more seeds than the large seeded genotypes but the small seed size did not improve the seed retention under water stress. Early flowering time provided a small relative advantage for the number of seeds produced by the small and medium seeded genotypes under water stress compared to the large seeded genotypes. However, it did not contribute to significantly better seed retention by the genotypes under water stress as the relative reduction was not strongly affected by flowering time. Among the genotypes with small and medium sized seeds, seed yield tended to be slightly higher in the earliest varieties. But the seed yield data suggests that differences in flowering time or seed size did not contribute significantly to differences in the seed yield of the genotypes under water stress. The high positive correlation between the relative reduction of seed number and of seed yield suggests

that seed number is an important determinant of the seed yield of the genotypes under water stress. Therefore, an ability to maintain seed number under water stress will contribute to better seed yield of the faba bean genotypes. The data also suggests that seed retention is very important for the yielding ability of the large seeded genotypes under limited water supply because the large seed size restricts the number of pods and seeds they can set.

Under rainfed conditions 100 seed weight was reduced only in Acc286 and Acc820. This could be because these genotypes experienced seed yield loss that was higher than the loss of seed numbers. For all other genotypes, the relative loss in seed yield was similar to the relative loss in seed numbers. The difference among the faba bean genotypes in their ability to partition pod dry weight to seeds was limited to large seeded genotypes. It represented the effect of flowering time on the ability of the genotypes to adjust their sink capacity and suggests that early flowering time may disadvantage this ability.

4.5 Summary

The results of dry matter accumulation and CGR of the genotypes at different stages of pod growth suggest that TDM could be used to evaluate the genotypic differences for seed yield under rainfed condition. The ability of a genotype to retain pods and maintain pod weight under stress, particularly for large seeded ones, was very important for seed yield.

Seed size and flowering time did not greatly affect the seed yield response of the genotypes under rainfed condition. However, they affected seed numbers and, to some extent, the ability to retain those seeds under water stress. These were

important contributing factors for the seed yield of the genotypes. Similarly, pod retention and maintaining pod weight under rainfed condition were influenced by seed size and flowering time. Therefore, seed size and flowering time could be exploited to manipulate seed and pod retentions and to providc better seed yield.

This conclusion points to the importance of reproductive growth of the faba bean for better yield under water stress. It would be appropriate to investigate the effect of water stress during the reproductive stage in an effort to improve the faba bean performance under such conditions.

Based on the results of this experiment it was decided to investigate the physiological response of faba bean genotypes to water stress in detail. It was decided that genotypes possessing a large range of flowering time and seed size be used for this purpose. The sensitivity to water stress needs to be studied at different growth stages for crop improvement. It was decided to use the reproductive stage as the reference stage because of the various factors discussed earlier.

Chapter 5

Screening of faba bean for response to water stress: Standardising the techniques

5.1 Introduction

The average yield of faba bean in Australia is only 1.3 t/ha (PulseAustralia, 2000). Variable rainfall is one of the important factors limiting the faba bean yield, contributing to its inconsistent yield levels across the years (Knight, 1994). The growing season of the crop in the southern Australia generally ends with the end of rains in spring and the development of terminal water stress (French, 1981, Loss and Siddique, 1994, Agung, 1995). This is also when the later stages of reproductive growth of faba bean occur. Faba bean is very sensitive to water stress at this stage. It loses turgor and wilts quickly in the field (Kassam, 1973, Finch-Savage and Elston, 1982). There are reports of drought during the reproductive stage reducing leaf area of faba bean, and resulting in lower pod weight (Grzesiak et al., 1989, Xia, 1994). Drought causes pod abscission, leading to lower pod retention (Grashoff, 1990a). Reports indicate that faba bean grain yield is positively linked to the number of pods and seeds retained (Katiyar and Singh, 1990, Stutzel and Aufhammer, 1992). Results of the field experiment conducted in Roseworthy in 1995 also support this. Water stress during the reproductive stage has been linked to reduced assimilate availability to sinks and reduced yield and yield components of faba bean (Pilbeam et al., 1990b). Therefore, it would be beneficial to develop varieties that can tolerate water stress in order to improve crop performance across the region.

Several physiological and growth characters such as stomatal conductance, leaf water potential, leaf area and dry matter production have been used to evaluate the drought response of crops. It is appropriate to mention the relevance of some of them for faba bean production here. Water stress causes leaf stomata to close and limits transpirational loss of water (Hsiao 1982). To some degree, stomatal closure and the consequent reduction of stomatal conductance help to improve water use efficiency of the faba bean (Nerkar et al., 1981). Plant water status affects seed yield of faba bean (Karamanos, 1984). It has been reported that midday water potential of faba bean is the most sensitive indicator of plant water status (Karamanos et al., 1982). Apart from affecting the plant water status, water stress also reduces leaf area production, increases leaf senescence and adversely affects stem dry matter accumulation of faba bean (Farah, 1981, Karamanos et al., 1982, Grashoff et al., 1990a). Dry matter production is known to significantly affect the yield performance of faba bean (Siddique et al., 1993, Agung, 1995). Therefore, it is appropriate to use physiological and growth characters to evaluate faba bean response to water stress.

It is necessary to identify a stage at which genotypes could be reliably screened for drought tolerance. The response of faba bean to water stress varies between the growth stages, and the reproductive stage is generally considered to be the most sensitive to yield (Pilbeam et al., 1990b, Xia, 1994). However, there are also reports that vegetative growth is more sensitive to water stress than seed set and seed growth stages (Plies-Balzer et al., 1995). The ability to identify drought tolerance traits at an early stage would not only be cost and time effective, it would also facilitate handling of a large number of accessions. Therefore, it is appropriate to compare the faba bean response to water stress at different growth stages to identify a suitable

stage for screening the genotypes and to examine the consistency of genotypic differences between the different growth stages.

It was decided to conduct a number of preliminary experiments to: (1) evaluate and standardise the techniques of measuring different physiological and growth parameters of the faba bean under water stress; and, (2) selecting an appropriate growth stage or growth stages to evaluate genotypes for response to stress. It was decided to stress the selected genotypes at the early vegetative and early podding stages, and to measure various relevant physiological, growth and yield characteristics.

5.2 Materials and methods

Three glasshouse experiments were conducted in order to accomplish the objectives outlined. The first of them involved stressing the faba bean genotypes at the vegetative stage; the second involved stressing at the early reproductive stage while the third combined stressing at the two stages in one experiment.

Table 5.1: Details of the genotypes used in the current series of experiments

Genotype	Seed size	Seed weight (g/seed)	Maturity group	Flowering time (Days after sowing
Fiord	Small	0.48	Early	66-70
Barkool	Small	0.52	Early	64-68
Acc165	Small	0.80	Early	62-66
Acc524	Small	0.48	Early	68-72
Acc482	Small	0.49	Medium	71-75
Acc973	Small	0.37	Late	94-100
Acc527	Medium	0.79	Medium	73-77
Icarus	Medium	0.83	Late	96-101
Acc286	Large	1.35	Early	52-56
Acc820	Large	1.98	Medium	68-73

Six of the genotypes tested in the field experiment carried out at Roseworthy in 1995 were chosen for the first two experiments along with four additional genotypes based

on their performance in breeding trials (Table 5.1). For the third experiment, Acc286, Fiord and Icarus were chosen for the differences in their seed size and flowering time. Fiord, the most widely adapted and high yielding genotype of Australia (at the time of the experiment), is small seeded and early flowering, Icarus is medium seeded and late flowering while Acc286 is large seeded and early flowering genotype. The number of genotypes was limited because of the wide differences among them for seed size, flowering time and yield potential, and also because of the exploratory nature of the experiment.

The growth conditions were determined to suit individual experiments. For the first experiment 1.5 kg soil was placed in pots of 150 mm diameter each, for the second experiment 4 kg soil was potted in pots of 200 mm diameter each and for the third experiment, 7 kg soil was potted in pots of 200 mm diameter each. The amount of soil potted was increased in the later experiments to accommodate bigger plants as the stressing was at the later stage of growth. In the first experiment the pots were lined with plastic bags to prevent water drainage, to measure the amount of water used. In the remaining experiments the pots were not lined and water was allowed to drain because of the difficulty of preventing water logging. The University of California soil mixture (UC soil) (Appendix 5.1) was used for potting. The soil was mulched with wood chips (300 g/pot) to reduce evaporation. The wood chips (300 g/pot) were also placed at the bottom of the pots before potting with soil in the last two experiments, to allow free draining of water.

Six pre-germinated seeds were planted in each pot. Three seedlings were retained in each pot in the first experiment and 4 seedlings in the remaining two. This difference was mainly due to the amount of soil used and the expected size of the plants at the

time of stressing and at the final harvest. To ensure adequate supply of nutrients to plants, a commercial slow release fertiliser (Osmocote) (Appendix 5.2) was applied at the rate of 100g per pot every 30 days of plant growth. The growth temperature in the glasshouse was maintained at 24°C/17°C day/night cycle. The plants were grown under natural daylight conditions. The first experiment was carried out from 24 April to 1 May 1997, the second from 2 June to 15 August 1997 and the third from 19 September 1997 to 16 January of 1998.

The plants were well watered up to the time that the stress treatment was imposed. Water stress was imposed by gradually reducing the amount of water supplied to the treatment pots. Pots were weighed daily and the difference between the successive pot weights was considered as the amount of water used. The amount of water supplied was reduced so that the total pot weight decreased by 10% per day of the original weight recorded at the start of stressing. Water was added to bring the pot weight to the required level if necessary. It took 12 days to reach 50% field capacity moisture level in the first experiment and 9 days in the second experiment. In the third experiment, during the vegetative stage it took 14 days to reach 50% field capacity and 10 days during the reproductive stage. To estimate the field capacity of the known dry weight of UC soil, pots were watered to saturation, covered, and allowed to drain freely for 48 hrs. The pots were then weighed and the amount of water contained was assumed to be the field capacity. The plants were allowed to experience stress for four days at a moisture content of 50% of field capacity before terminating the treatment. In the first experiment, plants were beginning to wilt at the end of stress period. Because faba bean is very sensitive to water stress, wilting, which occurs frequently in the field in spring, was considered to reflect adequate level of stress. Therefore, 4 days of stress at that moisture level was chosen for the

remaining two experiments. This method was followed to simulate the gradual development of stress as it occurs in the field. Unstressed control plants were watered adequately during the treatment period.

The genotypes were stressed at different physiological stages. In the first experiment stress was imposed when 3-4 fully opened leaves appeared on the main stem, The stage defined as early vegetative stage. In the second experiment stress was applied at the early podding stage, when the pods on the first two podding nodes attained 1cm length. At the completion of Experiment 1, it was considered that 3-4 leaf stage could be too early and therefore, 6-7 fully opened leaf stage was decided upon as the appropriate vegetative stage for later experiments. In the third experiment, genotypes were stressed at early podding stage also. The flower initiation of Acc286 occurred very early, therefore only Fiord and Icarus could be stressed at 6-7 leaf stage in this experiment. All the plants were harvested at the end of the stress treatment in the first two experiments. In the third experiment two plants from each pot were harvested at the end of each period of stress and the remaining two were carried through to maturity with adequate watering. The experiments consisted of three replications. Stress treatments had a corresponding non-stress treatment in each replication.

Mainly physiological responses to water stress, dry matter accumulation and seed yield and its components were recorded. In the first experiment specific leaf area (SLA), total dry matter (TDM), stomatal conductance, relative water content (RWC) and total amount of water used (during the period of stress only) were recorded at the end of stressing. Stomatal conductance and RWC of all the replications were taken on the same day in this experiment. In the second experiment leaf water potential was recorded in addition to the parameters recorded in Experiment 1, while water use

was not measured. Observations recorded in the third experiment included stomatal conductance, SLA and TDM during the stress, and, seed yield, seed number, pod yield, pod number and TDM at maturity. All the physiological observations were recorded on the youngest fully opened leaf between 12noon and 2pm. This time period was chosen because mid-day water potential of faba bean has been reported as the most sensitive indicator of plant water status (Karamanos, 1982). It is supported by similar observations regarding other crops (Rascio et al., 1987). The procedures described in Chapter 3 were followed while collecting the data.

In the first experiment, the RWC values were lower than expected given the severity of the stress and suggested an error in the methods. The discrepancy may have been because all the replications were harvested together. The time lag between harvesting the leaf discs and recording the fresh weight may have caused a loss of fresh weight of the leaf discs and an underestimation of the RWC. Therefore, it was decided that in the remaining experiments RWC would be measured one replication a day.

5.3 Results

5.3.1 Experiment 1: Response of the faba bean genotypes to water stress at the early vegetative stage

Water stress at the early vegetative stage significantly reduced stomatal conductance by 20% and leaf area by 14%. SLA significantly increased under water stress by 6%, which suggests that water stress reduced leaf weight more than the leaf area. Water stress also significantly reduced TDM and the amount of water used during the period of stress, both by 18%. The significant effect of water stress on different parameters suggests adequate stress had developed by the method of stressing used

in this experiment. However, the genotype and water stress interaction was not significant for any of these characters (Appendices 5.3 and 5.4).

The stomatal conductance of the genotypes differed significantly, but the data were highly variable and had a coefficient of variation (CV) of 41% (Appendix 5.3). This variability was attributed to the single observation recorded from each pot. Scrutiny of the raw data suggested that more measurements must be made for each treatment in each replication. Therefore, it was decided to record four readings of stomatal conductance per pot in the future experiments. Similarly, the data of SLA was also variable (CV=23%). This was attributed to the low number of leaflets (10) included in the sub-sample used to estimate SLA. Therefore, it was decided to increase the number of leaflets used in the sub-samples to 15 in the next experiments.

The leaf area and TDM of the genotypes were positively correlated with the amount of water used by the genotypes (during the period of stressing) (Figures 5.1 and 5.2). The TDM and leaf area of the genotypes were positively correlated (Figure 5.3).

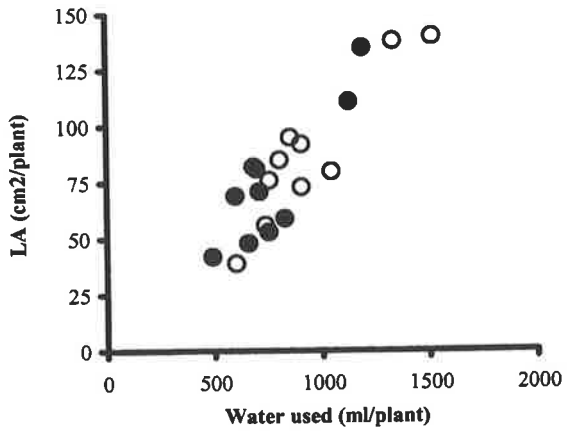

Fig 5.1: The relationship between water use and leaf area production by ten faba bean genotypes when stressed at early vegetative stage (r=+0.86 under stress, r=+0.92 under non-stress, n=10, p<0.01; Closed circles – Stress, Open circles – Non stress)

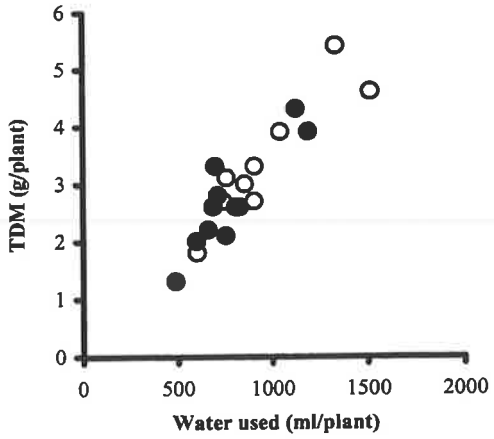

Fig 5.2: The relationship between water use and dry matter production by ten faba bean genotypes when stressed at early vegetative stage (r=+0.88 under stress, r=+0.91 under non-stress, n=10, p<0.01; Closed circles – Stress, Open circles – Non stress)

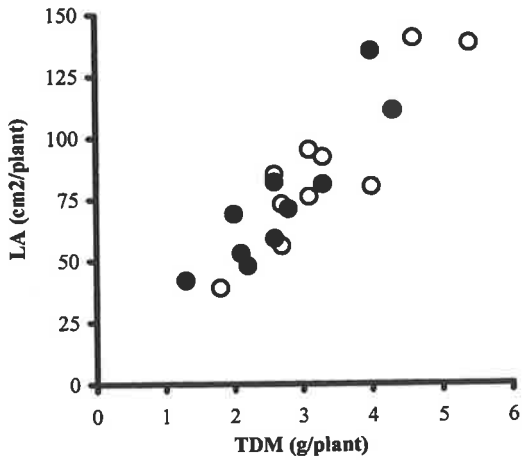

Fig 5.3: The relation between total dry matter production and leaf area produced by the genotypes at the early vegetative stage(r=+0.90 under stress, r=+0.89 under non-stress, n=10, p<0.01; Closed circles – Stress, Open circles – Non stress)

5.3.2 Experiment 2: Response of the faba bean genotypes to water stress at the early podding stage

Water stress significantly reduced stomatal conductance by 18%, RWC by 12% and water potential by 9% at the early podding stage (Appendix 5.5). This indicates sufficient level of stress had developed due to the treatment. Unlike at the early

vegetative stage, water stress did not significantly affect SLA and TDM (CV of 15% and 10% respectively). This is contrary to the earlier reports that recorded reduction in TDM when faba bean is subjected to water stress during the reproductive stage (Siddique et al., 1993, Agung, 1995). The interaction of water stress with genotypes was significant for stomatal conductance alone (Table 5.2). Among the genotypes only Acc165 and Acc286 recorded a significant reduction of stomatal conductance under stress.

The water stress and genotype interaction was significant for SLA but not for TDM (Table 5.3). Only Acc165 and Acc286 showed significant difference of SLA under stress at the early podding stage. Following on from the observations of the previous experiment, 15 leaflets were included in the sub-sample used to measure SLA. However, the data still were variable (CV=20%). Therefore, it was decided to include 20 leaflets in the sub-sample for SLA in the next experiment.

5.3.3 Experiment 3: Response of the faba bean genotypes to water stress at the early vegetative and early podding stages

5.3.3.1 Early vegetative stage

Water stress at the early vegetative stage significantly reduced stomatal conductance during the stress on the 4th day of reduced water supply (46%) and also at the end of the stress period (88%) (Table 5.4). It also significantly reduced TDM recorded both at the end of the stress period (30%) and at maturity (21%). However, water stress did not significantly affect SLA at this growth stage. The SLA data still contained high variability (CV=18%). Therefore, it was decided to increase the number of leaflets to 30 in the sub-sample used to measure SLA at the early podding stage.

Table 5.2: Effect of water stress during the early podding stage on leaf water relations of ten faba bean genotypes

Genotype			Average conductance (mol/m²/s)			RWC (%)	WP (MPa)
			Non stress	Stress	Average		
Small seed size							
	Early flowering	Fiord	1060	1346	1202	76	-0.39
		Barkool	712	838	774	79	-0.37
		Acc 165	1504	826	1174	79	-0.39
		Acc 524	1008	828	918	81	-0.40
	Medium flowering	Acc 482	986	622	804	76	-0.41
	Late flowering	Acc 973	916	748	832	80	-0.39
Medium seed size							
	Medium flowering	Icarus	1380	1086	1232	78	-0.41
	Late flowering	Acc 527	1174	920	1046	79	-0.40
Large seed size							
	Early flowering	Acc 286	1512	718	1116	80	-0.40
	Medium flowering	Acc 820	756	1054	904	78	-0.38
Average			**1100**	**898**			
Least significant difference (p=0.05)							
	Genotype			**292**		**non-significant**	**-0.02**
	Water level			**130**		**1**	**-0.01**
	Genotype X Water level			**414**		**non-significant**	**non-significant**

Table 5.3: Effect of water stress during the early podding stage on the specific leaf area (SLA) and dry matter production (TDM) of ten faba bean genotypes

Genotype			SLA (cm^2/g)			TDM (g/plant)
			Non stress	Stress	Average	
Small seed size	*Early flowering*	Fiord	348	382	365	12.0
		Barkool	407	334	371	12.2
		Acc 165	374	473	424	16.2
		Acc 524	454	447	451	12.4
	Medium flowering	Acc 482	441	389	415	12.1
	Late flowering	Acc 973	436	396	416	9.4
Medium seed size	*Medium flowering*	Acc 527	322	338	330	13.9
	Late flowering	Icarus	459	421	440	10.2
Large seed size	*Early flowering*	Acc 286	320	456	388	17.5
	Medium flowering	Acc 820	394	349	371	15.6
Least significant difference (p=0.05)						
Genotype				**57**		**1.3**
Water level				**non-significant**		**non-significant**
Genotype X Water level				**80**		**1.8**

The non-significant interaction on the fourth day of stressing on the stomatal conductance of Fiord and Icarus (Table 5.4) suggests that the varieties responded similarly to stress. At the end of the stressing period (13 days), the interaction between genotype and water stress was significant for stomatal conductance. The difference between genotypes under stress was not significant but Fiord had a significantly higher conductance than Icarus under non-stress. The stomatal conductance of both the genotypes under stress was extremely low, with Fiord suffering 91% relative reduction and Icarus, 84%. The stomatal conductance was very low compared to that of Experiment 1 (Appendix 5.4). It suggests the plants were under very high stress in this experiment even with the same methodology of imposing stress as in the earlier experiment. It should be mentioned here that while the first two experiments were conducted from May to August, the third experiment was conducted from September to January. Therefore, third experiment received more sunlight and had a higher evaporation than the others.

Water stress at the early vegetative stage significantly affected seed yield and its parameters at maturity (Table 5.5). It significantly reduced number of pods (45%), pod yield (46%), the number of seeds (55%) and seed yield (48%). Also, the interaction effect of water stress and genotype was significant for seed yield and its components (Table 5.5). Fiord recorded a 70-75% reduction in its seed yield and yield components under stress and Icarus recorded 19-25% reduction. While Icarus produced more pods and seeds than Fiord only under stress, it produced higher pod and seed yields than Fiord under both unstressed and stressed conditions.

Table 5.4: Effect of water stress during the early vegetative stage on average stomatal conductance, specific leaf area (SLA) and dry matter production (TDM) of two contrasting faba bean genotypes

Genotype	Average conductance (mol/m^2/s)					SLA (cm^2/g)	TDM (g/pl)	
	Pre-stress	4th day of stressing	End of stressing				End of stressing	At maturity
			Non-stress	Stress	Average			
Fiord	1378	998	1478	128	802	41.4	3.3	46.3
Icarus	1096	790	992	166	578	41.0	3.1	54.2
Average of								
Non stress		**1158**		**1234**		**39.8**	**3.7**	**56.0**
Stress		**630**		**146**		**42.5**	**2.6**	**44.5**
Least significant difference (p=0.05)								
Genotype	**122**	**non-significant**		**150**		**non-significant**	**non-significant**	**non-significant**
Water level		**288**		**148**		**non-significant**	**0.75**	**11.0**
Genotype X Water level		**non-significant**		**212**		**non-significant**	**non-significant**	**non-significant**

5.3.3.2 Early podding stage

Water stress at the early podding stage significantly reduced stomatal conductance on the 4th day of stress by 37% and at the end of the stress period (18 days) by 81% (Table 5.6). It also significantly reduced TDM by 33%, both at the end of the stress period and at maturity. As at the early vegetative stage in this experiment, stress did not significantly affect the SLA at the early podding stage. However the data, estimated using 30 leaflets, contained an acceptable level of variability (CV=12%). The stress treatment resulted in a 36% reduction in the number of pods at the end of stress period, but did not significantly affect the pod weight. The significant effect of water stress on most of the parameters suggests that sufficient level of stress had developed. Generally, the interaction between genotype and water stress was non-significant, suggesting that all the genotypes responded similarly to the stress that had developed.

Genotypes differed significantly for the stomatal conductance during the early podding stage. By the 4th day of stress the conductance had significantly reduced in Fiord and Acc286 but not in Icarus. Although the interaction was non-significant, a similar trend was observed at the end of stress (Table 5.6). This response was different from that during the early vegetative stage (Table 5.4). In contrast to Experiment 2, on the 4th day of stressing, there was a significant reduction in stomatal conductance under water stress in Fiord and Acc286. At the end of the stress treatment, all the three genotypes had very low stomatal conductance under stress. This trend was similar to that at the early vegetative stage in the same experiment (Table 5.4). The genotypes did not differ for SLA during early podding, and water stress had no significant effect on the SLA (Table 5.6).

Table 5.5: Effect of water stress at the early vegetative stage on the seed yield and yield components of two contrasting faba bean genotypes

Genotype	Pod number/pl			Pod yield (g/pl)			Seed number/pl			Seed yield (g/pl)		
	N.S.	Stress	Average	N.S	Stress	Average	N.S	Stress	Average	N.S	Stress	Average
Fiord	31	9 (71%)	20	35.5	10.0 (72%)	22.8	64	16	40 (75%)	29.7	8.3 (72%)	19.0
Icarus	27	22 (19%)	25	43.6	32.6 (25%)	38.1	47	34	40 (28%)	33.3	24.3 (25%)	28.8
Average of *Non-stress* *Stress*	**29** **16**			**39.5** **21.3**			**55.2** **24.9**			**31.5** **16.3**		
LSD (p=0.05)												
Genotype	**non-significant**			**6.3**			**non-significant**			**5.3**		
Water level	**4**			**6.0**			**8.0**			**5.1**		
Genotype X Water level	**6**			**8.9**			**12**			**7.5**		

Data in the brackets are the relative reductions under stress
LSD – Least significant difference
N.S. – Non-Stress

Table 5.6: Effect of water stress during the early podding stage on average stomatal conductance, specific leaf area (SLA), pod weight and dry matter production (TDM) of the three contrasting faba bean genotypes

Genotype	Average conductance (mol/m^2/s)							SLA (cm^2/g)	Pod weight (g/pl)	Pod number/pl	TDM (g/pl)	
	Pre-stress	4th day of stressing			End of stressing						End of stressing	At maturity
		N.S.	Stress	Average	N.S.	Stress	Average					
Acc 286	816	958	420	690	1104	324	714	263	12.4	7	12.5	35.1
Fiord	1160	1126	770	948	996	106	552	267	10.7	18	8.5	32.7
Icarus	696	640	534	586	592	74	334	306	14.3	10	12.4	33.6
Average of												
N.S.		**904**			**898**			**257**	**12.9**	**14**	**13.2**	**40.5**
Stress		**574**			**168**			**300**	**12.1**	**9**	**8.9**	**27.1**
LSD (p=0.05)												
Genotype	**n.s.**	**114**			**150**			**n.s.**	**n.s.**	**4**	**n.s.**	**n.s.**
Water level		**92**			**122**			**39**	**n.s.**	**3**	**2.9**	**2.7**
Genotype X Water level		**160**			**n.s.**			**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**

LSD = Least significant difference
N.S. = Non-stress
n.s. = non-significant

TDM excludes pod weight

Similarly, TDM of the genotypes during this stage and at maturity were also not significantly different and water stress had no significant effect on TDM (Table 5.6). Genotype and water stress interaction was non-significant for both pod number and pod yield at the end of stress (Table 5.6).

Water stress at the early podding stage significantly reduced seed yield and yield parameters at maturity (Table 5.7). It reduced the number of pods produced (33%) and the pod yield (36%). It also resulted in a 33% reduction in the number of seeds produced and 38% reduction of the seed yield. The significant effects of water stress again suggest a sufficient stress having developed during the experimentation.

The genotypes differed significantly for the final number of pods and seeds produced per plant but not for the pod and seed yield (Table 5.7). Although water stress at the early podding stage reduced yield, all the genotypes showed a similar reduction. The differences in the seed size affected the number of seeds and pods produced by the genotypes but the differences in their flowering time did not (Table 5.7). For example, the small seeded Fiord produced higher number of seeds and pods than the large seeded Acc286 and the medium seeded Icarus.

5.4 Discussion

Water stress at the early vegetative and the early podding stages significantly affected various physiological and growth characters. It also greatly reduced seed yield and yield components. This suggests that the methodology used to impose stress had created sufficient level of stress in the experiments and the rate of soil drying appeared to be reproducible. It should also be noted that the methodology of

Table 5.7: Effect of water stress at the early podding stage on the final yield and yield components of the three contrasting faba bean genotypes

Genotype	Pod number/pl			Pod yield (g/pl)			Seed number/pl			Seed yield (g/pl)		
	Non-stress	Stress	Average	Non-stress	Stress	Average	Non-stress	Stress	Average	Non-stress	Stress	Average
Acc 286	11	6 (45%)	8	24.8	15.0 (40%)	19.9	16	9 (44%)	12	22	12 (45%)	17
Fiord	26	18 (31%)	22	25.1	16.8 (33%)	20.9	32	26 (19%)	29	21	13 (38%)	17
Icarus	17	11 (35%)	13	24.9	16.3 (35%)	20.6	25	18 (28%)	21	20	13 (35%)	16
Average of												
N.S.		**18**			**24.9**			**24**			**21**	
Stress		**12**			**16.0**			**16**			**13**	
LSD (p=0.05												
Genotype		**2**			**non-significant**			**4**			**non-significant**	
Water level		**2**			**3**			**3**			**3**	
Genotype X Water level)		**non-significant**			**non-significant**			**non-significant**			**non-significant**	

Data in the brackets are the relative reductions under stress

LSD – least significant difference

creating stress was designed to control the total soil moisture level in a manner repeatable across the growth stages and experiments. Such a method provides highly contrasting water regimes and biological effects, which are acceptable for stress research (Nerkar et al., 1981).

The stomatal conductance data initially contained high variability, because a single observation was recorded per pot. Subsequently four observations were recorded per pot and this reduced the variability associated with the stomatal conductance data. Generally Fiord recorded higher conductance than Icarus and Acc286, particularly under stress while Acc286 recorded higher conductance than Fiord particularly under non-stress condition. The interaction between water stress and genotypes on the conductance was not significant in all the experiments. However, this could be due to the high sensitivity of stomatal conductance to water stress, as evident in the large reductions in conductance of the genotypes under stress. It has been reported that faba bean genotypes close their stomata only after reaching threshold level of stress (leaf water potential of -0.6 to –0.7 MPa), which may result in a sudden loss of conductance, as seen in the current experiments (Agung, 1995).

Leaf area was recorded in the first experiment but in the later experiments SLA was preferred instead as it integrates both leaf area and leaf dry matter. Also, measuring SLA from the sub-samples of leaves was more practical and time saving than measuring the leaf area of the whole plants. The interaction of stress with the genotypes was not significant for SLA. This was mainly because of the high variability in the SLA data. The size of the sub-sample was increased successively among the experiments to reduce the variability. The SLA values at the early podding stage were 5 to 10 times higher than those at the early vegetative stage. In

the absence of any report on the SLA of the faba bean for comparison, the unusual increase could only be attributed to the bigger leaf size of the genotypes at this stage compared to the smaller leaves at the early vegetative stage.

Leaf water potential, the amount of water used and RWC were also examined for suitability to estimate plant water status. Water stress significantly reduced the first two characters and the genotypes differed significantly for them. However, the interaction of water stress and genotypes was not significant, suggesting that the genotypes responded similarly to the stress for leaf water potential and the amount of water they used during the stress period. Water stress significantly reduced RWC but its interaction with the genotypes was not significant. Lack of genotypic difference in faba bean for leaf water characteristics has been reported earlier, although faba bean leaves are known to loose their turgor when RWC reaches 65%-75% (Grashoff and Verkerke, 1991, Agung, 1995). This was attributed to the lack of the ability of faba bean to maintain osmotic potential under water stress (Grashoff and Verkerke, 1991).

The RWC and the leaf water potential recorded in this experiment agree with earlier reports. For example, Agung and McDonald, (1998), reported that RWC of faba bean genotypes declined from 90% at the beginning of the growing season to 76%-80% after flowering, under rainfed cultivation. Nerkar et al., (1981), reported RWC of the faba bean genotypes ranged from 90% under full water supply to 50% when stressed at 61% field capacity of the soil for one week. The leaf water potential of the genotypes recorded in this experiment were lower than those reported by Grashoff and Verkerke, (1991). They reported midday leaf water potential to be -1.2 MPa under stress in a glasshouse study. Reports from field studies show that faba bean leaf water potential goes down to -0.7 MPa to -1.6 MPa (Karamanos et al., 1982,

Riccardi and Steduto, 1988). However, Agung, (1995) reported that water potential of faba bean genotypes ranged from -0.3 MPa to -0.7 MPa in the field, and in a glasshouse study, from –0.4 MPa- under full watered condition to –1.6 MPa under stress. Although the leaf water potential data recorded in the current experiment are slightly lower than the earlier reports, there is variation in the actual value of leaf water potential among the reports. It suggests that actual data depends upon the stress conditions prevalent in a given experiment.

Water stress significantly reduced TDM at both the early vegetative and podding stages except in Experiment 2. This conforms to earlier reports (Grashoff, 1990a). The interaction of genotypes and water stress was non-significant for TDM, suggesting that the three genotypes responded similarly to stress. Interestingly, the variability in the TDM data was lower during the early vegetative stage (CV=12.7% in Experiment 1 and CV=15% in Experiment 3) than during the early podding stage (CV=28% in Experiment 2 and CV=31% in Experiment 3). TDM was based on three plants in Experiment 1, four plants in Experiment 2 and two plants in Experiment 3. Therefore, the number of plants harvested may not be the reason for variability in the TDM data. It is possible that faba bean being cross-pollinated, growth of individual plants may vary even within a single seed lot of any given genotype (Duc, 1997). It is likely that such differences in growth and pod set would cause differences in TDM, mainly at the later stages of growth. Variation in autofertility would also contribute to differences in pod-set, and therefore seed yield, among plants of a genotype, particularly for plants grown in a glasshouse (J. Paull, pers.comm.). Visual observations at the time of podding stage harvests also confirmed such differences.

It was expected that water stress at the early vegetative stage would not affect the final yield and its components, because no reproductive growth is formed. Contrary to the expectations, water stress at the 6-7 leaf stage significantly affected seed yield and its components of the genotypes in Experiment 3. This stage was chosen for stressing because, at the completion of Experiment 1, the 3-4 leaf stage was considered being too early. This was based on visual observation of the size of the seedlings in the experiment. However, Fiord being early flowering may have begun flower initiation by 6-7 fully opened leaf stage. The severe reduction of the yield components of Fiord due to water stress at 6-7 leaf stage provides reason to believe that the genotype may have already initiated flowering. Therefore, effectively only Icarus was stressed at the early vegetative stage, rendering the comparison of the effect of water stress at the two stages on the seed yield of the genotypes improper.

Water stress at the early podding stage significantly affected the number of pods produced by the genotypes, but not their pod weight. This is at variance with Pilbeam et al., (1990a,b), who reported that seed yield and all its components of faba bean were adversely affected by water stress at this stage. The trend was seen at maturity also, where water stress at the podding stage significantly affected the numbers of pods and seeds produced by the genotypes but not the pod weight or seed yield. It indicates that although seed yield of the faba bean is not directly affected by water stress, ability of the genotypes to retain pods and seeds is important in maintaining the seed yield under stress. In the field experiment of 1995, conducted in Roseworthy, water stress significantly reduced seed yield of the genotypes apart from the number of pods and seeds produced. However, in that experiment also, the results indicated that the ability of the genotypes to retain pods and seeds is important in maintaining the seed yield under water stress.

5.5 Summary

In these exploratory experiments faba bean genotypes were water stressed at early vegetative and early podding stages. A major objective was to evaluate the ability of various physiological and growth characters to screen the response of faba bean genotypes to water stress. It was to test and modify the techniques of data collection to reduce any variability that might arise in the results of the parameters. The other aim was to evaluate the suitability of these two growth stages for such a screening.

The method of creating stress used in the current study, based on the soil moisture level, developed sufficient stress to affect different physiological, growth, and yield characters of the faba bean genotypes. It was also reproducible across experiments. Therefore, it was decided to adopt the technique for future work.

The stomatal conductance was found to be very sensitive to water stress and inherently variable as a result. However, increasing the number of observations recorded per replication offset this. RWC and leaf water potential values recorded in these experiments agreed with the earlier reports. Initial data of RWC were very low. This was attributed to the time lag between harvesting the leaf sample and measuring the fresh weight. The problem was overcome by limiting the number of samples harvested for RWC to one replication per day.

Initial SLA data was variable, which was controlled by increasing the size of the leaf sub-sample used. The cross-pollinated nature of faba bean could introduce differences in growth among the individual plants of a genotype, grown from a single

seed lot. It could contribute variability to the TDM and related data. The problem may be partially overcome by sowing seeds of similar size.

Because of the early flowering nature of genotypes such as Acc286, any delay beyond 3-4 leaf stage would make it impossible to stress different genotypes at vegetative stage. Technically only Icarus was stressed at early vegetative stage in Experiment 3. Inability to stress all the genotypes at the vegetative stage rendered comparing the two growth stages for response of the genotypes to water stress impossible. As a result, it was decided to stress faba bean genotypes at both the stages in the future experiment for an appropriate comparison.

Chapter 6

Response of faba bean to water stress: Evaluation of physiological traits for genotype selection

6.1 Introduction

Although Mediterranean climate of southern Australia is well suited to faba bean, its seed yield in the region has been inconsistent (Knight, 1994, Mwanamwenge, 1998). This is mainly attributed to variable rainfall. Terminal drought is a characteristic feature of the region and hence the chances of the crop suffering stress during the reproductive stage are very high (French, 1981, Richards, 1991). Faba bean is known to be susceptible to water stress during the reproductive stage (Grashoff, 1990a, Stutzel and Aufhammer, 1992, Xia, 1994). This observation is supported by the data from the Roseworthy field experiment of 1995 and from the experiments described in Chapter 5. Since faba bean cultivation has expanded beyond the high rainfall areas in southern Australia where it has been most commonly grown, development of varieties that can produce better and more stable yields under water stress is necessary for the development of the industry.

Crop improvement for better performance under drought involves combining high yielding ability and stress tolerance traits for stability of yields in the varieties under cultivation in a given region. Therefore, a major aspect of crop improvement is to identify traits linked to an ability to maintain yield under stress. As described in Chapter 5, several physiological and growth characters are used for identifying such

traits in crops. It is also necessary to identify a growth stage at which genotypes could be reliably screened for tolerance traits. Based on a survey of literature, a field experiment at Roseworthy (1995) and the preparatory experiments described in Chapter 5, stomatal conductance, relative water content (RWC), water potential, osmotic potential, specific leaf area (SLA) and total dry matter (TDM) production were identified as possible traits to be evaluated. Since grain yield is susceptible to water stress during the reproductive stage of faba bean, it is a suitable stage to identify any tolerance traits that may explain differences in yield under field conditions, and to compare the suitability of any other growth stages for such an evaluation. Practical considerations demand that screening be done at an early stage so that resources can be better utilised and a large collection of genotypes can be assessed. Therefore, the seedling stage is an attractive candidate to compare with the reproductive stage.

Experiments were designed to (1) evaluate the physiological response of the selected faba bean genotypes to water stress at two growth stages and, (2) compare the results of this evaluation with the agronomic performance in order to establish the relevance of the glasshouse study for the field performance. No information is available on tolerance traits of faba bean and on growth stage suitable to screen the genotypes for such traits. Therefore, this work aimed at identifying drought tolerance traits among the physiological responses of the genotypes to water stress and also to identify a growth stage at which the genotypes could be screened for these genotypes.

6.2 Materials and methods

For the physiological evaluation, a glasshouse study was conducted using fifteen diverse genotypes. Agronomic evaluation was carried out in the field at Roseworthy

with six selected genotypes. Gtain yield data from faba bean breeding trials sown different locations, spread over a number of years was used for comparisons. The genotypes for the glasshouse study were chosen from the ones tested in the earlier experiments and from the breeding program based on their performance in the field trials. They included the standard genotypes Fiord (small seeded, early flowering, high yielding and widely grown by the Australian farmers at the time of experiment), Icarus (late flowering medium seed size) and Acc286 (very early flowering and large seed size). The details of the genotypes tested in these experiments are provided in Table 6.1.

Table 6.1: Details of the genotypes used in the current experiments

Genotype	Seed size	Seed weight (g/seed)	Maturity group	Flowering time (Days after sowing)
Fiord	Small	0.48	Early	66-70
Acc524	Small	0.48	Early	68-72
Acc868	Small	0.67	Early	65-69
Acc610	Small	0.71	Early	63-69
Acc482	Small	0.49	Medium	71-75
Acc973	Small	0.37	Late	94-100
Acc278	Medium	0.82	Early	65-70
Acc611	Medium	0.98	Early	62-69
Acc722	Medium	0.99	Medium	71-75
Acc974	Medium	0.80	Medium	75-79
Icarus	Medium	0.83	Late	96-101
Acc735	Medium	0.97	Late	80-87
Acc287	Large	1.24	Early	54-60
Acc649	Large	1.32	Early	62-67
Acc286	Large	1.35	Early	52-56
Acc664	Large	1.47	Early	65-68
Acc979	Large	1.01	Late	75-79
Acc766	Large	1.52	Late	84-88

Details of the glasshouse experiment

The glasshouse experiment was sown on 26 April and harvested on 12 October 1998. Seeds of uniform size were pre-germinated before sowing. Seven kg soil was potted in pots of 200 mm diameter. The UC soil mixture was used for potting (Appendix 5.1). Ten pre-germinated seedlings were planted in each pot of which six were retained. To ensure an adequate supply of nutrients to plants, a commercial slow release fertiliser was applied at the rate of 100g per pot every 30 days of plant growth

(Appendix 5.2). Growth temperature in the glasshouse was maintained at 22°C/15°C day/night cycle. The plants were grown under natural daylight conditions.

Genotypes were stressed at early vegetative and early podding stages. Early vegetative stage was defined as the stage at which 3 to 4 leaves were fully opened on the main stem and early podding stage as that at which pods on the first two podding nodes were 1 cm long. Each stress treatment had an unstressed control, which received adequate water during the experiment. The experiment consisted of three replications and was laid out as randomised complete block design (RCBD).

The plants were well watered up to the time that the stress treatment was imposed. The technique of imposing stress described in Chapter 5 was adopted in the current experiment. In this experiment, it took 13 days to reach 50% field capacity at the early vegetative stage and 11 days during the early podding stage. The plants were subjected to 4 days of stress at this moisture level. The development of stress was confirmed by the visual wilting symptoms. The field capacity of the UC soil used in the experiment was determined as described in Chapter 5.

Stomatal conductance, RWC, leaf water potential, osmotic potential, SLA and TDM were recorded at the end of stress treatment as described in Chapter 3. All the physiological observations were recorded on the youngest fully opened leaf, between 12 noon and 2 pm. Osmotic potential at full turgor was calculated by multiplying RWC and the corresponding measured osmotic potential (Leport et al., 1998). It eliminates underestimation of the osmotic potential due to dilution when using the sap expressed from frozen and thawed tissue (Wenkert, 1980). Three plants were harvested from each pot at the end of stress treatment and the remaining three plants

were grown to maturity with adequate watering. At maturity TDM, pod number, pod weight, seed number and seed yield were recorded from the remaining three plants as described in Chapter 3.

Details of the field experiment

The field experiment was sown on 6 June and harvested on 13 November 1998 at Roseworthy, South Australia. Six genotypes selected for the experiment included Fiord, Icarus, Acc286, Acc287, Acc974 and Acc979. Sowing density was 25 seeds/m^2 for all the genotypes except for Acc286, a big seeded genotype that was sown at 20seeds/m^2. The genotypes were replicated 3 times. The experiment received no irrigation and was sprayed once each to control weeds and chocolate spot disease.

Observations were recorded beginning 108 days after sowing (DAS), which broadly coincided with the early podding stage, and on 115, 122 and 137 DAS. They included leaf water potential, leaf osmotic potential, TDM, pod number, pod weight, seed number and seed weight, as described in Chapter 3. Four plants were harvested at each observation. Pods were dissected and seeds were separated. At maturity ten plants were harvested for TDM, pod number, pod weight, seed number and seed yield. These parameters were converted to per m^2 by multiplying with the number of plants per m^2. All the plant parts were dried at 85°C for 48 hours to get dry weight.

Analysis of the data from the breeding trials

Data from the trials conducted by the faba bean breeding program at Waite Institute, Adelaide, were compared with the results of the glasshouse experiment. Yield data of only eight of the genotypes used in the glasshouse experiment were available from trials conducted in the southern Australia during 1994-97. Seed yield of the selected

genotypes at a site as well as the average yield of all the genotypes at that site (site mean yield) were used for analysis. Linear regressions were calculated for the relationship between the yield of each individual genotype in a site and the site mean yield across the years for all the sites (Finlay and Wilkinson, 1963). This relation averages the performance of the genotypes across different production environments ranging from favourable to unfavourable, and therefore can be used to evaluate their adaptability to a given cropping region. The regression coefficient (slope of the regression line) and Y-intercept of the regression equation were used to assess the response of genotypes to the environment of the cropping region. The data from the current glasshouse study was used with this analysis to compare the results of the glasshouse experiment to field performance of the genotypes.

6.3 Results

6.3.1 Physiological response of genotypes to water stress

Water stress was applied at the early vegetative and at the early podding stages. The data showed that the interaction of water stress with genotypes at both the stages significantly affected conductance, RWC, leaf water potential, osmotic potential, SLA and TDM (Tables 6.2-6.9). At both the stages water stress greatly reduced conductance (by 63% and 83% respectively) and RWC (15% and 20% respectively).

Stomatal conductance of this experiment was comparable to that of the earlier experiment (Table 5.2, Appendix 5.3). Acc610 recorded the highest conductance under stress at the early vegetative stage whereas Acc286, Acc973, Acc482 and Acc 735 recorded the lowest (Table 6.2). Fiord and Icarus recorded similar and moderately high conductance under stress at this stage. At the early podding stage, Acc868 recorded the highest conductance under stress while Icarus, Acc664, Acc722

Table 6.2: Effect of water stress at the early vegetative stage on the relative water content and average stomatal conductance of the faba bean genotypes

Genotype	Stomatal conductance ($mmol/m^2/s$)			Relative water content (%)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	1166	462	814	72	67	70
Acc524	1218	390	804	86	66	76
Acc868	1238	456	848	85	74	79
Acc610	1274	674	974	83	72	78
Medium flowering						
Acc482	988	310	650	76	73	75
Late flowering						
Acc973	1248	306	776	80	67	74
Medium seed size						
Early flowering						
Acc278	760	366	562	84	69	76
Acc611	1296	574	936	81	67	74
Medium flowering						
Acc722	1276	426	852	75	67	71
Late flowering						
Icarus	1226	460	844	80	62	71
Acc735	1092	314	702	77	67	72
Large seed size						
Early flowering						
Acc649	1162	494	828	81	70	76
Acc286	1106	286	696	83	66	74
Acc664	1110	474	792	82	65	74
Late flowering						
Acc766	1274	510	892	82	73	78
	1162	**434**		**80**	**68**	
LSD (p=0.05)						
Genotype		**88**			**4**	
Water level		**32**			**2**	
Genotype X Water level		**124**			**6**	
CV (%)		***9***			***8***	

Table 6.3: Effect of water stress at the early podding stage on the average stomatal conductance and relative water content of the faba bean genotypes

Genotype	Stomatal conductance ($mmol/m^2/s$)			Relative water content (%)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	602	134	368	80	69	75
Acc524	638	76	356	82	66	74
Acc868	784	218	502	85	63	74
Acc610	682	94	388	81	69	75
Medium flowering						
Acc482	434	140	288	80	70	75
Late flowering						
Acc973	566	64	316	82	69	76
Medium seed size						
Early flowering						
Acc278	452	120	286	81	64	72
Acc611	778	102	440	82	67	75
Medium flowering						
Acc722	616	60	338	80	61	71
Late flowering						
Icarus	530	58	294	82	58	70
Acc735	576	102	340	79	61	70
Large seed size						
Early flowering						
Acc649	664	86	376	80	62	71
Acc286	656	136	396	80	63	72
Acc664	520	60	290	79	67	73
Late flowering						
Acc766	586	84	336	80	66	73
	606	**102**		**81**	**65**	
LSD (p=0.05)						
Genotype		**32**			**5**	
Water level		**12**			**2**	
Genotype X Water level		**46**			**8**	
CV (%)		***8***			***7***	

and Acc973 recorded the lowest (Table 6.3). Fiord and Acc286 recorded moderately high conductance under stress at this stage. It was evident that the genotypic differences in conductance under water stress were not consistent across the growth stages even when the genotypes were significantly affected by the water stress.

Values of the RWC data at the early podding stage were comparable to that of the earlier experiment (Table 5.2). Acc868 recorded the highest RWC and Icarus the lowest under stress at the early vegetative stage (Table 6.2). At the early podding stage Acc482 recorded highest RWC, and Icarus the lowest (Table 6.3). The genotypes had different rankings for RWC under stress at the two growth stages as shown by the absence of significant rank correlation. Therefore, similar to the result with stomatal conductance, RWC of the genotypes under stress was also not consistent across the growth stages.

The leaf water potential under water stress decreased (60% at the early vegetative stage and 63% at the early podding stage), much more than the osmotic potential (6% and 38% respectively). This suggests a lack of osmotic adjustment to compensate the reduction in water potential. Such observations have been made earlier (Grahoff and Verkerke, 1991). At the early vegetative stage Acc611, Acc610, Fiord, Acc722 and Acc766 recorded the highest water potential under stress while Acc664, Icarus, and Acc482 recorded the lowest (Table 6.4). At the early podding stage Acc722 and Acc664 recorded the lowest water potential under stress while Fiord, Acc868 and Acc278 recorded the highest (Tables 6.5). The water potential at this stage was lower compared to that of the earlier experiment (Table 5.2). It suggests development of more severe soil drought in this experiment, which may be due to higher TDM. Although care was taken to avoid it, in this experiment the prevailing wilting may

Table 6.4: Effect of water stress at the early vegetative stage on the leaf water potential (WP) and osmotic potential (OP) of the faba bean genotypes

Genotype	WP (MPa)			OP (MPa)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	-0.34	-1.05	-0.70	-0.81	-0.84	-0.82
Acc524	-0.30	-1.14	-0.72	-0.73	-0.90	-0.81
Acc868	-0.34	-1.16	-0.75	-0.79	-0.88	-0.83
Acc610	-0.35	-0.99	-0.67	-1.00	-1.08	-1.04
Medium flowering						
Acc482	-0.52	-1.17	-0.84	-0.83	-0.81	-0.82
Late flowering						
Acc973	-0.44	-1.16	-0.80	-0.68	-0.99	-0.83
Medium seed size						
Early flowering						
Acc278	-0.61	-1.13	-0.87	-0.93	-0.84	-0.88
Acc611	-0.47	-0.97	-0.72	-0.93	-0.81	-0.87
Medium flowering						
Acc722	-0.48	-1.05	-0.77	-0.87	-0.80	-0.83
Late flowering						
Icarus	-0.56	-1.17	-0.87	-0.81	-1.01	-0.91
Acc735	-0.51	-1.26	-0.89	-0.84	-0.97	-0.90
Large seed size						
Early flowering						
Acc649	-0.39	-1.12	-0.75	-0.95	-0.85	-0.90
Acc286	-0.46	-1.16	-0.81	-0.75	-0.84	-0.79
Acc664	-0.61	-1.30	-0.96	-0.79	-0.91	-0.85
Late flowering						
Acc766	-0.42	-1.09	-0.76	-0.91	-0.89	-0.90
	-0.45	**-1.13**		**-0.84**	**-0.89**	
LSD (p=0.05)						
Genotype		**0.13**			**-0.11**	
Water level		**0.05**			**-0.04**	
Genotype X Water level		**0.18**			**-0.16**	
CV (%)		***11***			***10***	

Table 6.5: Effect of water stress at the early podding stage on the leaf water potential (WP) and osmotic potential (OP) of the faba bean genotypes

Genotype	WP (MPa)			OP (MPa)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	-0.58	-1.37	-0.97	-0.92	-1.61	-1.27
Acc524	-0.57	-1.46	-1.01	-1.03	-1.76	-1.39
Acc868	-0.61	-1.38	-0.99	-1.08	-1.78	-1.43
Acc610	-0.54	-1.52	-1.03	-1.15	-1.69	-1.42
Medium flowering						
Acc482	-0.61	-1.42	-1.01	-1.22	-2.05	-1.63
Late flowering						
Acc973	-0.55	-1.56	-1.06	-1.01	-1.80	-1.41
Medium seed size						
Early flowering						
Acc278	-0.56	-1.38	-0.97	-0.96	-1.57	-1.27
Acc611	-0.50	-1.52	-1.01	-0.95	-1.63	-1.29
Medium flowering						
Acc722	-0.60	-1.68	-1.14	-1.05	-1.94	-1.50
Late flowering						
Icarus	-0.58	-1.47	-1.03	-1.21	-1.99	-1.60
Acc735	-0.61	-1.54	-1.08	-1.27	-1.88	-1.57
Large seed size						
Early flowering						
Acc649	-0.48	-1.42	-0.95	-1.16	-1.64	-1.40
Acc286	-0.54	-1.58	-1.06	-1.26	-2.03	-1.64
Acc664	-0.58	-1.68	-1.13	-1.25	-1.87	-1.56
Late flowering						
Acc766	-0.59	-1.54	-1.07	-1.06	-1.73	-1.40
	-0.57	**-1.50**		**-1.11**	**-1.80**	
LSD (p=0.05)						
Genotype		**-0.04**			**-0.05**	
Water level		**-0.02**			**-0.02**	
Genotype X Water level		**-0.06**			**-0.08**	
CV (%)		***10***			***7***	

have been severe enough to affect accurate estimation of leaf water potential. Therefore, estimation of bulk leaf turgor at the early podding stage as the difference between water potential and osmotic potential provides negative values.

The stomatal conductance of the genotypes at similar water potential and RWC was lower at the early podding stage than at the earlier stage. The genotypes experienced a decrease in stomatal conductance similar to the decrease in plant water status (water potential and RWC). The conductance of all the genotypes declined at approximately –0.7 MPa water potential and 74%-78% RWC at both the stages (Figures 6.1-6.2). A similar relationship between stomatal conductance and water potential of faba bean in the field has been reported from Western Australia (Leport et al., 1998).

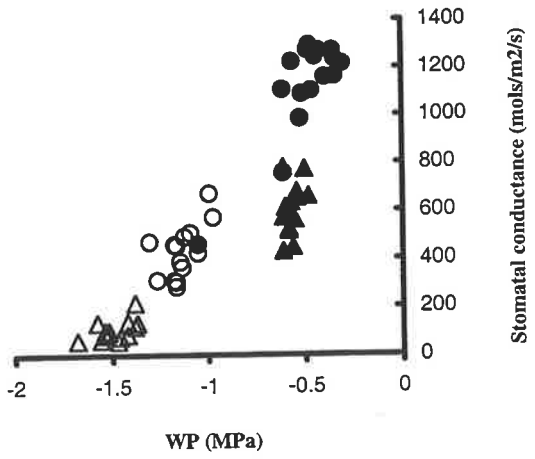

Figure 6.1: Relationship between stomatal conductance and leaf water potential of faba bean genotypes (Legend: Closed symbols – control, Open symbols – stressed; Circles – early vegetative stage, Triangles – early podding stage)

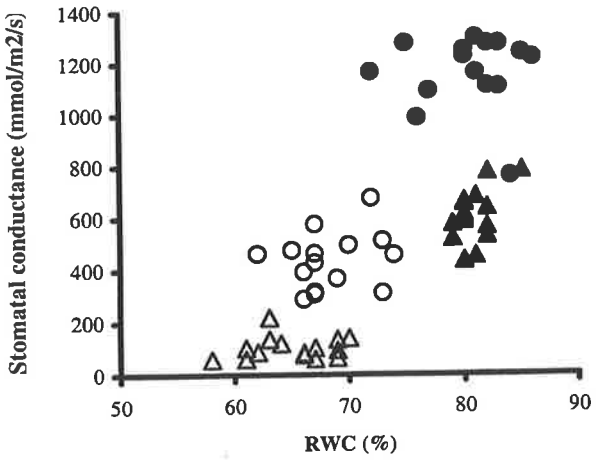

Figure 6.2: Relationship between stomatal conductance and RWC of faba bean genotypes (Legend: Closed symbols – control, Open symbols – stressed; Circles – early vegetative stage, Triangles – early podding stage)

Faba bean genotypes were unable to conserve plant water at the early podding stage under water stress. The relationship between RWC and water potential showed that when not stressed, the genotypes maintained similar RWC at the early podding stage at comparatively lower water potential than at the early vegetative stage (Figure 6.3).

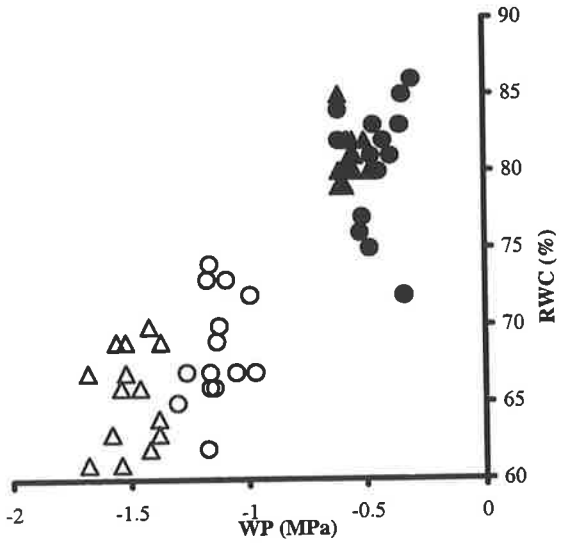

Figure 6.3: Relationship between RWC and water potential of faba bean genotypes (Legend: Closed symbols – control, Open symbols – stressed; Circles – early vegetative stage, Triangles – early podding stage)

It suggests an inability of faba bean to reduce RWC corresponding with the water potential declines at this stage. Therefore, faba bean genotypes were unable to respond to stress as it developed at the early podding stage. When stressed, the genotypes maintained lower RWC at the early podding stage at relatively higher water potential than at the early vegetative stage. Because faba bean lacks osmotic adjustment under water stress, this indicates a rapid loss in plant water content at the later growth stage. This could be a reason for the susceptibility of the reproductive stage of faba bean to water stress. Agung, (1995) reported the possibility that faba bean is unable to respond to water stress as it develops and, at a threshold water potential suddenly closes stomata, showing sharp decline in the conductance. The relationship between the stomatal conductance, RWC and water potential revealed in the current data support that argument.

At each of the two growth stages there were significant differences in the osmotic potential under stress. At the early vegetative stage, Acc610 recorded the lowest osmotic potential, Acc722 the highest, while Fiord and Acc286 recorded moderately high osmotic potential under stress (Table 6.4). At the early podding stage Acc482 recorded the lowest osmotic potential while Acc278 recorded the highest (Table 6.5). While Icarus recorded very low osmotic potential under stress at this stage, Fiord recorded very high osmotic potential. These data show that genotypic differences in water potential and osmotic potential under stress also were not consistent across the two growth stages.

Osmotic potential at full turgor (at 100% RWC) revealed that genotypes had no significant osmotic adjustment under stress at the two growth stages (Table 6.6). Osmotic potential at full turgor for the genotypes under stress (average -0.6 MPa)

Table 6.6: Osmotic potential of the faba bean genotypes at full turgor at the early vegetative and early podding stages

Genotype	Early vegetative stage (MPa)			Early podding stage (MPa)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	-0.58	-0.56	-0.57	-0.7	-1.1	-0.90
Acc524	-0.63	-0.59	-0.61	-0.8	-1.2	-1.00
Acc868	-0.67	-0.65	-0.66	-0.9	-1.1	-1.00
Acc610	-0.83	-0.78	-0.81	-0.9	-1.2	-1.05
Medium flowering						
Acc482	-0.63	-0.59	-0.61	-1.0	-1.4	-1.20
Late flowering						
Acc973	-0.54	-0.66	-0.60	-0.8	-1.2	-1.00
Medium seed size						
Early flowering						
Acc278	-0.78	-0.58	-0.68	-0.8	-1.0	-0.90
Acc611	-0.75	-0.54	-0.65	-0.8	-1.1	-0.95
Medium flowering						
Acc722	-0.65	-0.54	-0.60	-0.8	-1.2	-1.00
Late flowering						
Icarus	-0.65	-0.63	-0.64	-1.0	-1.2	-1.10
Acc735	-0.65	-0.65	-0.65	-1.0	-1.1	-1.05
Large seed size						
Early flowering						
Acc649	-0.77	-0.60	-0.69	-0.9	-1.0	-0.95
Acc286	-0.62	-0.55	-0.59	-1.0	-1.3	-1.15
Acc664	-0.65	-0.59	-0.62	-1.0	-1.3	-1.15
Late flowering						
Acc766	-0.75	-0.65	-0.70	-0.8	-1.1	-0.95
	-0.68	**-0.61**		**-0.9**	**-1.17**	
LSD (p=0.05)						
Genotype		**ns**			**ns**	
Water level		**ns**			**ns**	
Genotype X Water level		**ns**			**ns**	
CV (%)		***11***			***12***	
ns - statistically similar						

and control (average –0.7 MPa) were similar at the early vegetative stage. At the early podding stage, osmotic potential of the genotypes at full turgor differed between the water treatments with an average of –0.9 MPa under control and –1.2 MPa under stress. The difference of –0.3 MPa represents the osmotic adjustment and indicates that the genotypes lack significant osmotic adjustment. These values agree with the field data reported by Leport et al., (1998), where they observed that osmotic potential at full turgor averaged about –1 MPa for faba bean under irrigated as well as rainfed condition. Similar to the current results, they also observed that the osmotic potential at full turgor differed between irrigated and rainfed conditions only during the reproductive stage.

Significant differences in SLA occurred between the genotypes at both the stages. At the early vegetative stage Acc766 recorded the lowest SLA under stress while Acc611 recorded the highest. At this stage Icarus was among the genotypes with lowest SLA, and Fiord and Acc286 among the highest (Table 6.7). At the early podding stage, Acc735 recorded the lowest SLA under stress and Acc649, the highest. At this stage Fiord and Acc286 recorded higher SLA and that of Icarus was among the lowest (Table 6.8). Generally, early flowering genotypes had higher SLA at both the early vegetative and early podding stages than the mid and late flowering genotypes. Significant increase in SLA of the genotypes under water stress at the early vegetative stage indicates that stress reduced leaf weight more than the leaf area (Table 6.7). However, at the early podding stage, water stress resulted in a small but significant increase of SLA (Tables 6.8). A similar trend was observed in the preparatory experiments also (Table 5.3, Appendix 5.3).

In conclusion, while there were significant differences between the genotypes in all

the physiological parameters, they could not be grouped consistently across the growth stages based on the stomatal conductance, RWC, water potential, osmotic potential and SLA. This is supported by that Spearmen's correlation of rank in the stressed and non-stressed treatments was not significant for any of these characters at the early vegetative and early podding stages (r_s=-0.23 for stomatal conductance, r_s=+0.28 for RWC, r_s=+0.18 for water potential, r_s=+0.05 for osmotic potential and r_s=-0.29 for SLA). Therefore, the leaf water characters and SLA may not be useful tools to screen faba bean response to water stress.

At the early vegetative stage large seeded genotypes recorded the highest TDM under stress and the small seeded Acc611 the lowest (Table 6.7). At this stage the interaction between stress and genotypes was significant for TDM and some genotypes showed significant reduction of TDM. However, most of the genotypes had statistically similar TDM under stress indicating a similar response to stress by all of them at this stage. At the early podding stage, the large seeded genotype Acc649 recorded the highest TDM under stress and the small seeded Acc524 the lowest (Table 6.8). Fiord, a small seeded genotype, and Acc286, a large seeded genotype, also recorded high TDM at this stage while that of Icarus was among the lowest. The genotypes had significantly similar rankings for TDM under stress at the early vegetative and early podding stages (Spearman's rank correlation, r_s=+0.64, n=15, p=0.01). TDM of the genotypes under stress and non-stress conditions were significantly correlated (r=+0.74 at the early vegetative stage and r=+0.88 at the early podding stage, n=15, p<0.01). It indicates that the relative rankings of the TDM of the genotypes to water stress at the two stages were similar, and were not affected by the differences in seed size. In addition, the TDM of the genotypes under stress at the end of treatment at the early vegetative and early podding stages had similar relative

Table 6.7: Effect of water stress at the early vegetative stage on the specific leaf area (SLA) and total dry matter (TDM) of the faba bean genotypes

Genotype	SLA (cm^2/g)			TDM (g/plant)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	47	65	56	3.0	2.7	2.9
Acc524	51	67	59	3.3	2.4	2.8
Acc868	59	59	59	4.1	2.6	3.4
Acc610	51	66	58	4.1	2.8	3.4
Medium flowering						
Acc482	55	50	52	4.2	3.0	3.6
Late flowering						
Acc973	50	67	59	2.6	2.2	2.4
Medium seed size						
Early flowering						
Acc278	43	59	51	3.5	2.5	3.0
Acc611	55	70	63	2.3	1.6	1.8
Medium flowering						
Acc722	31	47	39	3.0	2.3	2.6
Late flowering						
Icarus	52	41	47	3.8	2.8	3.3
Acc735	53	51	52	3.7	2.5	3.1
Large seed size						
Early flowering						
Acc649	42	41	41	5.7	2.9	4.3
Acc286	45	65	55	4.5	3.2	3.8
Acc664	40	52	46	6.5	3.1	4.8
Late flowering						
Acc766	44	40	42	5.2	4.1	4.7
	48	**56**		**4.0**	**2.7**	
LSD (p=00.5)						
Genotype		**9**			**0.8**	
Water level		**3**			**0.3**	
Genotype X Water level		**13**			**1.2**	
CV (%)		***11***			***9***	

Table 6.8: Effect of water stress at the early podding stage on the specific leaf area (SLA) and total dry matter (TDM) of the faba bean genotypes

Genotype	SLA (cm^2/g)			TDM (g/plant)		
	Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size						
Early flowering						
Fiord	188	239	214	37.4	27.1	32.3
Acc524	252	241	247	24.2	16.2	20.2
Acc868	180	196	188	25.5	17.4	21.5
Acc610	250	210	230	27.8	19.3	23.5
Medium flowering						
Acc482	208	194	201	27.9	24.0	25.9
Late flowering						
Acc973	218	187	203	29.5	26.1	27.8
Medium seed size						
Early flowering						
Acc278	219	183	201	22.4	20.7	21.6
Acc611	239	200	220	22.4	16.7	19.6
Medium flowering						
Acc722	258	229	243	27.8	25.2	26.5
Late flowering						
Icarus	228	195	212	30.7	21.0	25.9
Acc735	209	179	194	24.8	18.4	21.5
Large seed size						
Early flowering						
Acc649	232	245	239	41.0	30.0	35.5
Acc286	208	225	216	39.5	28.3	33.9
Acc664	198	228	213	38.2	27.6	32.9
Late flowering						
Acc766	202	209	206	39.3	28.3	33.8
	219	**211**		**30.6**	**23.1**	
LSD (p=0.05)						
Genotype		**6**			**3.1**	
Water level		**2**			**1.1**	
Genotype X Water level		**9**			**4.3**	
CV (%)		***6***			***10***	

rankings as their corresponding TDM at maturity (r_s=+0.41 at the early vegetative stage and r_s=+0.85 at the early podding stage; n=15, p=0.01). These relations show that the response of TDM to water stress was consistent across the growth stages and the faba bean genotypes could be ranked consistently on this basis. Therefore, TDM may be suitable for screening the faba bean response to water stress. TDM at the early podding stage was higher than in the earlier study (Table 5.3), indicating a higher dry matter accumulation in the current experiment. This may also have contributed to the lower water potential of the genotypes in the current experiment. TDM at this stage was, however, comparable to that of the individual plants in the Roseworthy field experiment, 1995 (Table 4.1).

Interaction of water stress and the genotypes for TDM was significant at both the stages (Tables 6.7-6.8). The data shows that generally water stress at the early vegetative stage did not result in a large reduction in TDM at maturity (Table 6.9). On the other hand, reductions of the TDM at maturity of the genotypes stressed at the early podding stage were generally large, ranging from 28% to 57%. At maturity TDM in the unstressed treatment was statistically similar to that of the early vegetative stage stress in all the genotypes except Acc610 and Acc611. However, TDM of the genotypes stressed at the early podding stage was significantly lower than that of the unstressed control in all genotypes. In the field experiment of Roseworthy, 1995, TDM at 116 DAS, which generally coincided with podding stage, was also significantly lower under rainfed condition. Therefore this data suggests that the TDM of faba bean at the early podding stage is sensitive to stress.

Table 6.9: Effect of water stress at the early vegetative stage (S1) and at early podding (S2) on the total dry matter (g/plant) of the faba bean genotypes at maturity

Genotype	Non-stress	Stress treatment		Average	Relative reduction at maturity due to	
		S 1	S 2		S 1 (%)	S 2 (%)
Small seed size						
Early flowering						
Fiord	28.9	26.4	16.3	23.9	9	44
Acc524	22.9	18.4	10.9	17.4	20	52
Acc868	26.3	23.4	11.9	20.6	11	55
Acc610	26.5	20.1	13.5	20.0	24	49
Medium flowering						
Acc482	26.4	23.9	15.3	21.9	9	42
Late flowering						
Acc973	35.1	33.7	19.8	29.5	4	44
Medium seed size						
Early flowering						
Acc278	25.6	22.6	13.4	20.5	12	48
Acc611	26.8	19.6	11.4	19.2	27	57
Medium flowering						
Acc722	27.9	24.3	14.1	22.1	13	49
Late flowering						
Icarus	37.0	33.5	20.8	30.4	9	44
Acc735	29.1	26.2	16.2	24.0	10	44
Large seed size						
Early flowering						
Acc649	35.4	30.9	21.0	29.1	13	41
Acc286	34.2	30.1	19.1	27.8	12	44
Acc664	37.6	31.5	20.4	29.8	16	46
Late flowering						
Acc766	33.4	31.2	24.1	29.6	7	28
	30.2	**26.4**	**16.5**			
LSD (p=0.05)						
Genotype			**1.8**			
Water level			**0.8**			
Genotype X Water level			**6.2**			
CV (%)			***8***			

6.3.2 Effect of water stress on the pod number, pod weight, seed number and seed yield of the faba bean genotypes

The interaction of water stress and the genotypes was significant for pod number, pod weight, seed number and seed yield at maturity (Tables 6.10-6.13). The seed yield and yield components of most of the genotypes grown under control conditions were statistically similar to those when stressed at the early vegetative stage. On the other hand, water stress at the early podding stage significantly reduced seed yield and yield components of all the genotypes at maturity. Therefore, the results indicate that the grain yield is very susceptible to water stress at early podding and also that faba bean genotypes respond differently to water stress.

When stressed at the early vegetative and early podding stages, Fiord produced the highest number of pods and Acc649, Acc664 and Acc766 the lowest (Table 6.10). The genotypes had similar rankings for pod number as well as for relative reductions of pod number when stressed at the two growth stages (r_s=+0.91 and r_s=+0.70 respectively; n=15, p=0.01), which indicates the susceptibility of the genotypes to water stress for pod production was consistent at both the growth stages. Acc649, Acc664, Acc610, Icarus, Acc482, Acc611 and Acc735 showed large relative reductions in pod number when stressed at the early vegetative stage. Since these genotypes belong to different maturities, differences in phenology may not have contributed to such a reduction of pod weight. Although stressing commenced at 3-4 leaf stage, it continued for 17 days. Therefore, some of these genotypes may have initiated flowering by the end of stress period, leading to the reduction in pod numbers.

When stressed at the early vegetative and early podding stages, Fiord produced the

Table 6.10: Effect of water stress at the early vegetative stage (S1) and at early podding (S2) on the pod number (per plant) of the faba bean genotypes at maturity

Genotype	Non-stress	Stress treatment		Average	Relative reduction at	
		S 1	S 2		S 1 (%)	S 2 (%)
Small seed size						
Early flowering						
Fiord	7.7	7.3	4.3	6.4	5	44
Acc524	6.3	6.3	2.7	5.1	0	57
Acc868	3.7	3.4	2.3	3.6	8	38
Acc610	6.0	3.7	2.0	3.9	38	67
Medium flowering						
Acc482	8.3	6.3	4.0	6.2	24	52
Late flowering						
Acc973	5.7	5.4	4.0	5.4	5	30
Medium seed size						
Early flowering						
Acc278	5.3	5.0	3.3	4.9	6	38
Acc611	5.0	4.0	2.0	3.7	20	60
Medium flowering						
Acc722	3.0	3.0	2.0	2.7	0	33
Late flowering						
Icarus	4.0	3.0	2.0	3.0	25	50
Acc735	3.3	2.7	2.0	2.7	18	39
Large seed size						
Early flowering						
Acc649	3.7	2.0	1.0	2.2	46	73
Acc286	4.0	4.0	2.0	3.3	0	50
Acc664	3.3	2.0	1.0	2.1	39	70
Late flowering						
Acc766	2.0	2.0	1.7	2.2	0	15
	4.8	**4.0**	**2.4**			
LSD (p=0.05)						
Genotype		**0.8**				
Water level		**0.2**				
Genotype X Water level		**1.0**				
CV (%)		***10***				

Table 6.11: Effect of water stress at the early vegetative stage (S1) and at early podding (S2) on the seed number (per plant) of the faba bean genotypes at maturity

Genotype	Non-stress	Stress treatment		Average	Relative reduction at	
		S 1	S 2		S 1 (%)	S 2 (%)
Small seed size						
Early flowering						
Fiord	24.1	19.7	7.8	17.2	18	68
Acc524	18.7	13.8	5.9	12.8	26	68
Acc868	12.3	11.4	4.3	9.4	7	65
Acc610	15.1	9.5	3.4	9.3	37	77
Medium flowering						
Acc482	21.4	21	7.1	16.5	2	67
Late flowering						
Acc973	17.5	17.2	6.2	14.0	2	65
Medium seed size						
Early flowering						
Acc278	17.1	15.8	4.9	12.6	8	71
Acc611	10.9	7.4	3.0	7.1	32	72
Medium flowering						
Acc722	10.3	7.3	2.0	6.5	29	81
Late flowering						
Icarus	8.6	7.4	3.1	6.4	14	64
Acc735	9.0	8.2	3.5	6.9	9	61
Large seed size						
Early flowering						
Acc649	8.0	5.8	2.0	5.3	28	75
Acc286	9.7	7.5	3.1	6.8	23	68
Acc664	6.9	5.2	2.0	4.7	25	71
Late flowering						
Acc766	6.3	5.3	2.2	4.6	16	65
	13.1	**11.0**	**4.0**			
LSD (p=0.05)						
Genotype			**0.7**			
Water level			**0.3**			
Genotype X Water level			**1.3**			
CV (%)			***9***			

highest number of seeds per plant while Acc664, Acc649, Acc766 and Acc722, produced the lowest (Table 6.11). This trend was similar to that of pod number. The seed numbers of all the genotypes were significantly lower when stressed at early podding. The genotypes had similar rankings for seed number as well as for relative reductions of seed number when stressed at the two growth stages (r_s=+0.96 and r_s=+0.77 respectively; n=15, p=0.01), which indicates that the sensitivity of the seed production of the genotypes to water stress was consistent across the growth stages.

Acc482 and Fiord recorded the highest pod weight when stressed at the early vegetative and early podding stages, while Acc649, Icarus, Acc722, and Acc664 recorded the lowest (Table 6.12). The genotypes had similar rankings for pod weight and for relative reductions of pod weight when stressed at the two growth stages (r_s=+0.86 and r_s=+0.85 respectively; n=15, p=0.01). Also, they had similar rankings for pod number and pod weight under stress at the two growth stages (r_s=+0.79 at the early vegetative stage and r_s=+0.71 at the early podding stage, n=15, p=0.01). Hence the pod number and pod weight response of the genotypes had consistency among the two growth stages.

The genotypes differed significantly for yield under water stress at both the growth stages. Acc482 and Fiord recorded the highest seed yield under stress at both stages while Icarus, Acc649, Acc722 and Acc868 recorded the lowest (Table 6.13). The genotypes had similar rankings for seed yield as well as for yield reductions when stressed at the two stages (r_s=+0.85 and r_s=+0.84 respectively; n=15, p=0.01). The ranking of the genotypes for seed number was similar to that for seed yield under stress at both the growth stages (r_s=+0.72 at the early vegetative stage and r_s=+0.76 at the early podding stage; n=15, p=0.01). Therefore the yield response of the

Table 6.12: Effect of water stress at the early vegetative stage (S1) and at early podding (S2) on the pod weight (g/plant) of the faba bean genotypes at maturity

Genotype	Non-stress	Stress treatment		Average	Relative reduction at	
		S 1	S 2		S 1 (%)	S 2 (%)
Small seed size						
Early flowering						
Fiord	11.1	9.0	3.3	7.8	19	70
Acc524	9.5	7.0	2.8	6.4	26	71
Acc868	5.9	5.1	1.5	4.1	14	75
Acc610	8.7	5.7	1.4	5.3	34	84
Medium flowering						
Acc482	10.4	9.4	3.6	7.8	10	65
Late flowering						
Acc973	8.3	7.5	2.8	6.2	10	66
Medium seed size						
Early flowering						
Acc278	10.1	7.8	2.4	6.8	23	76
Acc611	9.7	6.0	2.2	6.0	38	77
Medium flowering						
Acc722	7.6	5.1	1.4	4.7	33	82
Late flowering						
Icarus	5.7	5.1	1.8	4.2	11	68
Acc735	6.4	5.5	1.9	4.6	14	70
Large seed size						
Early flowering						
Acc649	7.3	5.0	1.6	4.6	32	78
Acc286	9.4	7.2	2.0	6.2	23	79
Acc664	7.8	5.2	1.6	4.8	33	79
Late flowering						
Acc766	7.4	6.3	1.9	5.2	15	74
	8.4	**6.5**	**2.1**			
LSD (p=0.05)						
Genotype		**1.1**				
Water level		**0.5**				
Genotype X Water level		**2.5**				
CV (%)		***12***				

Table 6.13: Effect of water stress at the early vegetative stage (S1) and at early podding (S2) on the seed yield (g/plant) of the faba bean genotypes at maturity

Genotype	Non-stress	Stress treatment		Average	Relative reduction at	
		S 1	S 2		S 1 (%)	S 2 (%)
Small seed size						
Early flowering						
Fiord	13.6	11.3	4.4	9.7	17	68
Acc524	11.3	9.1	3.8	8.1	19	66
Acc868	8.2	6.9	2.2	5.7	16	73
Acc610	10.9	7.4	2.0	6.7	32	82
Medium flowering						
Acc482	13.2	12.2	4.8	10.1	8	64
Late flowering						
Acc973	10.5	9.6	3.7	7.9	9	65
Medium seed size						
Early flowering						
Acc278	12.8	10.4	3.4	8.9	19	73
Acc611	11.6	7.5	2.9	7.3	35	75
Medium flowering						
Acc722	9.8	6.9	1.9	6.2	30	81
Late flowering						
Icarus	7.6	6.8	2.5	5.6	11	67
Acc735	8.6	7.3	2.9	6.3	15	66
Large seed size						
Early flowering						
Acc649	10.0	6.8	2.4	6.4	32	76
Acc286	12.1	9.2	3.0	8.1	24	75
Acc664	10.7	7.0	2.4	6.7	35	78
Late flowering						
Acc766	9.8	8.5	2.8	7.0	13	71
	10.7	**8.5**	**3.0**			
LSD (p=0.05)						
Genotype		**1.1**				
Water level		**1.0**				
Genotype X Water level		**3.8**				
CV (%)		***10***				

genotypes was consistent under stress at the two growth stages.

Seed yield of the genotypes was significantly correlated with seed number, pod number and pod weight when stressed at the early vegetative (r=+0.86, r=+0.83 and r=+0.99 respectively; n=15, p<0.01) and early podding stages (r=+0.87, r=+0.82 and r=+0.99 respectively; n=15, p<0.01). The ranking of the genotypes for seed yield under stress was similar to that of seed number, pod number and pod weight when stressed at the early vegetative (r_s=+0.71, r_s=+0.79 and r_s=+0.99 respectively; n=15, p=0.01) and early podding stages (r_s=+0.75, r_s=+0.71 and r_s=+0.99 respectively; n=15, p=0.01). Therefore, the effects of water stress affecting the formation, retention and development of pods and seeds will significantly influence the seed yield of the faba bean genotypes. Data from the Roseworthy field experiment of 1995 also illustrated that an ability to maintain pod weight and retain seeds under water stress was important for better seed yield (Sections 4.3.3, 4.4.2). Seed yields of the genotypes, stressed at the early vegetative and early podding stages, were significantly correlated with each other (r=+0.92, n=15, p<0.01) and had significant rank correlation (r_s=+0.85; n=15, p=0.01). Similarly relative reductions of the seed yield at the two instances of stresses were significantly correlated (r=+0.85 n=15, p<0.01) and had significant rank correlation with each other (r_s=+0.84; n=15, p=0.01). Therefore, it may be said that the yield of faba bean genotypes showed similar relative sensitivities to water stress at the two growth stages. Seed yields under unstressed conditions were significantly correlated with the seed yields when the genotypes were stressed at the two stages (r=+0.80, and r=+0.69 respectively; n=15, p<0.01). However, it was not correlated with the reductions of the seed yields of the genotypes stressed at the two stages. This suggests that the response to water stress in seed yield of the genotypes was independent of their yield potential.

Data showed that the susceptibility of the seed yield, seed number, pod weight and pod number of the genotypes to stress was consistent between the early vegetative and early podding stages. This is illustrated by the significant rank correlations for these parameters and their relative reductions under stress at the two growth stages. Consistency is important in choosing early vegetative stage to screen the faba bean response to stress as against the susceptible early podding stage.

By fitting a continuous distribution (normal distribution) to the relative reduction of the seed yield when stressed at the early podding stage, genotypes were grouped into three classes as tolerant (<69% reduction), intermediate (69-75% reduction) and susceptible (>75% reduction) based on the standard deviation of the distribution. Tolerant group includes Acc482, Acc973, Acc735, Acc524, Icarus and Fiord. The intermediate group includes Acc766, Acc868, Acc278, Acc611 and Acc286. The susceptible group includes Acc649 Acc664, Acc722 and Acc610.

Stomatal conductance, osmotic potential and specific leaf area were not correlated with seed yield and yield components of the genotypes when stressed at the early vegetative or early podding stages. RWC and leaf water potential of the genotypes, when stressed at the early vegetative stage were not correlated with their seed yield seed number, pod weight and pod number. However, when stressed at the early podding stage, RWC of the genotypes was significantly correlated with pod number, pod weight, seed number and seed yield at maturity ($r=+0.51$, $r=+0.58$, $r=+0.55$ and $r=+0.55$ respectively, $n=15$, $p=0.05$). Leaf water potential of the genotypes stressed at the early podding stage, was significantly correlated with seed number ($r=+0.58$; $n=15$, $p=0.05$), pod number, pod weight and seed yield also ($r=+0.47$, $r=+0.44$ and

r=+0.45; n=15, p=non-significant). It should be noted that these correlations explain only 19%-36% of the variability. However, they indicate that an ability to maintain high plant water content during the early podding stage, which is a drought sensitive stage, may result in better seed yields of the faba bean genotypes. Significant correlation between leaf water potential and seed number of the genotypes stressed at this stage implies that water stress during podding mainly affected seed production. Water stress may have affected seed production either by restricting further seed formation or by causing seed abortion or both. This is critical for large seeded genotypes, which produce only limited number of pods and seeds.

TDM of the genotypes when stressed at the early vegetative and early podding stages were not correlated with the seed yield and yield components. The relative reduction of TDM of the genotypes when stressed at the early vegetative stage was highly correlated with the relative reductions in pod number (r=+0.72, stressed at the early vegetative stage and r=+0.66, stressed at the early podding stage; n=15, p=0.01) and in seed yield (r=+0.56, p=0.05, stressed at the early vegetative stage and r=+0.47, n.s., stressed at the early podding stage; n=15; Figures 6.4-6.5; Appendix 6.1). However, the relative reductions in TDM of the genotypes stressed at the early podding stage did not show such correlations. Also relative reductions of the TDM of the genotypes stressed at these two stages were not correlated with each other. Therefore the relative sensitivity of the TDM of the genotypes stressed at the early vegetative stage was similar to the sensitivity of their seed yield when stressed at early podding also. These correlations suggest that relative reductions of the TDM when stressed at the early vegetative stage, as a trait, may be useful in screening the ability of the genotypes to sustain seed yield under stress.

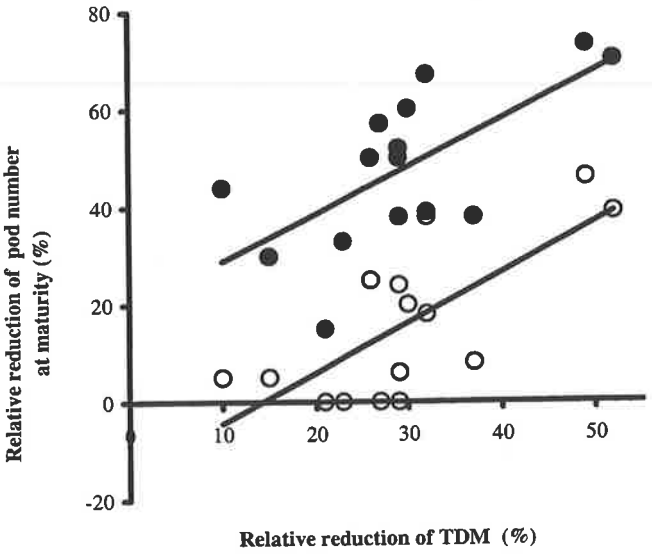

Figure 6.4 : Relationship between relative reduction of TDM of the genotypes at the end of stressing at early vegetative stage with the relative reduction of pod number under the two stresses (Open circles – stressed at the early vegetative stage; Closed circles – stressed at the early podding stage)

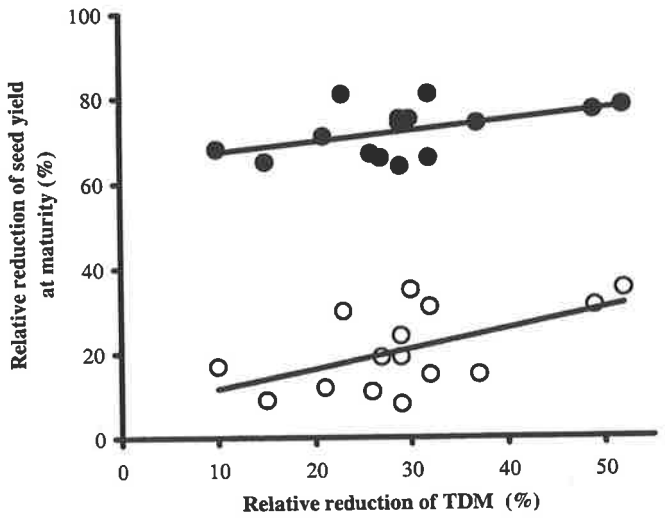

Figure 6.5 : Relationship between relative reduction of TDM of the genotypes at the end of stressing at early vegetative stage with the relative reduction of seed yield under the two stresses (Open circles – stressed at the early vegetative stage; Closed circles – stressed at the early podding stage)

The data showed that when stressed at the early vegetative stage, the late flowering genotypes recorded lower relative reduction of pod weight, seed number and seed yield than the early flowering genotypes. It suggests that the late flowering genotypes were less susceptible to water stress at the early vegetative stage than the early flowering ones. This response may be associated with the phenology of the genotypes where flowering buds may have formed in early flowering genotypes but not in the late flowering genotypes. This assumes importance in selecting the genotypes for very early sowing time. The argument is supported by the data from the Roseworthy field experiment of 1995. In that experiment it was observed that while early flowering genotypes produced more seeds than the late flowering ones, early flowering time did not lower the sensitivity to water stress, because all the genotypes experienced similar relative reduction of the seed number (Table 4.5).

Although the late flowering genotypes recorded lower relative reduction of seed yield than the early flowering ones, they did not necessarily produce higher yields because of the lower sensitivity to stress. Therefore, it appears that sensitivity of the seed yield to water stress and the absolute yield level under stress are different in the faba bean genotypes, suggesting the possibility that even low yielding genotypes may have traits that can be used for crop improvement under water stress.

Generally the small seeded genotypes produced more pods and seeds than the large seeded genotypes under both the stresses and control (Tables 6.10-6.11). This trend was observed in the Roseworthy field experiment of 1995 also (Table 4.3). Although small seeded genotypes recorded higher pod weight and seed weight per plant than the large seeded genotypes, these differences tended to be marginal (Tables 6.12-6.13). The results indicate that large seeded genotypes compensate for lower number

of pods and seeds through higher seed weight.

6.3.3 Agronomic evaluation of the faba bean genotypes

There were no significant differences among the genotypes for leaf water potential during the reproductive growth in the field (Figure 6.6). Similarly, the genotypes did not significantly differ for osmotic potential during the reproductive growth except on 115 DAS, when Acc286 recorded significantly lower osmotic potential than the other genotypes (Figure 6.6). In the glasshouse experiment also, Acc286 recorded lowest osmotic potential when stressed at early podding (Table 6.5). Leaf water potential and osmotic potential of the genotypes were generally parallel to each other (except on 115 DAS). Water potential and osmotic potential were not correlated with dry matter, seed yield and yield components, during the reproductive growth and at maturity. This suggests a lack of osmotic adjustment among the genotypes in response to developing water stress under the field conditions. It should be mentioned that at early podding, water potential and osmotic potential in glasshouse were lower than values in the field at comparable growth stage.

Genotypes differed significantly for vegetative dry matter (VDM, consisting of stem and leaf), pod wall dry weight, seed weight and pod number during the reproductive growth (Appendices 6.2-6.5). VDM generally declined sharply coinciding with a linear increase in the seed weight (Figure 6.6). This phase also coincided with large decline in leaf water potential, indicating that the genotypes experienced rapidly developing stress during the reproductive stage. The two large seeded genotypes Acc286 and Acc287, showed large decline of the pod wall weight along with a linear increase in the seed weight. Acc974 and Acc979 recorded only small declines but the pod wall dry weight of Fiord and Icarus did not decline (Figure 6.6). These

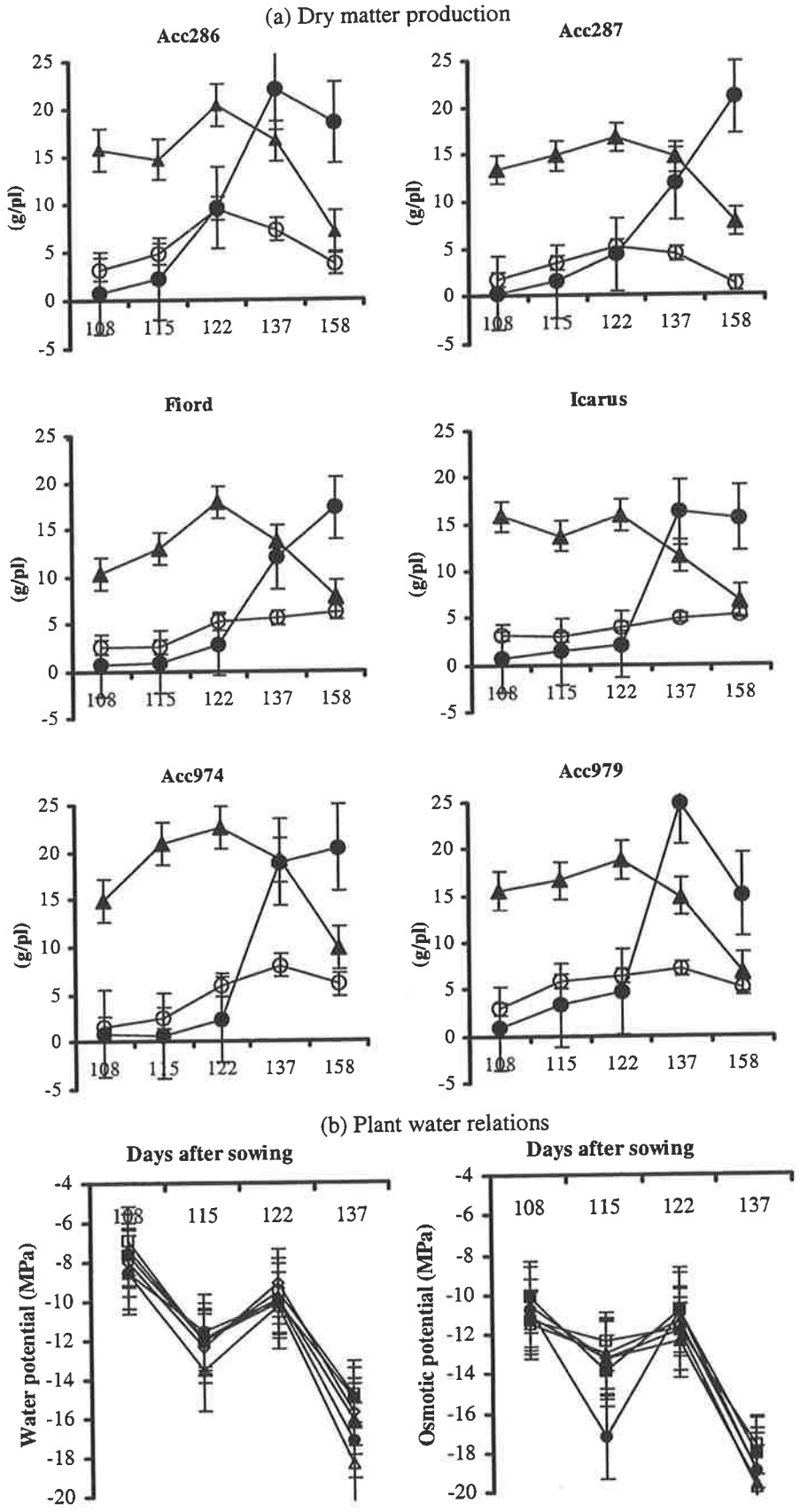

Figure 6.6 (a) Vegetative dry matter (▲), pod wall dry weight (○), seed weight (●)of six faba bean genotypes grown in the field at Roseworthy and (b) leaf water potential and osmotic potential Acc286 (●), Acc287 (○), Fiord (▲), Icarus (Δ), Acc974 (■) and Acc979(□)

results indicate Acc286 and Acc287 may have remobilised the reserves from pod wall to growing seeds more than the others, which may have been due to their early flowering time and large seed size. Acc287 and Fiord continued accumulation of seed weight beyond 137 DAS. However, seed weight of Acc286 and Acc979 declined after 137 DAS whereas Icarus and Acc974 maintained their seed weight. Therefore, the genotypes differed for seed weight accumulation but the differences were not influenced by their seed size or flowering time.

Table 6.14: Vegetative dry matter (VDM), seed yield and yield components of the selected faba bean genotypes at maturity

Genotype	VDM (g/m^2)	Pod number/m^2	Pod weight (g/m^2)	Seed number/m^2	Seed yield (g/m^2)
Acc286	106	141	332	235	277
Acc287	157	192	444	305	421
Acc974	195	244	528	495	408
Acc979	138	173	402	293	301
Fiord	234	300	699	994	516
Icarus	137	185	418	379	311
LSD (0.05)	**33**	**58**	**86**	**83**	**69**

The genotypes differed significantly for seed yield, seed number, pod weight and pod number at maturity (Table 6.14). Fiord recorded the highest seed yield, seed number, pod weight and pod number, while Acc286 recorded the lowest. A similar trend was observed with VDM at maturity. However, because of complete leaf shedding, VDM at maturity consisted solely of stems. The data did not reveal any definitive trend dependent upon the seed size or flowering time of the genotypes for seed yield and its components. Seed yield was significantly correlated with seed number, pod number and pod weight per m^2 ($r=+0.85$, $p=0.05$, $r=+0.91$ and $r=+0.92$ respectively, $n=6$, $p<0.01$). This shows that high yield of faba bean under dry land condition is associated with the ability to produce and retain large number of pods and seeds as well as to maintaining pod weight. This result is similar to the results from the Roseworthy field experiment of 1995.

6.3.4 Analysis of the data from the breeding trials

Analysis of the yield data of the selected genotypes from the breeding trials from 1994 to 1997 is presented in Table 6.15. Small and medium seeded genotypes (approximately 0.5–1.0 g/seed) which flower early to medium (by about 60-75 DAS), generally yielded higher than the site mean yield. This could be attributed to their ability to produce more pods and seeds than the large seeded genotypes (Table 6.10-6.11). Although early flowering time appeared to be important for the better yield performance of the genotypes in the region, earliness in itself does not seem to confer higher yields, as seen in the case of Acc286, and Acc278.

Table 6.15: Analysis of the yield data of selected faba bean genotypes grown in different locations and from 1995-1997 in the southern Australian region

Genotype	Slope	Y-intercept (kg/ha)	Mean seed yield (kg/ha)		Flowering time	Seed size
			Genotype	Site		
Icarus	0.89	-111	1518	1820	Late	Medium
Acc973	0.96	-213	1775	2063	Late	Small
Acc482	0.96	231	1998	1827	Medium	Small
Acc286	1.08	-69	1584	1523	Early	Large
Acc278	1.08	-36	1871	1756	Early	Medium
Acc610	1.08	41	2245	2035	Early	Small
Acc611	1.04	135	2066	1855	Early	Medium
Acc524	0.9	129	1861	1853	Early	Small
Fiord	1.11	116	2075	1842	Early	Small

The genotypes could not be ranked consistently across the glasshouse study and the breeding trials based on the seed yield. However, relative reduction of the seed yield of the genotypes stressed at the early podding stage in the glasshouse study and the slope of the regression equation of the yield in the breeding trials had similar rankings (r_s=0.62, n=9, p=0.01), and the two were significantly correlated (r=+0.66, n=9, p=0.05; Figure 6.7). Therefore, the sensitivity of seed yield to water stress at the early podding stage and the stability of the genotypes to field environment were similar. Hence the current data is consistent with the field data.

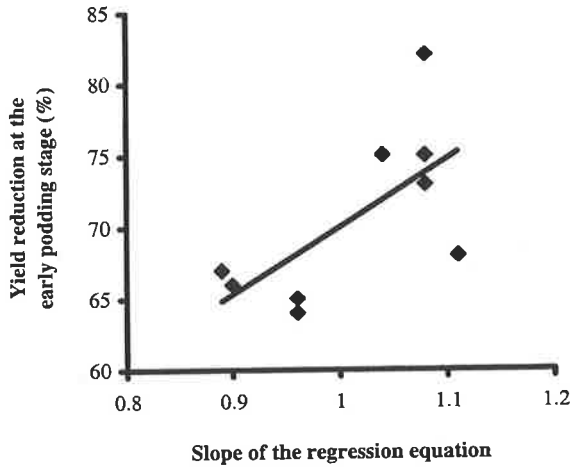

Figure 6.7: Relationship between the seed yield reduction of the faba bean genotypes when stressed at the early podding stage in the glasshouse study and the yield stability observed in the breeding trials in southern Australia

6.4 Discussion

Faba bean genotypes were subjected to water stress at the two growth stages to evaluate the practical utility of the physiological and growth responses for screening in a breeding program. Accordingly, the results will be discussed here with an emphasis on screening for crop improvement under water stress.

The genotypes differed significantly for stomatal conductance, which was drastically reduced by water stress at both the growth stages. However, it was not correlated with seed yield, seed number, pod weight or pod number. The genotypes experienced decrease in the conductance similar to the decrease in plant water status under water stress at both the growth stages (Figures 6.1-6.2). Leport et al., (1998) reported similar relationship between the conductance and water potential of several cool season legumes including faba bean in the field trials from Western Australia. As discussed in section 5.4 (Tables 5.4, 5.6; Appendices 5.3, 5.5), and by Agung,

(1995), faba bean tends to maintain normal conductance until a threshold level of stress develops, and suddenly closes stomata. Therefore stomatal conductance is of limited use in evaluating faba bean response to water stress.

The data showed that faba bean lacks osmotic adjustment under water stress. Levels of osmotic potential at full turgor at the early podding stage observed in the glasshouse study (Table 6.6) were similar to those reported elsewhere from the field experiments (Leport et al, 1998). This indicates consistency and reliability of the data with respect to field performance reported elsewhere. Osmotic potential and water potential recorded in the Roseworthy field experiment were not correlated with the seed yield, seed number, pod weight and pod number. In the preparatory experiment also the interaction of genotypes and water stress was not significant for leaf water potential, indicating that the genotypes respond similarly to water stress as under unstressed control (Table 5.2). As a result, leaf water potential and osmotic potential may not be suitable to screen the faba bean genotypes for response to water stress.

High correlations of RWC and leaf water potential under stress at the early podding stage with seed yield, seed number, pod weight and pod number indicate that it is necessary to maintain higher plant water status at this stage for better seed yield. The genotypes maintained lower RWC at the early podding stage at comparatively higher water potential than at the early vegetative stage, which suggests an inability to respond to developing stress quickly and to conserve plant water (Figure 6.3). This is supported by the absence of interaction of water stress and the genotypes for RWC in the preparatory experiment (Table 5.2), and a general lack of osmotic adjustment. Therefore, RWC is not a good trait to screen the genotypes under water stress.

Specific leaf area under stress was not consistent between the growth stages and the genotypes showed different trends of SLA in response to water stress. It was also not correlated to seed yield. A similar trend was observed in earlier experiment also (Table 5.3, Appendix 5.3). It was not possible to consistently rank the genotypes using SLA under water stress at the early vegetative and early podding stages. Therefore, it may not be useful in screening faba bean response to water stress.

The TDM of the genotypes under stress at the two growth stages was not correlated with the seed yield but it was consistent between the growth stages. This is reflected by the similar rankings of the genotypes under stress at the early vegetative and early podding stages. However, high correlations between the relative reduction of the TDM due to water stress at the early vegetative stage and the relative reductions of pod number and of seed yield of the genotypes stressed at both the early vegetative stage and early podding stages is very important. It indicates that the sensitivity of the TDM to water stress at this growth stage was similar to the sensitivities of the pod number and seed yield of the genotypes at a later (early podding) stage. The data from the field experiment in Roseworthy (1995) showed that the ability to retain pods and maintain pod weight under rainfed condition contribute to better seed yield of the genotypes (Table 4.3, Figure 4.1). Data from the 1998 field experiment in Roseworthy also indicated that high yield under rainfed condition is associated with the ability of the genotypes to set large number of pods, seeds and to maintain the pod weight. Together, these relations indirectly suggest that reduction of TDM of the genotypes in response to stressing at the early vegetative stage, obtained under controlled conditions, is able to predict their response to water stress in the field. A more direct conclusion may be obtained in future work where glasshouse study is combined with field studies specifically targeted to drought stress. It was not possible

to include it in the current study because of the limited time available.

The susceptibilities of the seed yield, seed number, pod weight and pod number of the genotypes to water stress were consistent between the early vegetative and early podding stages. It suggests that the early vegetative stage is appropriate to screen the faba bean response to water stress. The data indicated that the initial seed size did not influence the response of the TDM of the genotypes to stress at both the stages. This eliminates the possibility of the TDM response to stress at the early vegetative stage being a function of the initial seed size. Therefore, the relative reduction of TDM measured at the early vegetative stage of growth may be used to screen faba bean response to water stress in the field.

Data from the glasshouse evaluation were compared with the yield data from breeding trials to analyse relationships between the glasshouse results and field performance. The analysis showed that it was not possible to consistently rank the genotypes across the two sets of data based on the seed yield. However, the reduction of the seed yield of the genotypes under water stress at the early podding stage in the glasshouse study was similar to the stability of their seed yield in the breeding trials, represented by the slope of the regression equation of the seed yield in trials across southern Australia. This is important because the reduction of the seed yield of the genotypes under stress at the two growth stages in the glasshouse study were significantly correlated ($r=+0.85$, $n=15$, $p<0.01$). Also, the reduction of the TDM at the early vegetative stage and of seed yield at both the growth stages under water stress were significantly correlated. Therefore, drought tolerance of faba bean, measured as reduction of TDM at the early vegetative stage in the glasshouse study, was related to the stability of the genotypes across sites, but not necessarily to high

yields. Hence, response of TDM to water stress at this stage may be validly used to screen the faba bean genotypes for the yield stability under drought in the field. This provides an easy and quick technique to screen the genotypes.

6.5 Summary

The data showed that faba bean is generally incapable of maintaining and conserving plant water status under drought. As a result, individual physiological parameters, although sensitive to water stress, are not reliable tools to screen the response of the genotypes to water stress. On the other hand, TDM may be a better indicator of drought response of the faba bean genotypes. It integrates all the physiological and metabolic responses to water stress. The analysis clearly demonstrated that TDM estimated at an early growth stage (3-4 open leaf stage) may be useful in assessing the ability of the faba bean to maintain seed yield under stress. Therefore, it needs to be further investigated. Future work should establish the validity of the response of TDM and its relation with the seed yield using a larger number of genotypes, both in the glasshouse and field experiments. Inheritance of the character should be studied so that it can be used to breed better yielding genotypes.

Chapter 7

Faba bean response to heat stress: Developing techniques to assess acquired thermal tolerance and heat shock proteins

7.1 Introduction

Faba bean is considered to be sensitive to water and high temperature stresses (Morgan et al., 1991; Turner et al., 1996). The crop is adversely affected during the reproductive stage if the mean temperature exceeds 20°C (Saxena et al., 1988). It looses turgor quickly under hot and dry conditions, leading to early closure of stomata and consequent reduction in CO_2 fixation (Chaves, 1991). High temperature reduces water potential of faba bean, injures photosynthesis and reduces growth (McDonald and Paulsen, 1997). Temperature stress inhibits flower initiation, hastens flowering and reduces the duration of pod filling (Evans, 1959; Dekhuijzen and Verkerke, 1986; DeCosta, 1997). Although seed growth rate may increase under high temperature, it may not be sufficient to offset shortened pod filling period that results in reduced individual seed weight (Agung, 1995).

A survey of literature revealed that plants possess some level of tolerance to elevated temperatures, inherent or acquired (Chen et al., 1982). When the plants are subjected to non-lethal temperatures above the optimum, they acquire an ability to tolerate lethal temperatures. Such ability is defined as acquired thermal tolerance (ATT) (Chen et al., 1982). The occurrence of sublethal chronic heat stress is more common in the field crops than of the occurrence of lethal temperatures. Metabolic changes

occurring under such conditions contribute more to the crop adaptation to stress than the inherent heat tolerance (Chen et al., 1982, Howarth et al., 1997). Therefore, ATT is more relevant to the field performance of the crops than their inherent tolerance. ATTs of sorghum measured in the glasshouse and field experiments were significantly correlated to each other and to the seedling growth under heat stress (Howarth et al., 1997). Significant correlation between the ATT and whole plant heat tolerance has been reported in soybean (Martineau et al., 1979) and wheat (Saadalla et al., 1990b, Shanhan et al., 1990). ATT of wheat is significantly correlated with the grain yield and its quality in the regions with hot climates (Shanahan et al., 1990; Fokar et al., 1998). ATT of wheat is also known to be consistent across the years, quantitatively heritable and is known to possess genetic variability for the character (Porter et al., 1995). Therefore ATT, a physiological measure of stress response, is correlated to the phenotypic responses of the genotypes to heat stress and also to the grain yield in the field. Hence it is a valid trait to screen the genotypes for differences under heat stress. However, no information is available regarding the ATT of faba bean and no method is available to assess its ATT.

Techniques to assess ATT should induce differences for the character among the genotypes and be able to quantify these differences. To develop ATT, plants require exposure to a high but sublethal temperature (Chen et al., 1982; Porter et al., 1994). Such a requirement is reported from both controlled environment and field studies (Gavuzzi et al., 1997; Howarth et al., 1997). Both detached leaf and intact plants have been commonly pre-treated to induce ATT (Li et al., 1991; Porter et al., 1994; Srinivasan et al., 1996; Fokar et al., 1998), followed by its quantification. Estimation of ATT involves exposing the pre-treated plant tissue to lethal high temperature and measuring the injury caused to the cell (Chen et al., 1982; Fokar et al., 1998). The

conditions used for pre-treatment and heat shock treatments cited in the literature vary among the crops, growth stage of evaluation and the plant material used (Sadalla et al., 1990a, Howarth et al., 1997; Marcum, 1998). Therefore, it is necessary to standardise the conditions for these treatments before genotypic differences in faba bean can be evaluated.

Available literature suggests that electrolyte leakage from plant tissue and reduction of 2,3,5-triphenyl-tetrazolium-chloride (TTC) are commonly used to evaluate ATT. Using these two measures, relative injury (RI) caused to the cells is calculated, which gives an indication of the extent of thermal tolerance the cells have developed under pre-treatment. Abiotic stresses like drought and extremes of temperature are known to affect the cell membrane integrity (Ristic et al., 1996). It is well documented that the ability of a cell to maintain the integrity of its membranes is important to tolerating many stresses (Ristic and Cass, 1993; Collins et al., 1995). The argument is that when cell membrane is stable, organelles remain intact and functional, providing tolerance to the stress (Chen et al., 1988). Hence a study of the membrane integrity under heat stress can potentially provide a measure of tolerance of faba bean to the stress. When cell membranes rupture, cell contents leak to the surrounding media (Collins et al., 1995). The increase in electric conductivity (EC) of the media provides a measure of the extent of leakage and therefore, of the damage to the cell membrane (Sadalla et al., 1990a,b; Srinivasan et al., 1996). This forms the basis for the EC test. This method of assessing ATT has been correlated to the phenotypic response of several crops to heat stress, including soybean (Martineau et al., 1979), sorghum, pearl millet (Howarth et al., 1997) and wheat (Saadalla et al., 1990b, Fokar et al., 1998). TTC reduction is attributed to the enzymes in the mitochondrial electron transport chain (Kalina and Palmer, 1968; Towill and Mazur, 1975). They

reduce TTC to red coloured formazan, indicating an intact electron transport chain and functional mitochondria. Hence it measures the ability of the cells to retain the functionality of the enzymatic systems under stress (Porter et al., 1994). The heat stressed tissue is incubated in TTC, and formazan production is quantified using spectrophotometer. TTC reduction has been correlated to grain yield of wheat and is shown to be quantitatively heritable (Porter et al., 1995, Ibrahim and Quick, 1999).

Plants synthesise unique proteins when subjected to stresses such as drought and heat (Vierling, 1991; Almoguera et al., 1993; Blumenthal et al., 1994; Waters et al., 1996). The role of these stress proteins, called heat shock proteins (HSP), has been the subject of much recent research (Howarth and Ougham, 1993; Nguyen et al., 1994; Ristic et al., 1996; Forreitor et al., 1997; Downs et al., 1998). The literature shows that generally low molecular weight (LMW) HSP (approximate molecular weight of 17 kD) are associated with protection of protein structure under heat stress while high molecular weight (HMW) HSP (approximate molecular weight of 70 kD) are associated with repair of the denatured proteins. Evidence for this has been reported in peas (Lee et al., 1995, Lee and Vierling, 2000), *Arabidopsis thaliana* (Forreiter et al., 1997), and wheat (Krishnan et al., 1989, Vierling and Nguyen, 1992, Joshi et al., 1997).

It is suggested that HSP are involved in the development of thermal tolerance in plants (Vierling, 1991). Correlations between HSP synthesis and ATT have been reported for several crops (Howarth and Kirsten, 1994; Schirmer et al., 1994; Joshi et al., 1997). Nguyen et al., (1992) reported that HSP production in the field was strongly correlated with thermal tolerance of the seedlings. Kimpel and Key, (1985) had observed that profiles of HSP messenger RNA accumulated in soybean seedlings

grown in the field were similar to those of the seedlings grown in the glasshouse. Production of LMW-HSP in wheat was strongly correlated to the degree of thermal tolerance of the seedlings (Vierling and Nguyen, 1992). In the same study the authors reported that seedling survival at HSP-inducing temperature was correlated to the ATT. Similar data has been reported for maize and sorghum (Jorgensen et al., 1992, Howarth and Kirsten, 1994). Genetic linkage between ATT and the expression of HSP gene family have also been reported in wheat and maize (Jorgensen and Nguyen, 1995, Joshi et al., 1997). Therefore, analysis of the profiles of HSP may be used to study the genotypic differences in the crop response to heat stress and is relevant to crop improvement. However, no information is available on the HSP of faba bean and information on techniques to study HSP is also completely lacking.

Relatively little is known about the response of faba bean to heat stress, its ability to develop ATT and HSP production. No information is available on the techniques to study ATT and HSP of the crop. Therefore it was decided to conduct experiments to: (1) develop techniques to quantify ATT in faba bean genotypes with a view of developing a screening method, and, (2) develop techniques to radiolabel HSP of faba bean genotypes and to separate them on single dimension gel.

In the field, high temperatures and water stress occur together and their effects cannot be separated, rendering it difficult to study the heat stress in isolation (Burke, 1990; Howarth and Ougham, 1993). Therefore heat stress should be assessed under controlled conditions, which is how the current study was conducted. It is difficult to visualise HSP on gels because they are produced in very minute quantities (Vierling and Nguyen, 1990). However, by labelling them with radioactivity HSPs can be separated on single dimension gels (Martino-Catt et al., 1993), which are

autoradiographed to visualise protein bands on X-Ray film (Jorgensen and Nguyen, 1995). Therefore, in the current study experiments were conducted to develop the techniques of measuring the ATT, and of labelling HSP with radioactivity under controlled conditions. ATT & HSP measurements were made on separate tissues. After standardising the techniques, selected genotypes were evaluated for differences in their ability to acquire tolerance to high temperature and for HSP synthesis.

7.2 Materials and methods

Genotypes were selected based on their seed yields in the breeding trials from across South Australia (Table 7.1). Different sets of genotypes were used in different standardisation experiments based on the availability of seeds and these are named along with the details of individual sets of experiments. Glasshouse experiments were conducted between July 1999 and February 2000. Seeds of uniform size were pre-germinated before sowing. Seven kg of UC soil mixture was potted in pots of 200 mm diameter each (Appendix 5.1). Ten pre-germinated seedlings were planted and six uniform seedlings were retained in each pot. To ensure adequate supply of nutrients to plants, a commercial slow release fertiliser (Osmocote) (Appendix 5.2) was applied at the rate of 100g granules per pot every 30 days of plant growth. There was no toxicity or deficiency of nutrients because of the application of osmocote and the plants were healthy. The growth temperature in the glasshouse was maintained at 22°C/15°C day/night cycle. Plants were maintained under natural daylight conditions. Plants were pre-treated at the 3-4 fully opened leaf stage in an enclosed growth cabinet for both EC and TTC reduction assays. All the experiments consisted of unstressed control and heat shock treatments for each of the genotypes; each treatment replicated four times.

Table 7.1: Details of the genotypes used in the current series of experiments

Genotype	Seed size	Seed weight (g/seed)	Maturity group	Flowering time (Days after sowing)
Fiord	Small	0.48	Early	66-70
Acc524	Small	0.48	Early	68-72
Acc1038	Small	0.77	Early	64-66
Acc165	Small	0.66	Early	63-66
Acc868	Small	0.67	Early	65-69
Acc610	Small	0.71	Early	63-69
Fiesta	Small	0.70	Early	65-69
Acc482	Small	0.49	Medium	71-75
Acc973	Small	0.37	Late	94-100
Acc278	Medium	0.82	Early	65-70
Acc611	Medium	0.98	Early	62-69
Acc527	Medium	0.79	Medium	73-77
Acc722	Medium	0.99	Medium	71-75
Acc974	Medium	0.80	Medium	75-79
Acc770	Medium	0.95	Late	79-82
Icarus	Medium	0.83	Late	96-101
Acc1056	Medium	1.18	Late	90-96
Acc735	Medium	0.97	Late	80-87
Acc779	Large	1.05	Early	57-61
Acc484	Large	1.06	Early	63-67
Acc287	Large	1.24	Early	54-60
Acc649	Large	1.32	Early	62-67
Acc286	Large	1.35	Early	52-56
Acc664	Large	1.47	Early	65-68
Acc979	Large	1.01	Medium	75-79
Acc617	Large	1.15	Medium	71-77
Acc683	Large	1.22	Medium	73-79
Acc820	Large	1.98	Medium	68-73
Acc766	Large	1.52	Late	84-88

7.2.1 Standardisation of pre-treatment and heat shock conditions to estimate ATT

The objective of pre-treatment is to expose plants for sufficient duration to non-lethal higher temperature, generally 10°C above optimum, to enable tolerance to lethal temperatures to develop. It was decided to test 25°C, 30°C, 35°C and 40°C for pre-treatment. Pre-treatment duration of 24 hours was chosen as it has been widely reported for several crops where pot-grown seedlings were tested (Onwueme, 1979, Chen et al., 1982, Porter et al., 1994, Fokar et al., 1998, Marcum, 1998). Plants should be pre-treated under dark and humid conditions to reduce transpirational cooling, which otherwise introduces variability in the data (Porter et al., 1994, Fokar

et al., 1998). In the current study plants were covered with loose plastic bags, which were sealed around the top of the pot to ensure high humidity. This minimises transpirational cooling, which otherwise introduces variability in the data. These plants were pre-treated in an enclosed growth cabinet for 24 hours in dark.

The different pre-treatment conditions were first tested with heat shocking at 50°C for 45 minutes, but this was subsequently reduced to 45°C (see p. 151). A range of high temperatures has been reported for heat shock treatment. For example Chen et al., (1982) heat shocked tomato, potato, soybean and beans at 50°C while Martineau et al., (1979) used 48.5°C to 50°C to heat shock soybean genotypes. There are reports of using 44°C to heat shock wheat (Blum & Ebercon, 1981; Shanahan et al., 1991). The objective of heat shock treatment is to injure the cell membrane of the pre-treated tissue sufficiently so that genotypic differences are identified.

The EC test was chosen to assess ATT because it has been widely used in several crops (Chen et al., 1982; Sapra & Anaele, 1991, Srinivasan et al., 1996; Fokar et al., 1998). A number of experiments have found that EC and whole plant thermal tolerance are correlated (Saadalla et al., 1990b). Significant correlations between EC results and field performance have also been reported (Saadalla et al., 1990b, Howarth et al., 1997, Fokar et al., 1998). There is also evidence that the genetic differences identified by the EC test are consistent across the years in both the field and glasshouse studies (Martineau et al., 1979, Shanahan et al., 1990).

7.2.1.1 Quantifying relative injury by measurement of EC (RI_{EC})

After pre-treatment, 10 leaf discs (0.72 cm^2) were punched from the youngest fully opened leaf, using a sharp core-borer, from each replication. They were collected in

10 ml snap-on capped polypropylene tubes with a drop of nano-pure water (NP). These airtight tubes maintain high humidity. The leaf discs were rinsed gently at least four times with NP water to remove electrolytes adhering to the cut edges. The tubes were capped and incubated in a water bath maintained at the heat shock temperature, for 45 minutes. After the treatment the tubes were removed, 10 ml of NP water added to and incubated at 10°C for 24 hours. A long incubation period allows proper diffusion of electrolytes from the tissue while low incubation temperature prevents tissue deterioration during long incubation (Martineau et al., 1979; Howarth et al., 1997).

After incubation, the tubes were kept at room temperature for 60 minutes to equilibrate with the ambient temperature. Then they were shaken gently by hand and the initial EC of the liquid medium was recorded using an LC84 conductivity meter (TPS Electronics Pty. Ltd., Brisbane, QLD, Australia). Shaking ensures uniform mixing of the electrolytes in the media and minimises measurement errors. The samples were autoclaved in the same fluid medium at 120 kPa pressure for 20 minutes. During autoclaving, the caps were removed and tubes were covered with aluminium foil to prevent boiling over of the fluid. The tubes were allowed to cool to room temperature. This generally took 2 hours. Then the tubes were vigorously shaken by hand and the total EC recorded. RI (RI_{EC}) was calculated using the formula provided by Tahir & Singh, (1993). A higher RI_{EC} indicates higher leakage of cell contents and therefore, a lower ATT.

$$RI_{EC}(\%) = \frac{\text{Initial EC}}{\text{Final EC}} X100$$

7.2.1.2 Quantifying relative injury using TTC reduction (RI_{TTC})

Experiments were conducted to compare TTC reduction assay with the EC test. The TTC reduction represents enzyme functionality of mitochondria (Porter et al, 1994; Fokar et al., 1998). Reports of the heritability of TTC reduction are also available (Porter et al., 1994; Ibrahim and Quick, 1999). However, clear relationship between RI_{EC} and RI measured by TTC (RI_{TTC}) has not been established yet, perhaps because they estimate different aspects of cell response to heat shock. Techniques used for TTC reduction assay were adapted from Porter et al., (1994) and Chen et al., (1982).

After pre-treatment of whole plants, duplicate sets of 10 leaf discs (0.72 cm^2) were punched from the youngest fully opened leaf, using a sharp core-borer, from each replication. They were collected in 10 ml snap-on capped polypropylene tubes with a drop of NP water, as with the RI_{EC} measurement. These airtight tubes maintain high humidity. The leaf discs were rinsed gently once with NP water. The tubes were capped and incubated in a pre-heated water bath maintained at the heat shock temperature for 45 minutes. Control samples were maintained at room temperature (20°C) for the same duration. After the heat shock treatment, 8 ml of TTC solution was added to each tube and the samples were vacuum infiltrated for 10 minutes. Vacuuming improves the infiltration of TTC solution through the cell membrane. Positive and negative vacuum was applied to enhance the infiltration. TTC solution contained 8 mg/ml TTC in 50 mM K_2PO_4 (pH 7.4) and 0.5 ml/l of Tween-20, a surfactant. The surfactant increases infiltration into the leaf tissue for a more uniform saturation of the cells with TTC. After vacuuming, the tissue was incubated in the TTC solution for 24 hrs in the dark at room temperature to allow reduction of TTC to formazan. After 24 hrs, the TTC solution was drained and the leaf discs were rinsed 4 times in NP water. They were incubated in 4 ml of 95% ethanol for 24 hrs in dark

at room temperature. Ethanol extracts formazan. The intensity of red colour was quantitated by reading the optical density of the incubation solution at 530 nm (OD_{530}) in a double beam spectrophotometer. The reference used was 95% ethanol. The incubation solution containing formazan was collected in 1.5 ml cuvettes and the cuvettes were vortexed before reading (10x4x45 mm). RI_{TTC} was expressed as follows:

$$RI_{TTC}(\%) = \frac{OD_{530} \text{ of the heat shocked sample}}{OD_{530} \text{of the control sample}} X100$$

During the initial experimentation on EC and TTC assay the leaf discs appeared to be damaged by heat shock treatment at 50°C at all the pre-treatment temperatures. Hence the heat shock temperature was reduced to 45°C. Subsequently, the data and visual observations showed that this regime of heat shock treatment was reliable and repeatable. Therefore, separate experiments to standardise the conditions for heat shock treatment were not necessary.

7.2.2 Standardising the HSP techniques

Experiments were conducted to measure recovery of total protein from the leaf tissue and to develop techniques to label the HSP. Duplicate sets of ten leaf discs from each replication were obtained in all these experiments, one set was used as the unstressed control while the other set was used as treatment or reference sample, and was subjected to heat stress. Techniques followed for total protein extraction from leaf tissue, protein pelleting and its quantitation were adopted from Lowry et al., (1951) Peterson et al., (1977, 1983) and Collins et al., (1995).

7.2.2.1 Techniques of protein extraction and quantitation

The samples were collected in 4 ml scintillation vials, liquid nitrogen was added and the leaf discs crushed to a fine powder using a microgrinder. 500 μl buffer containing 125 mM Tris-HCl (pH 6.8), 4% sodiumdodecylsulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol was added to the powder (Laemmli, 1970; Collins et al., 1995). The contents of the vial were mixed thoroughly and heated at 100°C for 3 minutes in boiling water. The tubes were then centrifuged at 15800 X g for 20 minutes and the clear supernatant was stored at –20°C. Lipids, SDS and 2-mercaptoethanol should be cleaned from this crude protein extract to avoid their interference with the protein quantitation. The method of Peterson, (1983) was slightly modified for this cleaning. Briefly, 10 μl of sodiumdeoxycholate (DOC) was added to100 μl of protein extract in an eppendorf tube, the mixture vortexed and allowed to stand at room temperature for 10 minutes. To this 10 μl of 72% trichloroacetic acid (TCA) was added and the tubes were centrifuged for 30 minutes at 15800 X g. The supernatant was completely removed from the pellet by pipetting. The protein pellet was dissolved in 100 μl of NaOH (1 N) by heating at 100°C for 5 minutes in a heating block and the volume was made up to 1 ml with NP water. Duplicate samples of 300 μl protein solution were used for protein quantitation using the Folin-Phenol method as described by Peterson, (1977, 1983) a modification of the method developed by Lowry et al., (1951). A standard curve was drawn using known quantities of bovine serum albumin (BSA) as the marker protein. The details of the reagents used in the quantitation are provided in Appendix 7.1.

To determine the extent of total protein extracted by this extraction technique, duplicate sets of ten leaf discs were obtained from each replication. To one set of the two leaf samples 0.1mg (100μg) bovine serum albumin protein (BSA) was added

before total protein (mg/dry weight) was extracted. This was considered as reference sample. BSA was not added to the other set of leaf discs, which was the control sample. Total protein contents of the reference samples were adjusted for the dry weight of control samples. Recovery of the protein was calculated using the formula:

$$\text{Recovery}(\%) = [\frac{Reference - Control}{0.1}] X\, 100$$

This method was chosen for its ease as it fits the criteria of quick method of evaluation. An alternative method is to test the actual recovery of the added BSA, which requires that it be individually separated from the rest of the protein mix. Although currently this would be cumbersome, time consuming and would not be quick, it is acknowledged that the method provides better estimation of protein recovery and should be further explored and simplified for adaptation.

7.2.2.2 Techniques of labelling HSP

Development of the techniques of labelling HSP included testing two methods, by placing leaf discs on filter paper moistened with labelled incubation buffer and by incubating leaf discs in the labelled buffer. The effect of vacuuming in labelling was also evaluated.

Filter paper technique without vacuuming

The method of labelling leaf discs was modified from Collins et al., (1995). Filter paper was placed on the lid of a plastic petri dish (55 x 14 mm). A hole was punched in the base of the petri dish using a hot steel needle. The filter paper was moistened with 500 μl incubation buffer (5 mM MES/KOH, pH 6.8), leaf discs were transferred on to it and the petri dish sealed with parafilm to make it water tight. Leaf samples were obtained from the youngest fully opened leaf from the plants at the early vegetative stage. Samples were either incubated on the bench (control) or heat

shocked in the pre-heated water bath at 45°C for 1 hr, 2hr or 3 hr. Generally the heat shock temperature used to quantify ATT is also used to induce HSP synthesis (Krishnan et al., 1989, Vierling, 1991, Howarth and Kirsten, 1994, Joshi et al., 1997). At the beginning of heat shock, 500 μl incubation buffer containing 20 μCi label was injected on to the leaf discs through the hole on the upper cover of the petri dish. Labelled buffer was added to both the control and treatment samples. Total volume of the incubation buffer was chosen to avoid submerging the leaf discs during treatment. After the heat shock treatment (at 45°C), leaf discs were immediately transferred to 4 ml scintillation vials and frozen in liquid nitrogen to arrest further labelling. Total protein was extracted, pelleted and the pellet dissolved as described earlier (7.2.2.1). Duplicate samples of 200 μl of protein solution were used to estimate total protein content. Triplicate samples of 50 μl of crude protein extract and of protein solution were mixed with 4 ml of ScintiVerse II Universal LS cocktail and incorporation of the radioactivity measured by scintillation.

Filter paper technique with vacuuming

Vacuuming is used extensively to increase incorporation of radioactivity into leaf protein (Krishnan et al., 1989, Vierling and Nguyen, 1990, Jorgensen and Nguyen, 1995). Therefore, leaf samples placed on filter paper were vacuumed prior to heat shocking. Vacuum was applied for 5 minutes, with alternating positive and negative vacuuming during the period to enhance the effect.

Labelling by placing the samples in labeled buffer

The experiments of labelling the samples on filter paper were followed by attempts to label the leaf discs without using filter paper. For this, the leaf discs were collected in 4 ml scintillation vials containing 50 μl of unlabelled buffer. After they were transferred to the laboratory, 100 μl of the labelled buffer was added to the vials. The

total volume of buffer, 150 μl, was sufficient to wet all the leaf discs, did not create any submergence problem and most of it was taken up by the leaf discs after vacuuming. Therefore, it was not necessary to remove excess buffer before beginning the heat shock treatment. These samples were vacuumed prior to treatment. Vacuum was applied for 5 minutes, with alternating positive and negative vacuuming during the period to enhance the effect. The vials were capped and incubated either at room temperature (control samples) or heat shocked at 45°C (treatment samples) in a constant temperature water bath. After the incubation, the vials were frozen in liquid nitrogen to arrest further labelling. Total protein was extracted, pelleted and pellet dissolved as described earlier (7.2.2.1). Duplicate samples of 200 μl of protein solution were used to estimate total protein content. Triplicate samples of 50 μl of crude protein extract and of protein solution were mixed with 4 ml of ScintiVerse II Universal LS cocktail and incorporation of the radioactivity measured by scintillation.

Amount of label used

Amount of radioactivity used for labelling HSP depends upon the stage of labelling, plant tissue and the amount of tissue used. For example Howarth et al., (1997) labelled 2 days old seedlings of pearl-millet and sorghum with 20 μCi radioactivity. On the other hand, Krishnan et al., (1989) used 100 μCi radioactivity to label 25 mg leaf tissue of wheat seedlings at 2 leaf stage. While testing mungbean, Collins et al., (1995) labelled 1 g of hypocotyl sections with 50 μCi radioactivity. Therefore, it was decided to begin the evaluations with 20 μCi of radioactivity. The most common label cited in literature, S^{35}Methionine, was chosen for the experiments (Vierling and Nguyen, 1990, Collins et al., 1995 Joshi et al., 1997).

Protein separation and assessment

Proteins in the crude extract were separated on one-dimensional SDS-polyacrylamide gel (SDS-PAGE), containing 10% resolving gel (29:1 cross linked) overlaid with 5% stacking gel (Sambrook et al., 1989). Crude extracts of control and heat shocked samples of each genotype were separated in adjacent lanes. Equal amounts of radioactivity (10^5 DPM/lane) were loaded in lanes and gels were run with standard protein markers (SeeBlue™ Pre-Stained Standards, Novex, SanDiego, CA, USA). Gels were stained with Coomassie Blue R-250 and dried on Whatman 3 MM filter paper with vacuum and heat (55°C) in a gel drier. Labelled proteins were visualised by autoradiography on Kodak XAR-5 film (not pre-flashed prior to exposure). The films were exposed to gels for 10 days at –70°C. Relative distances of the movement of the bands (Rf value) were used to represent proteins. Rf value was ratio of the distance of the bottom of a protein band from the bottom of the well to the distance of the bottom of the dye front from the bottom of the same well.

7.3 Results

7.3.1 Standardisation of the ATT techniques

In this study, ATT and RI have been used interchangeably although ATT is inversely proportional to RI. This is because essentially, RI is an estimate through which ability of the genotypes to develop thermal tolerance is estimated.

7.3.1.1 Determining the pre-treatment temperature on whole plants

Icarus and Acc 286 were used in the initial experiments to determine the pre-treatment temperature. Leaf samples were heat shocked at 50°C for 45 minutes and

the RI_{EC} was measured. The data was highly variable (CV=30%) (Table 7.2) due largely to the variability in the replication data of 25°C and 40°C pre-treatment temperatures. Generally, ATT development occurs approximately 10°C above the optimum growth temperature (Chen et al., 1982). Average temperature of the growing season in southern Australia during the early part of cropping season is less than 20°C. Therefore, while 40°C may have been too high for pre-treatment 25°C may not be high enough. Hence these two temperatures were not used further. Consequently, 30°C and 35°C were tested next. After achieving low variability in the data using Icarus and Acc 286, 21 selected genotypes were pre-treated at the two temperatures. Leaf samples were heat shocked at 45°C for 45 minutes. The results are presented in Table 7.3.

Table 7.2: Effect of range of pre-treatment temperatures on the RI_{EC} of faba bean genotypes

Pre-treatment temperature	Replication	RI_{EC} (%)	
		Icarus	Acc286
25°C	1	34	19
	2	72	16
	3	70	44
	Average	*59*	*27*
30°C	1	10	19
	2	12	23
	3	16	25
	Average	*13*	*22*
35°C	1	38	12
	2	29	15
	3	29	21
	Average	*32*	*16*
40°C	1	45	42
	2	55	50
	3	66	34
	Average	*55*	*42*

The data contained an acceptable level of variability (CV=11%). There was significant genetic variation in average RI_{EC}, which ranged from 22% to 75%. The two pre-treatment temperatures significantly affected RI_{EC} of the genotypes. Except Acc482 and Acc735, all the genotypes recorded significantly different RI_{EC} under the

Table 7.3: Effect of 30°C and 35°C pre-treatment temperatures on the RI_{EC} (%) of faba bean genotypes

Genotype	RI_{EC} (%) at pre-treatment temperature		
	30°C	35°C	Average
Small seed size			
Early flowering			
Fiord	57	30	44
Acc524	14	69	42
Acc868	52	37	45
Acc610	36	49	43
Medium flowering			
Acc482	41	36	39
Late flowering			
Acc973	47	63	55
Medium seed size			
Early flowering			
Acc278	75	16	46
Acc611	47	59	53
Acc974	15	62	59
Medium flowering			
Acc527	35	18	27
Acc722	23	62	43
Late flowering			
Icarus	32	60	46
Acc735	22	27	25
Large seed size			
Early flowering			
Acc286	63	49	56
Acc287	37	7	22
Acc649	42	23	33
Acc664	14	40	27
Medium flowering			
Acc617	9	38	24
Acc820	47	17	32
Acc979	47	56	52
Late flowering			
Acc766	70	80	75
Average	**39**	**43**	
LSD (p=0.05)			
Genotype		***7***	
Temperature		***2***	
Genotype * Temperature		***9***	
CV (%)		***11***	

two pre-treatment temperatures. Acc278 recorded the highest and Acc617 recorded the lowest RI_{EC} at 30°C, whereas Acc766 recorded the highest and Acc287 the lowest RI_{EC} at 35°C. The RI_{EC} of the genotypes under the two pre-treatment temperatures were not correlated and their rankings were not correlated, indicating that the temperatures affected the genotypes differently. Genotypes showed visual signs of leaf burning at the end of 24 hours of pre-treatment at 35°C but not at 30°C (Appendix 7.2). While some genotypes recorded a reduction of RI_{EC} at 35°C compared to that at 30°C, others recorded an increase. Therefore it was inferred that 35°C may have injured the cells, and hence was not suitable for pre-treatment.

The consistency of the RI_{EC} produced with a 30°C pre-treatment temperature was tested further. Genotypes were chosen on the basis of their RI_{EC} and visual symptoms (Appendix 7.2). The resulting RI_{EC} contained low variability (CV=6%) (Table 7.4). High positive correlation between the RI_{EC} in Tables 7.3 (at 30°C) and 7.4 ($r=+0.86$, $n=5$ $p>0.05$) indicates that the data is consistent across the experiments. Therefore, the work confirms that 30°C is an appropriate pre-treatment temperature.

Table 7.4: Effect of pre-treatment at 30°C on the RI_{EC} (%) of faba bean genotypes

Genotype	RI_{EC} (%)
Fiord	41
Icarus	31
Acc527	33
Acc735	36
Acc766	60
LSD (p=0.05)	*4*
CV (%)	*6*

7.3.1.2 Assessment of pre-treating leaf discs

Experiments were next conducted on pre-treating leaf discs to compare with the results from pre-treating the whole plants (7.3.1.1). Data from testing leaf discs alone

is presented in Table 7.5. The genotypes differed considerably for RI_{EC}, with Acc1056 recording the lowest of 54% and Acc286 and Acc287, the highest of 72%, but were statistically non-significant. This could be attributed to the variability in the results (CV=20%) that makes it difficult to identify smaller genotypic differences. Therefore, using the leaf discs for pre-treatment introduced unacceptably high variability in the data. Also, this RI_{EC} of the genotypes was not correlated with that at 30°C in Table 7.3. Further tests were conducted to confirm this hypothesis, by comparing pre-treatment of leaf discs with the pre-treatment of whole plant. Results are provided in Table 7.6.

Table 7.5: RI_{EC} (%) of the faba bean genotypes estimated by pre-treating the leaf discs

Genotype	RI_{EC} (%)
Fiord	71
Icarus	62
Acc165	60
Acc278	70
Acc286	72
Acc287	72
Acc482	64
Acc484	55
Acc766	71
Acc1056	54
LSD (p=0.05)	*non-significant*
CV (%)	*20%*

Table 7.6: RI_{EC} (%) of the faba bean genotypes estimated by pre-treating the leaf discs and whole plants

Genotype	RI_{EC} (%) by pre-treating	
	Detached leaf discs	Whole plant
Fiord	26	54
Icarus	21	44
Acc527	61	63
Acc735	53	44
Acc766	57	86
LSD (p=0.05)		
Genotype	*6*	
Plant part used	*4*	
The interaction	*8*	
CV (%)	*11*	

The RI_{EC} consisted of acceptable variability. The plant part used for pre-treatment significantly affected RI_{EC} of the genotypes. However the RI_{EC} of the genotypes when whole plants and leaf discs were pre-treated were not correlated, indicating that the methods affected genotypes differently. When this is considered with the high variability of the data in Table 7.5, it may be said that leaf discs were unable to provide appropriate pre-treatment to faba bean genotypes. On the other hand, RI_{EC} of the whole plant pre-treatment in this experiment was highly correlated with that in Table 7.4 and 7.3 (r=+0.86, and r=+0.81 respectively, n=5, p=>0.05). It indicates that pre-treatment of the whole plants generates consistent RI_{EC} across the experiments.

7.3.1.3 Comparison of the EC and TTC reduction assays

The TTC reduction assay (RI_{TTC}) was compared to the EC test, using the same genotypes as in Table 7.6. They were pre-treated at 30°C for 24 hrs in dark. Results are presented in Table 7.7. The method of estimation significantly affected RI of the genotypes. RI_{EC} and RI_{TTC} were highly correlated (r=+0.93, n=5, p=0.05) indicating that both the methods provide similar rankings for RI of the genotypes.

Table 7.7: RI (%) of the faba bean genotypes measured by TTC reduction assay and EC test

Genotype	RI (%) measured by	
	TTC assay	EC test
Fiord	77	54
Icarus	72	44
Acc527	80	63
Acc735	77	44
Acc766	85	86
LSD (p=0.05)		
Genotype	***14***	
Test method	***9***	
The interaction	***non-significant***	
CV (%)	***21***	

Because the data was variable (CV=21%), TTC reduction was tested further to study the total variability it generates in the data. The results are presented in Table 7.8.

The genotypes differ widely for RI_{TTC} with Icarus recording the lowest (17%) and Acc735, the highest (77%). However, the high variability (CV=23%) rendered results non-significant. The RI_{TTC} in Tables 7.7 and 7.8 were not correlated (r=0.46, n=5), indicating inconsistency in the results across experiments. Therefore, TTC reduction assay was not used to quantify RI in the final evaluations of the genotypes.

Table 7.8: RI (%) of the faba bean genotypes measured by TTC reduction assay

Genotype	RI_{TTC} (%)
Fiord	26
Icarus	17
Acc527	37
Acc735	77
Acc766	55
LSD (p=0.05)	*non-significant*
CV (%)	*23*

7.3.2 Evaluation of the genotypic differences for ATT

A protocol was developed to measure RI_{EC} based on the results of the standardisation work (Appendix 7.3). Using the protocol, RI_{EC} of twenty-six faba bean genotypes was measured. The variability in the data was acceptable (CV=11%). Results showed that the genotypes differed significantly for RI_{EC} (Table 7.9). Acc617 recorded the lowest RI_{EC} of 27% and Acc766 the highest RI_{EC} of 77%. RI_{EC} of Fiord (53%) was of midrange. These results were significantly correlated with the RI_{EC} recorded earlier with pre-treatment at 30°C (Table 7.3) (r=+0.60 and r_s=+0.64, n=17, p=0.01), indicating that the data was consistent. Generally, the seed size and flowering time of the genotypes did not contribute to the differences for the RI_{EC}.

RI_{EC} of the genotypes were compared with their field performance in the breeding trials in the low yielding sites (<1 t/ha average site yield) in southern Australia

Table 7.9: ATT – final evaluation EC test

Genotype	RI_{EC} (%)
Small seed size	
Early flowering	
Fiord	53
Fiesta	46
Acc165	51
Acc524	27
Acc610	45
Acc1038	25
Medium flowering	
Acc482	47
Late flowering	
Acc973	57
Medium seed size	
Early flowering	
Acc278	68
Acc611	30
Medium flowering	
Acc527	29
Acc722	42
Acc974	32
Late flowering	
Icarus	41
Acc770	76
Acc1056	57
Large seed size	
Early flowering	
Acc286	56
Acc287	45
Acc484	62
Acc664	70
Acc779	73
Medium flowering	
Acc617	23
Acc683	49
Acc820	54
Acc979	67
Late flowering	
Acc766	77
LSD (p=0.05)	*8*
CV (%)	*11*

(Table 7.10), to assess its relevance to the field condition. These sites were those most prone to high temperature stress. The comparison revealed that RI_{EC} was not correlated with the seed yields of the genotypes in the field. However, it was significantly correlated with the responsiveness of the genotypes to the environment,

represented by the coefficient of regression (slope) of the seed yields (r=+0.62, n=12, p<0.05). Therefore, cell membrane integrity of the genotypes estimated in this study was correlated with the stability of their seed yield in the low yielding production environment of the region.

Table 7.10: Analysis of the yield data of selected faba bean genotypes grown in low yielding (<1 t/ha) locations and from 1995-1997 in the southern Australian region

Genotype	Slope	Y-intercept	Mean seed yield (kg/ha)		Flowering time	Seed size
			Genotype	Site		
Fiord	1.05	27.9	632	575	Early	Small
Fiesta	0.59	278.4	565	488	Medium	Small
Acc165	1.01	170.3	697	518	Medium	Small
Acc524	0.73	239.6	610	505	Early	Small
Acc482	1.04	140.0	788	624	Medium	Small
Acc278	1.00	30.7	613	582	Early	Medium
Acc611	0.53	354.2	614	488	Early	Medium
Acc974	1.40	-220.2	647	624	Medium	Medium
Icarus	0.81	-14.4	451	575	Late	Medium
Acc286	1.17	-135.6	386	447	Early	Large
Acc617	0.39	146.2	336	483	Medium	Large
Acc979	1.50	-168.7	589	510	Medium	Large

7.3.3 Standardisation of the HSP techniques

7.3.3.1 Protein recovery

This work began with achieving high recovery of the total protein content with the extraction technique described in section 7.2.2. Several experiments were conducted using genotype Acc527, where total protein of the leaf discs was extracted and the extent of recovery calculated. A typical replication data presented in Table 7.11 contained low variability (CV=2%). It shows that high percentage recovery of total protein was achieved (average 86%) and confirms that the method was reliable and consistent.

Table 7.11: Extent of recovery of the total protein content of leaves provided by the method of extraction being used

Sample	Tissue dry wt (mg)	Total protein Extracted (mg)	Protein content (adjusted for dry weight)	Recovery (%)
Control	4.6	1.66	1.66	92
Reference	4.2	1.60	1.75	
Control	4.3	1.46	1.46	86
Reference	3.7	1.33	1.55	
Control	3.8	2.14	2.14	77
Reference	2.4	1.40	2.22	
Control	2.8	1.59	1.59	90
Reference	2.5	1.44	1.68	

7.3.3.2 Standardising techniques of labelling HSP

Filter paper technique

First, the incorporation of radiolabel in leaf discs placed on filter paper was tested. The incorporation of radioactivity was very low (Table 7.12). This was confirmed by autoradiography where no signal could be detected on X-Ray films. It was inferred that the leaf discs were unable to take up labelled buffer. Therefore, in the subsequent experiments samples were vacuumed to increase the intake of labelled buffer. Again radioactivity supplied was 20 μCi. However, there was no increase in the incorporation of label in the total protein (Table 7.13). Autoradiography confirmed this. It should be noted that Collins et al., (1995) used this method to label hypocotyls of mung beans (*Vigna radiata* L.) in their experiments. Therefore, it was concluded that the method is not suitable for labelling leaf discs of faba bean.

Labelling by placing the samples in labeled buffer

Labelling the leaf discs by placing them in labelled buffer was then tested using Acc683 and Acc766. Each sample was supplied with 20 μCi of the label. The

Table 7.12: Labelling the total protein with 20 μCi S^{35}Methionine by placing leaf discs on filter paper without using vacuum

Heat shock and Labelling duration	Treatment	DPMs in protein solution from 10 μl of crude extract
1 hr	Control	1104
	Heat shock	909
2 hr	Control	1139
	Heat shock	1400
3 hr	Control	1315
	Heat shock	1336
DPM – Disintegrations per minute		

Table 7.13: Labelling the total protein with 20 μCi S^{35}Methionine by placing leaf discs on filter paper using vacuum

Heat shock and Labelling duration	Treatment	DPMs in protein solution from 10 μl crude extract
1 hr	Control	810
	Heat shock	693
2 hr	Control	850
	Heat shock	811
3 hr	Control	873
	Heat shock	840
DPM – Disintegrations per minute		

Table 7.14: Labelling the total protein with 20 μCi S^{35}Methionine by placing leaf discs in 4 ml scintillation vial using vacuum

Heat shock and Labelling duration	Treatment	DPMs in protein solution from 10 μl crude extract	
		Acc683	Acc766
1 hr	Control	69624	38771
	Heat shock	39228	29428
2 hr	Control	95942	54078
	Heat shock	62696	48217
3 hr	Control	106759	31754
	Heat shock	78879	45224
DPM – Disintegrations per minute			

Table 7.15: Labelling the total protein with 2 μCi S^{35}Methionine by placing leaf discs in 4 ml scintillation vial using vacuum

Heat shock and Labelling duration	Treatment	DPMs in protein solution from 10 μl crude extract
1 hr	Control	1841
	Heat shock	2256
2 hr	Control	2755
	Heat shock	3285
3 hr	Control	4772
	Heat shock	3678
DPM – Disintegrations per minute		

samples in the scintillation vials were vacuumed and then heat shocked. The results showed that this method achieved higher level of incorporation of radioactivity in total protein (Table 7.14). The data also showed that heat shock treatment affected the incorporation of radioactivity into total protein content of faba bean genotypes.

Amount of label used

Because of the high cost associated with using 20 μCi of label per sample for a large number of samples, it was decided to test the feasibility of using a lower amount of radioactivity. Therefore, an experiment was conducted where Acc766 was labelled with 2 μCi of radioactivity. The results are presented in Table 7.15. The data showed that incorporation of label in total protein was reasonable even with 2 μCi of radioactivity. Therefore, it was inferred that to evaluate the HSP synthesis of the faba bean genotypes, labelling with 10 μCi radioactivity might be adequate. It was also decided that two hours of labelling provided adequate incorporation of radioactivity in the total protein based on autoradiographs (Plate 7.1).

7.3.4 HSP synthesis by the faba bean genotypes

On the basis of evaluation of the genotypes for ATT, ten genotypes representing the entire range of RI_{EC} were selected for studying the HSP synthesis. The genotypes were: Fiord, Fiesta, Icarus, Acc287, Acc524, Acc527, Acc617, Acc766, Acc973 and Acc974. The protocol developed to radiolabel (Appendix 7.4) and separate the HSP on single dimension SDS-PAGE based on the standardisation work was used in this experiment. Rf values of different protein bands averaged from 3 gels are presented in Tables 7.16 and 7.17. The Rf data of the molecular markers is also presented in Table 7.16. This provides a perspective of the molecular weight of the HSP synthesised in this study. It shows that low molecular weight HSP family (17 kD, Rf

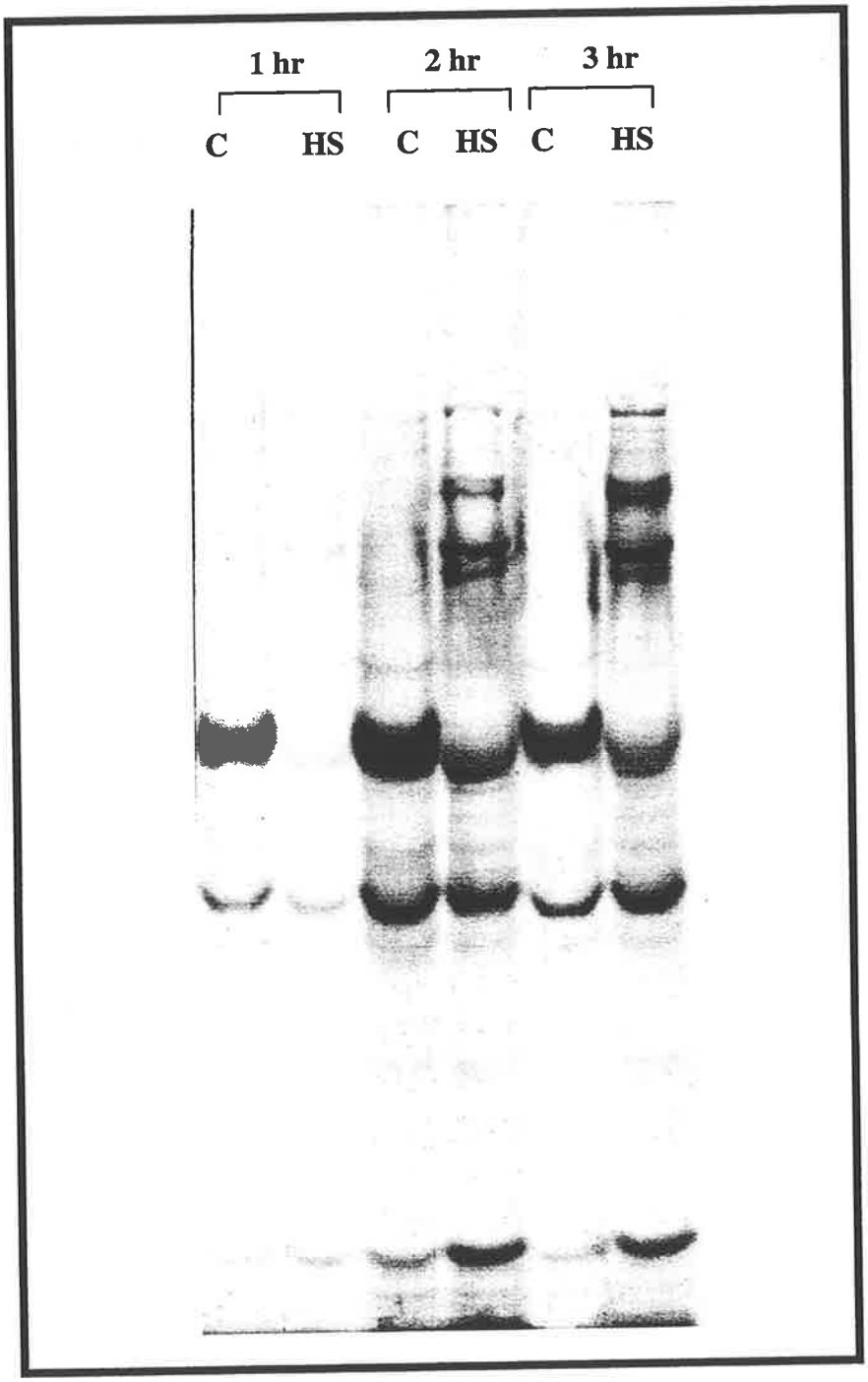

Plate 7.1: Effect of different durations of heat shock on the incorporation of radio-activity into faba bean HSPs

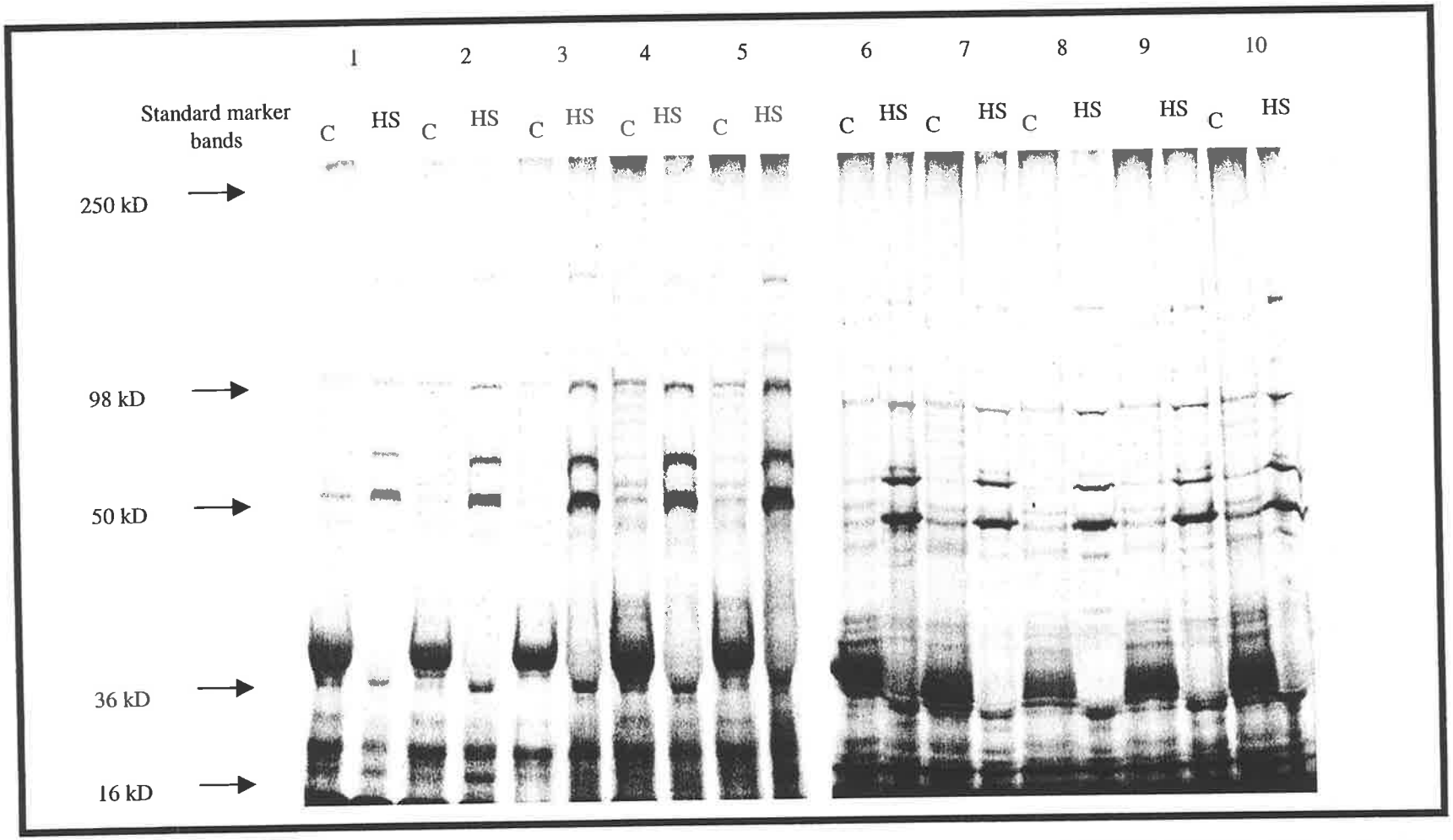

Plate 7.2: Autoradiograph of labelled HSPs of selected faba bean genotypes on single dimension SDS-gel (1–Fiord; 2-Icarus; 3-Fiesta; 4-Acc287; 5-Acc527; 6-Acc617; 7-Acc524; 8-Acc766; 9-Acc973; 10-Acc974; C – Control; HS – Heat shock)

Table 7.16: Pattern of HSP synthesis compared to unstressed control by the selected faba bean genotypes under heat stress, and Rf value of the molecular markers

Genotype	RI% (EC)	Rf value of the protein bands											
		0.984	0.935	0.822	0.693	0.669	0.535	0.520	0.421	0.386	0.299	0.181	0.157
Acc617	23	X	X	X	–	X	X	X	X	X	X	X	X
Acc524	27	X	X	X	–	X	X	X	X	X	X	X	X
Acc527	29	X	X	X	X	X	X	X	–	X	X	X	X
Acc974	32	X	X	X	–	X	X	X	X	X	X	X	X
Icarus	41	X	X	X	X	X	X	X	–	X	X	X	X
Acc287	45	X	X	X	–	X	X	X	–	X	X	X	X
Fiesta	46	X	X	X	–	X	X	X	–	X	X	X	X
Fiord	53	X	X	X	X	X	X	X	–	X	X	X	X
Acc973	57	X	X	X	–	X	X	X	X	X	X	X	X
Acc766	77	X	X	X	X	X	X	X	X	X	X	X	X
HSP (kD)		~17		~36					~70				

"X" HSP produced

"–" HSP not produced

Marker size (kD)	Rf value
250	0.024
98	0.403
50	0.612
36	0.801
16	0.985

Table 7.17: Pattern of suppression of protein synthesis compared to unstressed control by the selected faba bean genotypes under heat stress

Genotype	RI% (EC)	Rf value of the protein bands											
		0.916	0.785	0.756	0.724	0.505	0.419	0.417	0.409	0.383	0.364	0.224	0.168
Acc617	23	√	√	√	√	√	–	√	√	–	–	–	–
Acc524	27	√	√	√	√	√	–	√	√	–	–	–	–
Acc527	29	√	√	√	√	√	√	√	√	√	√	√	√
Acc974	32	√	√	√	√	√	–	√	√	–	–	–	–
Icarus	41	√	√	√	√	√	√	√	√	√	√	√	√
Acc287	45	√	√	√	√	√	√	√	√	√	√	√	√
Fiesta	46	√	√	√	√	√	√	√	√	√	√	√	√
Fiord	53	√	√	√	√	√	√	√	√	√	√	√	√
Acc973	57	√	√	√	√	√	–	√	√	–	–	–	–
Acc766	77	√	√	√	√	√	–	√	√	–	–	–	–

"√" ***Protein suppressed under heat stress***

"–" ***Protein not suppressed under heat stress***

value ~0.984), which is related to protective role under heat stress, was separated only as a single band (Plate 7.2). The high molecular weight HSP family (70 kD, Rf value ~0.421), related to repairing of the proteins damaged by heat stress, was separated relatively better. The data showed that heat shock induced production of several HSP in the genotypes relative to control treatment, and that it also inhibited production of some proteins. However, the pattern appeared to be common to most of the genotypes. Although the genotypes differed for the production of two HSP (Rf value 0.693 and 0.421), the differences were not correlated to their RI_{EC}. A similar trend was observed for the proteins suppressed by the heat shock (Rf value 0.419, 0.383, 0.364, 0.224 and 0.168). Therefore, the protein synthesis of the faba bean under heat stress may not be related to its ability to acquire thermal tolerance.

7.4 Discussion

Crop improvement for the Mediterranean climate of southern Australia requires development of genotypes capable of yielding well under both drought and heat stresses. To accomplish this, it is necessary to identify genotypes having traits of tolerance to the stresses and utilising those traits in breeding programs. Two traits often associated with tolerance to heat stress are ATT and HSP. This is the first study of ATT and HSP of faba bean. Major emphasis was laid on developing techniques to quantify ATT and to radiolabel HSP of faba bean. Because of the short period of time available for this study, the results of the evaluations could not be compared with plant growth responses under controlled conditions. In a number of trials over a range of crops ATT has been correlated with growth and grain yield under high temperature stress in the field and glasshouse trials. Therefore, it is valid to examine the ATT even without post-stress data on the whole plant growth and yield.

Low variability and high consistency of the RI_{EC} of the genotypes pre-treated at 30°C across the experiments and absence of signs of leaf burning indicated that it was an appropriate pre-treatment. Although pre-treating at 35°C provided significant RI_{EC} that contained low variability, evidence of leaf burning showed that it was too high for pre-treatment. Therefore, 30°C was chosen to be the pre-treatment temperature. The samples were heat shocked at 45°C for 45 minutes. It is pertinent to note that, while no prior-information is available on the faba bean, pre-treatment temperature depends upon the crop, the growth stage and production environment. For example, Chen et al., (1982) pre-treated tomato and potato at 35°C while Li et al., (1991) pre-treated common bean at 37°C. Wheat was pre-treated at 34°C by Fokar et al., (1998). Saadalla et al., (1990a) pre-treated 10-14 day old spring wheat seedlings at 34°C while Porter et al., (1994, 1995) pre-treated the 7 days old seedlings of the same crop at 37°C. Howarth et al., (1997) pre-treated pearl millet seedlings at 43°C because they were targeting their evaluation to the semiarid tropical environment, where soil temperature during the emergence of the seedlings is very high.

Pre-treating the whole plants consistently provided RI_{EC} containing low variability. Lack of correlation between the RI_{EC} of pre-treated leaf discs and of pre-treated whole plant suggests the two methods affect the ATT differently. However, pre-treating the leaf discs introduced high variability in RI_{EC}. As a result, this method cannot identify small differences between the genotypes for RI_{EC}. Therefore it was concluded that whole plants should be pre-treated. The reason for testing leaf discs was that it uses fewer resources and provides a method by which to pre-treat the field grown plants. A possibility for the use of detached leaves for pre-treatment has been reported in common bean (Li et al., 1991). However, in that study they pre-treated trifoliate leaves kept in water. Therefore, more testing needs before detached leaves

can be used for pre-treatment in faba bean.

Selected genotypes were evaluated for RI_{EC} using the protocol developed. The data was compared with the seed yield of the genotypes in the low yielding sites from the breeding trials in southern Australia. This revealed that while the two were not correlated, RI_{EC} and the stability of the genotypes in the field, (as represented by the slope of the regression between the average yields of genotypes and the sites) were significantly correlated. Hence it may be inferred that the cell membrane integrity of the faba bean under high temperature stress is related to its yield stability in the low yielding sites of southern Australia. It should be mentioned that the breeding trials were not specifically targeted for heat stress evaluation. ATT estimated in the current study is a measure of the cell membrane integrity under heat stress. The seed yield of faba bean in the field may be more related to the ability of the cells to maintain functionality than the membrane integrity under heat stress. Cell functionality can be estimated by TTC reduction assay. However, owing to the high variability in the RI_{TTC} during standardisation it was not used in the final evaluation.

It is more appropriate to compare ATT with the post-stress data on the growth and seed yield of the genotypes under controlled conditions, as well as with the seed yield from the field trials specifically targeted for heat stress. Because of the shortage of time, the current study does not have these data. Therefore, it is not possible to interpret the lack of correlation between RI_{EC} and the seed yields of the genotypes in the breeding trials.

The method of protein extraction was tested for consistency and high level of extraction before using in the study. The standardisation work of HSP concentrated

on developing a technique to label them with radioactivity, this being the first such work in faba bean. Two methods were tested – placing leaf samples on a filter paper moistened with labelled incubation buffer and placing them directly in labelled incubation buffer. The label used was S^{35}Methionine. Data showed that adequate incorporation of radioactivity in protein was achieved by placing the leaf samples directly in 150 μl of incubation buffer containing 10 μCi of S^{35}Methionine and with the use of vacuuming. Label was introduced at the beginning of the heat shock treatment and hence heat shock and labelling were of same duration. This condition was chosen for the ease of handling radioactivity. Alternatives were deliberately not tested. The data on incorporation of radioactivity and the autoradiograph suggested two hours as the appropriate labelling duration (Figure 7.1).

Analysis of genotypes that covered the full range of RI_{EC} revealed that the pattern of protein synthesis under heat stress was generally common to all the genotypes. This is contrary to the reports on some other crops. For example, Blumenthal et al., (1990) reported that wheat genotypes differed for HSP production, and that this difference was related to the differences in their thermal tolerance (measured as coleoptile growth). Krishnan et al., (1989) reported that wheat genotypes significantly differed for low molecular weight HSP (16, 17 and 26 kD) and this difference was correlated with ATT. Similar results have been reported for sorghum (Howarth and Kirsten, 1994). Therefore, lack of genotypic differences in faba bean for HSP synthesis may be the reason for the protein pattern not being correlated to the RI_{EC} of the genotypes in the breeding trials. It should be noted that in the current study, HSP were separated on 10% separating gel. Better separation can be achieved by the use of different concentrations of separating gels and by using gradient gels. It was not attempted in this evaluation because of lack of time. Without complete separation of all the HSP,

it is not possible to conclude on the lack of correlation of the pattern of protein synthesis under heat stress with RI_{EC}.

7.5 Summary

ATT and HSP are two traits associated with tolerance of plants to heat stress and are useful in crop improvement programs. This is the first study to develop techniques to quantify ATT and to radiolabel the HSP of faba bean. The aim was to provide a base for efforts to improving the performance of faba bean in the Mediterranean climate of southern Australia.

Electric conductivity, a measure of cell membrane integrity under heat stress, was chosen to quantify ATT. Techniques of measuring ATT of faba bean involved standardising the pre-treatment conditions, heat shock treatment and achieving consistent RI_{EC} that contained low variability. ATT is inversely proportional to RI_{EC}. The protocol is described in Appendix 7.3.

ATT of the faba bean genotypes assessed by this technique was significantly correlated with the stability of the yields but not with their seed yield in the breeding trials. This suggests the integrity of cell membranes under heat stress is important for maintaining yield levels of faba bean in the field and provides evidence for the applicability the current data to the field conditions. It is very difficult to assess the genotypic response to heat stress in the field. This difficulty warrants a comparison of ATT with the post-stress performance for dry matter accumulation and seed yield of the genotypes after pre-treatment to validate the applicability of RI_{EC} as a measure of ATT to the field performance. Because of the limited time available for the study, these data were not colleted. However, this protocol can form the basis for further work to compare the ATT of faba bean with the post-stress performance in the

controlled conditions. Field trials specifically targeted to assess the response to heat stress may be combined with the glasshouse evaluation of ATT using this protocol to further validate the trait for field performance. The protocol may also be used to measure ATT of the field grown plants. One potential method for this purpose would be to test using the detached leaf, kept in water, for pre-treatment. This might obviate the difficulty of pre-treating field grown plants and also avoid the variability in the data introduced by pre-treating the leaf discs. Therefore, the current protocol can be very much useful in the crop improvement program of faba bean targeted to high temperature regions.

The work on HSP consisted of radiolabelling the total protein and to separate it on single dimension SDS-PAGE. The objective was to develop a system of identifying unique pattern of protein synthesis under heat shock on a gel. Such ability is required to identify markers and use them to screen a large number of genotypes very quickly. Current work was intended to provide a base for this approach for crop improvement. The protocol is described in Appendix 7.4.

The pattern of protein synthesis of the genotypes observed in the present evaluation was not correlated with their ATT and seed yield. Any inference of this outcome is limited by the method of separation of HSP followed in the current study. Efficient separation using a combination of gradient gels, separating gels with different concentrations and fractionation of total protein prior to separating on gels would yield more useful data for a definitive inference. As mentioned elsewhere, the current study was limited to developing techniques to label the HSP of faba bean with radioactivity so that it can be separated on single dimension SDS gel because of limited time available. However, the protocol developed by this study allows for

efficient labelling of faba bean HSP. Therefore, it can be used to further study the pattern of protein synthesis of faba bean under heat stress and to elucidate the genotypic differences for HSP. Such a study forms the basis to determine the role of HSP in development of thermal tolerance in faba bean and in better seed yield under field conditions. The protocol can also be used to study the inheritance of HSP pattern and thereby be useful in breeding improvement of the crop targeted to high temperature cropping environment of southern Australia.

Chapter 8

General Discussion

In Australia, faba bean is grown in the Mediterranean climate of southern and western cropping zones (Perry, 1994). Faba bean is generally not irrigated, and is likely to experience drought and high temperatures during the reproductive stage in most years (French, 1981). Its average yields in Australia are only 1.3 t/ha (Pulse Australia, 2000). The high variability of its seed yield and low average yields are attributed to variations in rainfall (Knight, 1994). Hence it is necessary to improve the faba bean yields under the terminal drought and heat stresses of the region.

Improving crop yield under stress involves combining tolerance to stress with high yield potential. The first step in this programme is to identify suitable traits and to examine the level of genetic variability for most of those traits. However, such work in faba bean is scarce. Although there have been a number of studies on the faba bean response to water and heat stresses, these have used only a limited number of genotypes. There has been little work to examine the usefulness of physiological traits in a diverse set of genotypes. The current study was designed to address this gap. The major aims were to develop protocols to screen the faba bean genotypes for responses to drought and heat stresses, primarily using physiological and growth parameters. The potential of using heat shock protein production under heat stress was examined. This discussion will specifically address the work on developing protocols to screen the faba bean response to water and heat stresses. Limitations of the current study will also be addressed where pertinent. A brief account of the future line of work arising out of the current project will also be provided.

8.1 Developing screening protocol for drought response

The work began by assessing the extent of genotypic variations in faba bean for growth and seed yield under rainfed condition in the field in 1995. The genotypes chosen represented the wide variations in the seed size and flowering time present in faba bean. Following this, the techniques of measuring the physiological and growth responses of the genotypes to water stress were standardised. Selected genotypes were then assessed for response to water stress in a glasshouse study. A field experiment was also conducted simultaneously in this regard in 1998. The results from glasshouse study were compared with the seed yields of the genotypes from the breeding trials conducted across southern Australia by the faba bean breeding program, located at the Waite Institute. A brief account of the whole project follows.

8.1.1 Assessing the genotypic variation for growth and seed yield under rainfed condition in the field

The field evaluation of 1995 (Chapter 4) identified the reproductive stage of faba bean as being the most susceptible to water stress. This was revealed by large reduction in TDM, and CGR of all the genotypes under rainfed conditions at this stage, and also from the reductions in pod number and seed yield. The reductions in the rate of photosynthesis, stem dry matter accumulation and growth rate of faba bean under water stress contribute to the loss of dry matter (Green et al., 1986, Grzesiak et al., 1989). Water stress is known to reduce leaf size, increase leaf senescence and thereby reducing longevity of the canopy (Farah, 1981, Finch-Savage and Elston, 1982, Karamanos et al., 1982).

The correlation of the TDM at 116 DAS with the seed yield under rainfed condition in this study confirms the reports of dry matter production significantly affecting the

seed yield of faba bean (Siddique et al., 1993). Therefore, this correlation indicated that an ability to produce and maintain dry matter under water stress at the podding stage contributes to better yield performance of the genotypes in the field. Hence it is possible to use sensitivity of TDM to water stress as an indicator of the seed yield performance. In this regard, it should be noted that although large seeded genotypes recorded higher TDM and CGR early in the growth under rainfed condition, this did not result in higher seed yields and or sensitivity to drought. Similarly, the flowering time did not influence the TDM accumulation in the field.

The data on pod production from the field experiment showed that water stress continued to affect pod retention late into the reproductive growth of faba bean. The literature also shows that faba bean seed yield is strongly dependent upon the number of pods produced (Katiyar and Singh, 1990, Silim and Saxena, 1992, Agung, 1995). Further, the data showed that while seed size affected the number of pods and seeds produced by the genotypes, the flowering time had no major influence on them. However, flowering time affected the reduction of pod number of the genotypes under rainfed conditions. Early flowering time allowed lower reduction of pod numbers of small seeded genotypes and for large seeded genotypes late flowering time provided this benefit. Hence seed size and flowering time can influence the reduction in the number of seeds produced by faba bean. Several reports show that reduction in seed yield of faba bean is associated with reduction in the number of seeds produced (Farah, 1981, Adisarwanto, 1988, Stutzel and Aufhammer, 1992). The high pod abscission under water stress is reported to increase the reduction in seed number (Grasshoff, 1990a). Therefore, the results of the 1995 field trial show that an opportunity may exist to exploit the differences in the seed size and flowering time of the faba bean to obtain higher pod retention and more seeds under rainfed

conditions. This is important considering that the data also revealed the existence of genotypic differences for seed yield under rainfed condition in faba bean.

8.1.2 Standardising the techniques of measuring different growth and physiological responses of the genotypes to water stress

Based on the results of the field experiment of 1995, it was decided to study the response of faba bean genotypes, representing a large range of flowering time and seed size, to water stress. The reproductive stage was chosen to study the genotypic responses to drought because of the importance of this phase to yield and to compare the responses at any other growth stage with it.

Apart from the TDM and specific leaf area (SLA), the stomatal conductance, leaf water potential, osmotic potential and RWC were chosen to evaluate the response of faba bean to water stress. Faba bean is generally reported to lack the osmotic adjustment mechanism (Grasshoff and Verkerke, 1991, Agung, 1995). However, most of the reports on faba bean have studied only a restricted number of genotypes. Considering the extent of variation in seed size and flowering time, it was decided to evaluate the genotypic differences of faba bean for osmotic potential and RWC in this study.

An appropriate growth stage has to be identified at which to reliably evaluate the genotypic response to drought. The Roseworthy field experiment (1995) showed that reproductive stage is sensitive to drought, which is supported in the literature as well (Pilbeam et al., 1990b, Xia, 1994). However, there are also reports of vegetative stage being more sensitive than the seed set and seed growth stages (Plies-Balzer, 1995). For a breeding program to be time and cost efficient, the stage of screening

should be early in the growth. Hence early vegetative stage would be ideal and it was decided to evaluate the genotypes at both the vegetative and reproductive stages. Experiments were conducted under controlled conditions to standardise the methodology of imposing water stress and of measuring different physiological responses (Chapter 5).

Water stress was imposed gradually by controlled reduction of the soil moisture content. This was achieved by reducing the amount of water supplied to the stress treatment pots so that the total pot weight decreased by 10% per day of the original weight recorded at the start of stressing. The plants were stressed at this level of soil moisture for 4 days. This method was followed to simulate gradual development of stress as it occurs in the field. The method creates highly contrasting soil water regimes and biological effects. It took 9-14 days to reach 50% field capacity moisture level in different experiments, depending upon the growth stage and therefore, the total length of the stress period was approximately 13-18 days. The methodology had created sufficient level of stress consistently as was evident by its significant effect on various physiological and growth characters at both the early vegetative and early podding stages and on seed yield, seed number, pod yield and pod number of the genotypes (Chapter 5).

The techniques of measuring stomatal conductance, water potential and RWC for faba bean were optimised. The trials described in Chapter 5 showed that high sensitivity of stomatal conductance, RWC and water potential to water stress make the data inherently variable. This was overcome by increasing the number of replications per treatment pot and/or by increasing the size of the sample. For example, the initial conductance data was based on single observation per treatment

and contained high CV%. By increasing the number of observations per treatment to 4, the variability was brought down to acceptable level (CV<10%). The data indicated that only a limited number of samples could be tested per day for RWC and water potential. When all the replications were harvested on a single day, the data of these parameters contained high CV%. This was attributed to the time lag between harvesting the leaf disc and recording their fresh weight for RWC, and to the time lag between recording the water potential of different samples. That all the observations had to be recorded within 2 hrs (between 12noon and 2pm) also put a constraint on the number samples that could be tested for all the parameters. This was resolved by limiting the number of samples tested each day to single replication. The glasshouse trials (Chapter 5) showed that, on an average a total of 15 to 20 samples (from all the genotypes) could be tested by a single person for water potential, RWC and stomatal conductance in a day within 2 hrs.

Although leaf area was recorded initially, SLA was preferred because it integrates both leaf area and leaf weight under stress. Method of estimating the SLA with a sub-sample of leaves was time efficient and more practical than recording the leaf area of the whole plants. Initial SLA data contained large variability, which was controlled by increasing the size of sub-sample to 30 leaflets. The trials also showed that TDM data could contain high variability across the experiments irrespective of the number of plants used for estimation. Such variability may be attributable to the partial cross-pollinated nature of faba bean, whereby individual plants of a genotype derived from a single seed lot may still be variable in size (Duc, 1997). This problem may be partially managed by sowing seeds of similar size from a single seed lot.

Data on the severe reduction of seed yield of the genotypes when stressed at 6-7 leaf

stage suggested that this may not be appropriate as vegetative stage for all the genotypes. The early flowering genotypes such as Acc286 and Fiord may initiate flowering by this time while late flowering genotypes such as Icarus and Acc973 may still be in the vegetative phase. It should also be noted that the stress treatment continues for up to two weeks. As a result, although genotypes may be at vegetative stage at the start of stressing, some may initiate flowering before the end of the treatment. Therefore, to ensure that the genotypes of different flowering times are all at the vegetative stage during the entire stress treatment period, it was decided to commence stressing when 3-4 fully opened leaves appeared on the main stem. Stressing at the early podding stage was used to compare the responses at the two stages, so that an appropriate stage for screening may be determined.

8.1.3 Evaluation of the growth and physiological characters for screening faba bean response to water stress

Stomatal conductance, leaf water potential, osmotic potential, RWC, SLA and TDM were evaluated as possible criteria to screen for drought tolerance. The data showed that faba bean genotypes differed significantly for all the physiological response to water stress (Chapter 6). However, they were not useful as traits to screen the genotypes for crop improvement under water stress, the details of which are discussed below.

It appeared that faba bean maintained normal stomatal conductance even when plant water stress developed, and closed stomata when the stress reached a threshold. This resulted in a sudden decline of the conductance. This has been reported elsewhere (Kassam, 1973, Agung, 1995). Stomatal conductance did not influence the yield or the ability of the genotypes to maintain yield under stress. Therefore, it can be said

that conductance cannot be used as a screening trait for crop improvement under drought. The relationship between the stomatal conductance and water potential at the two growth stages in this evaluation was similar to the ones reported from the field trials from Western Australia, which also has a Mediterranean climate (Leport et al., 1998). It supports the applicability of the results of the glasshouse study to the field situation.

While genotypes differed for leaf water potential and osmotic potential in the glasshouse evaluation, in the field trial in Roseworthy (1998) there was no significant difference among the genotypes (Chapter 6). The two parameters were not correlated to the seed yield or the ability of the genotypes to maintain it under stress in both the glasshouse and field evaluations. Also, osmotic potential did not influence dry matter accumulation in both the studies. It should be noted that the levels of osmotic potential of the genotypes recorded in the glasshouse study were similar to those reported in the field trials by Leport et al., (1998), validating the usefulness of the current data to the field conditions. However, the results of the evaluations, including the preparatory experiment (Table 5.2), suggest that the faba bean genotypes respond similarly to water stress and that faba bean lacks osmotic adjustment mechanism to cope with stress. This confirms earleir reports for faba bean which used a smaller number of genotypes (Grashoff and Verkerke, 1991, Agung, 1995). This contrasts with other legumes such as chickpea and lupin that possess a significant level of osmotic adjustment (Turner, et al. 1987, Morgan, et al., 1991). On the other hand, it was reported that soybean cultivars did not possess osmotic adjustment under water stress (Turner, et al., 1978). Clearly, the importance of osmotic adjustment in the drought tolerance varies among the legumes. Osmotic adjustment does not appear to contribute to the adaptability of faba bean to the southern Australian region and

hence osmotic potential may not be used as a screening tool to identify drought tolerance of faba bean genotypes.

The significant correlation of water potential and RWC under stress at the early reproductive stage with the seed yield, seed number, pod yield and pod number in the glasshouse experiment only indicate the susceptibility of the growth stage to water stress. The relationship between RWC and water potential under water stress at this stage suggests that faba bean is unable to respond to the developing stress and to conserve plant water. This response is similar to that of stomatal conductance observed in this evaluation and suggested elsewhere (Agung, 1995). These results provide an insight into the poor adaptability of faba bean to the southern Australian climate. The large variations in yields could be mainly attributed to the inability of the genotypes to conserve water by responding to the developing plant water deficit early. Although Nerkar et al., (1981) reported that stomatal closure under water stress may reduce water use and help faba bean to conserve moisture, the evidence to the contrary is substantial in literature. Therefore, water potential and RWC cannot be used as selection traits to improve crop performance in the region.

Ranking of the genotypes for SLA under water stress was inconsistent between the growth stages. Also, it was not correlated with the seed yield, seed number, pod yield and pod number or the ability of the genotypes to maintain any of these under water stress. Hence SLA is not a suitable trait of drought tolerance in faba bean.

Although TDM at both the early vegetative and early podding stages was not correlated with the seed yield of the genotypes, there was consistency in its response between the two growth stages. This was evident from the similar rankings of the

genotypes for TDM under stress at these growth stages. Important result in this regard is that the relative reduction of the TDM under water stress at the early vegetative stage was correlated to the relative reductions of the pod number and seed yield of the genotypes at the early podding stage. The data from the field evaluations of 1995 and 1998 indicated that high yield of faba bean genotypes under rainfed conditions was associated with the number of pods and seeds they set, and the ability to maintain pod weight under increasing water stress. The strong influence of pod number and seed number of faba bean on its yield is well documented (Adisrwanto, 1988, Katiyar and Singh, 1990, Silim and Saxena, 1992, Agung, 1995). Therefore, it can be inferred that the reduction of TDM under water stress at the early vegetative stage could be used to assess the sensitivity of the faba bean seed yield to water stress at a later stage (early podding) as well.

The consistency of the reduction under water stress of TDM, the seed yield and pod number at the two growth stages, and the significant relationships between them in this study are very useful. The reduction of the seed yield of the genotypes under water stress at the early podding stage observed in the glasshouse study was similar to the stability of their seed yield in the breeding trials. This provides evidence of relevance of the current results to the field conditions. Therefore, sensitivity of TDM at the early vegetative stage itself could be used to assess the faba bean response to water stress. The data from the glasshouse study also showed that the individual seed size did not influence the response of the TDM to water stress at both the early vegetative and early podding stages. This result removes the possibility of the wide variation in the seed size among the faba bean genotypes interfering with the early TDM response to water stress. Therefore, the evaluations suggest that the reduction of TDM under water stress, at the early vegetative stage (3-4 fully opened leaves),

can be used to assess the ability of the faba bean to maintain seed yield under stress. This being an early growth stage, and TDM being easy to measure, the results provide an easy and quick technique to screen the genotypes.

The data showed that the effect of water stress on the seed yield is independent of the yield potential of the genotypes (Section 6.3.2). Therefore, incorporation of stress tolerance traits should improve and stabilise the faba bean yield in the field. Essentially, crop improvement can only be achieved by combining the ability to sustain yield under stress with high yields in the genotypes under cultivation in a given production environment.

In general, the evaluation of growth and physiological responses of the genotypes to water stress indicated that plant water relations and stomatal conductance are unlikely to contribute to the adaptability of faba bean to the southern Australian region. The fact that TDM was a suitable screening trait also suggests that the approach to faba bean improvement under water stress should target the whole plant response rather than individual characters.

Findings of the current study provide a foundation for further work. In future, work can be carried out to further establish the validity of the TDM response under controlled conditions to the field. Evaluating a large collection of genotypes for TDM response under controlled condition, and correlating it with the field trials specifically targeted to water stress would be the next step in this direction. It was not possible to accomplish this goal in the current study because of the limited time available. It would also be useful to evaluate the inheritance of the TDM response elucidated in this study. This would allow the response to be used to breed better

yielding faba bean genotypes targeted to the southern Australian region.

8.2 Developing screening protocol for response to high temperature stress

No information was available on the faba bean response to high temperature stress in the literature. The time available for the study was limited. Therefore, this work was confined to developing techniques to study the ability of the faba bean to acquire thermal tolerance and the pattern of protein synthesis under heat stress. The ATT and HSP are the two common traits used to study the crop response to heat stress.

8.2.1 Acquired thermal tolerance

The literature shows that the conditions of pre-treatment and heat shock treatment should be standardised for each crop and for the specific growth stage of evaluation (Chen et al., 1982, Li et al., 1991, Saadalla et al., 1990a, Shanahan et al., 1990). Literature also shows that ATT can be quantified by measuring EC or TTC reduction (Chen te al., 1982, Srinivasan et al., 1996, Fokar et al., 1998). Accordingly the work began with standardising the pre-treatment and heat shock conditions, followed by comparing the methods of quantifying ATT. Selected genotypes were evaluated for ATT using the protocol thus developed (Appendix 7.3).

The data showed that the genotypic difference for ATT exists in faba bean. While it was not correlated with the grain yield of the genotypes in the low yielding sites of southern Australia, ATT was significantly correlated with the stability of their yield in those breeding trials. These trials were not specifically heat stress experiments and therefore comparison of the yield data with the ATT may not be appropriate. Due to shortage of time no specific field trials could be conducted in the current study and

therefore, data from the breeding trials had to be used for comparison. However, the results provide evidence for the validity of ATT of faba bean to the field conditions. Sublethal temperatures occur in the field frequently. Metabolic changes occurring under such conditions contribute to the development of ATT, making it relevant to the response of crops in the field (Chen et al., 1982, Howarth et al., 1997). ATT is shown to be correlated with the growth and grain yield of several crops under high temperature, both in the glasshouse and field trials. The reports include sorghum (Howarth et al., 1997), soybean (Martineau et al., 1979) and wheat (Saadalla et al., 1990b, Shanahan et al., 1990, Fokar et al., 1998). Therefore, ATT is correlated to the phenotypic responses of crops including grain yield, under heat stress. Consistency of ATT across years and its quantitative heritability has been reported in wheat (Porter et al., 1994, 1995). Hence ATT could be used in breeding programs to improve crop performance under heat stress.

The protocol developed in this study provides the first framework for studying the ATT of faba bean. In future, it can be used to obtain ATT and the post-stress growth and yield of the genotypes under controlled conditions. This will provide appropriate parameters for comparing the ATT. Also, such data can be used in conjunction with the specifically targeted field trials designed for heat stress evaluation, to validate the relevance of ATT to the field response of faba bean. Future work should evaluate the suitability of different growth stages, including reproductive stage for screening. This provides an opportunity to test and use ATT, which involves simple and quick protocol developed in this study, in faba bean improvement programs.

8.2.2 Heat shock proteins

The work on HSP of faba bean was limited to developing a protocol to incorporate

radioactivity in HSP and to separate them on a single dimension SDS gel. This is the first such work in faba bean. The study showed that the leaf samples should be placed directly in contact with labelled incubation buffer and subjected to vacuuming prior to heat shock treatment for appropriate incorporation of radioactivity into HSP. Vacuuming is generally employed to increase incorporation of radioactivity into leaf protein (Krishnan et al., 1989, Vierling and Nguyen, 1990, Jorgensen and Nguyen, 1995). The trials also showed that by using the current method, 10 μCi of the label (S^{35} Methionine) provided with sufficient incorporation and proper separation of HSP on a SDS gel, through autoradiography.

After the protocol for labelling HSP was standardised, ten genotypes selected using their RI_{EC}, were tested for pattern of protein synthesis under heat shock. All the genotypes produced HSP while some proteins were inhibited under heat shock. However, the patterns were generally similar in all the genotypes. A comparison indicated that the pattern of HSP and RI_{EC} were not correlated. This is contrary to reports for several crops where production of specific HSP and ATT were correlated (Blumenthal et al., 1990, Vierling and Nguyen, 1992, Howarth and Skot, 1994, Schirmer et al., 1994, Joshi et al., 1997). The lack of correlation could be because the use of 10% separating gel in the current study may not have achieved complete separation of all the HSP. Better separation could be achieved through a combination of the use of gradient gels, separating gels of different concentration, and protein fractionation prior to gel separation. Lack of time prevented these avenues being explored and the current study was limited to achieving labelling of HSP and its preliminary separation on single dimension SDS-gel.

Although correlation between the HSP production and ATT has been reported in

several crops, it is not yet established if the two are causally related. Secondly, there are no reports available so far of ATT of any crop being improved by using HSP production as a trait to select parental lines. It may be possible that HSP are of little use in crop improvement under heat stress despite its correlation with ATT in several crops. However, HSP research itself is relatively new in agriculture and it is only recently that significant advances were made in this field. This is the first study on the HSP of faba bean. When these facts are considered together with the apparently inadequate separation of HSP in the current study, it would not be appropriate to infer on the absence of relationship between HSP pattern in the current study and the RI_{EC}, and the seed yield from the breeding trials. However, it should be noted that adequate separation of HSP has been achieved using the 10% SDS-gel in several crops including wheat (Krishnan et al., 1987, Vierling and Nguyen, 1992), mung bean (Collins et al., 1995) and sorghum (Howarth and Skot, 1994).

The current protocol allows for efficient labelling of HSP and therefore provides the first framework for future analysis of response of protein synthesis by faba bean under heat stress. This is fundamental to study the role of HSP in developing ATT in faba bean and for its better yield in the field. HSP is can be used as markers in breeding programs to identify parental lines, although no reports are available yet. The current protocol of labelling allows screening large number of genotypes quickly. It provides a tool to study the inheritance of HSP pattern and its genetic control, which is very useful in breeding programs. Further work on the protocol itself might involve testing the effect of pre-treatment on the HSP production. This would provide another perspective on the relationship between HSP and ATT.

8.3 Summary

8.3.1 Highlights of the current study

Important findings of the current project are summarised below.

- This is the first study to evaluate the physiological responses of a relatively large number of faba bean genotypes representing the wide range of seed size and flowering time to drought and heat stress.
- This is the first study on the ATT and HSP of faba bean
- Faba bean improvement program for drought stress of southern Australia should target the whole plant response, not the individual physiological characters.
- It is possible to use the relative reduction of TDM of the faba bean genotypes under water stress at the 3-4 fully opened leaf stage to screen for the differences in the sensitivity of their seed yield in the field.
- The technique of creating water stress described in the current study provides data valid for the field conditions.
- The genotypic difference for ATT exists in faba bean
- Cell membrane integrity of faba bean under heat stress is correlated with the stability of their seed yield in the Mediterranean climate of southern Australia.
- The protocol developed to incorporate radioactivity into leaf protein allows for efficient labelling of HSP of faba bean

8.3.2 Future work arising out of the current study

The following are a few suggestions for work arising from the current project.

- Combining the evaluation of TDM response of faba bean genotypes under controlled conditions with field trials in low rainfall sites.
- Evaluating the inheritance of the TDM response and ATT obtained in the current

study in segregating populations to test the utility of the characters in breeding programs targeted to southern Australia.

- Evaluating the effect ATT of faba bean on the post-stress growth and yield performance of the genotypes under the controlled conditions.
- Combining the evaluation of ATT of faba bean genotypes under controlled conditions with field trials specially targeted to high temperature sites.
- Further studying the pattern of HSP synthesis in faba bean by using more efficient methods of protein separation such as protein fractionation and gradient gels. This is necessary to clarify the existence of genotypic differences in faba bean for HSP and to assess the advantage of including it in a crop improvement program.

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Appendix 3.1

Monthly average rainfall and temperature data for Roseworthy agriculture farm

Month	1995				1998				Long-term average			
	Rainfall (mm)	Rainy days	Temperature (°C)		Rainfall (mm)	Rainy days	Temperature (°C)		Rainfall (mm)	Rainy days	Temperature (°C)	
			Maximum	Minimum			Maximum	Minimum			Maximum	Minimum
January	18.8	7	31.4	17.4	43.2	2	30.6	13.8	22	4	30.1	15.2
February	25.4	5	30.7	16.9	2.6	1	30.2	12.8	20	3	29.4	15.3
March	14.2	3	25.1	13.1	6.4	3	28.0	12.4	19	4	26.9	13.6
April	39.4	11	20.5	11.3	58.2	11	20.9	8.8	38	8	23.3	11.4
May	37.6	9	17.8	9.5	10.0	8	20.1	7.8	49	11	18.4	8.7
June	58.0	14	16.0	8.8	58.4	16	15.7	6.3	54	13	16.3	6.9
July	97.0	23	13.6	7.5	54.8	13	13.8	4.6	49	14	14.7	6.4
August	16.7	9	17.8	6.6	15.4	18	15.3	6.2	52	15	15.6	6.1
September	24.5	7	19.3	6.9	44.4	15	20.8	8.6	44	12	18.1	7.1
October	49.2	8	23.6	10.4	14.6	10	22.7	7.4	41	10	22.2	9.2
November	7.6	4	26.9	11.8	35.8	7	26.1	9.7	27	6	25.4	11.5
December	8.1	5	26.0	11.3	0.0	0	29.6	12.4	24	5	27.8	13.4

(Rural Services Department, Roseworthy Agricultural College, SA, Australia)

Appendix 4.1 : Layout of field trial in paddock E7 at Roseworthy, in 1995

Replication – 1				Replication – 2				Replication - 3			
G-2	G-4	G-6	G-5	**G-3**	**G-4**	**G-8**	**G-7**	G-8	G-2	G-6	G-4
G-3	G-8	G-1	G-7	**G-2**	**G-5**	**G-1**	**G-6**	G-1	G-3	G-7	G-5
G-1	**G-5**	**G-7**	**G-8**	G-5	G-6	G-8	G-2	**G-6**	**G-5**	**G-4**	**G-7**
G-4	**G-6**	**G-2**	**G-3**	G-1	G-4	G-3	G-7	**G-3**	**G-2**	**G-8**	**G-1**

North

Irrigated plots are shaded

Genotypes:

Genotypes			
G-1	Fiord	G-5	Acc527
G-2	Icarus	G-6	Acc617
G-3	Ascot	G-7	Acc820
G-4	Acc286	G-8	Acc973

Appendix 4.2:

Date of 50% flowering of the genotypes in the field trial at Roseworthy, 1995

Genotype	Date of 50% flowering	
	Date	DAS
Fiord	1 September	59
Ascot	4 September	62
Acc973	20 September	78
Acc527	11 September	69
Icarus	25 September	83
Acc286	20 August	47
Acc820	4 September	62
Acc617	11 September	69
DAS – days after sowing		

Appendix 5.1:

Details of preparation of the University of California (UC) soil mixture used in the experiments (Plant Research Centre, Waite)

1200 litres of Golden-grove sand is sterilised at 100°C for 30 minutes. To this 750 litres of peat is added and mixed for 4 minutes. Extra water can be added at this stage. The mixture is left for 20 minutes. After that, the following fertilisers are added and mixed in for 4 minutes:

Calcium Hydroxide	1000 g
Lime	1800 g
Nitrophoska (15-4-12)	1500 g

The mixture is expected to have a pH of 6.8

Appendix 5.2: Details of Osmocote

Osmocote contains following nutrients:

Total Nitrogen (N)	17%
Nitrate	8.2%
Ammonium	8.8%
Total Phosphorus (P)	1.6%
Water soluble	1.3%
Citrate soluble	0.3%
Total Potassium (K)	8.7%
(Potassium Sulphate, Water soluble, no chlorine)	
Total Sulphur (S)	4.5%
(Sulphate)	
Inert fillers	3.8%
Organic resin coating	9.5%
(based on vegetable oils)	

Appendix 5.3: Average stomatal conductance, leaf area and specific leaf area (SLA) of the ten faba bean genotypes as affected by water stress during the early vegetative stage

Genotype		Leaf conductance (mol/m^2/s)			Leaf area (cm^2/plant)			SLA (cm^2/g)		
		Non-stress	Stress	Average	Non-stress	Stress	Averag	Non-stress	Stress	Average
Small seed size										
Early flowering	Fiord	1188	954	1070	92	82	87	51	56	53
	Barkool	1238	1004	1112	85	69	77	59	61	60
	Acc 165	1230	936	1084	95	71	83	60	54	57
	Acc 524	1556	1102	1330	39	42	41	47	75	61
Medium flowering	Acc 482	1604	1652	1628	76	59	68	46	42	44
Late flowering	Acc 973	1266	1204	1236	56	48	52	42	41	41
Medium seed size										
Medium flowering	Acc 527	2000	1664	1832	80	81	80	47	75	46
Late flowering	Icarus	1444	598	1022	73	53	63	49	49	49
Large seed size										
Early flowering	Acc 286	1626	1264	1446	140	135	138	68	73	71
Medium flowering	Acc 820	798	282	540	134	111	125	55	54	54
		1370	**1090**		**87**	**75**		**52**	**55**	
Least significant difference (p=0.05)										
Genotype		**248**			**5**			**12**		
Water level		**111**			**10**			**non-significant**		
Genotype X Water level		**non-significant**			**non-significant**			**non-significant**		

Appendix 5.4: Total dry matter (TDM) of and the amount of water used (WU) by the ten faba bean genotypes as affected by water stress during the early vegetative stage

Genotype		TDM (g/plant)			WU (ml/plant)		
		Non-stress	Stress	Average	Non-stress	Stress	Average
Small seed size							
Early flowering	Fiord	3.3	2.6	2.9	907	686	797
	Barkool	2.6	2.0	2.3	805	600	702
	Acc 165	3.1	2.8	2.9	855	712	784
	Acc 524	1.8	1.3	1.6	602	492	547
Medium flowering	Acc 482	3.1	2.6	2.8	829	757	793
Late flowering	Acc 973	2.7	2.2	2.5	738	660	699
Medium seed size							
Medium flowering	Acc 527	4.0	3.3	3.6	1044	698	871
Late flowering	Icarus	2.7	2.1	2.4	906	754	830
Large seed size							
Early flowering	Acc 286	4.6	4.0	4.3	1516	1192	1354
Medium flowering	Acc 820	5.4	4.3	4.8	1335	1127	1231
		3.3	**2.7**		**947**	**775**	
Least significant difference (p=0.05)							
Genotype		**0.5**			**121**		
Water level		**0.2**			**55**		
Genotype X Water level		**non-significant**			**non-significant**		

Appendix 5.5:

Effect of water stress on average leaf conductance, relative water content (RWC) and leaf water potential (WP) of faba bean at the early podding stage

Treatment	Conductance ($mol/m^2/s$)	RWC (%)	WP (MPa)
Non-stress	550	84	-0.38
Water stress	449	74	-0.42
Relative reduction (%)	***18***	***12***	***9***
LSD (p=0.05)	***207***	***non-significant***	***non-significant***

Appendix 6.1: Effect of water stress at the early vegetative (S1) and at early podding stages (S2) on the relative reduction of total dry matter of the faba bean genotypes recorded at the end of the treatment period

Genotype	Relative reduction (%) under stress treatment	
	S 1	S 2
Small seed size		
Early flowering		
Fiord	10	28
Acc524	27	33
Acc868	37	32
Acc610	32	31
Medium flowering		
Acc482	29	14
Late flowering		
Acc973	15	12
Medium seed size		
Early flowering		
Acc278	29	8
Acc611	30	25
Medium flowering		
Acc722	23	9
Late flowering		
Icarus	26	32
Acc735	32	26
Large seed size		
Early flowering		
Acc649	49	27
Acc286	29	28
Acc664	52	28
Late flowering		
Acc766	21	28

Appendix 7.1: Reagents used in protein quantitation

CTC reagent: 0.1% (w/v) $CuSO_4.5H_2O$, 0.2% (w/v) sodium (or potassium) tartrate, 10% (w/v) Na_2CO_3. The Na_2CO_3 was dissolved in about one-half the final volume and added slowly to a solution of copper sulfate tartrate (also about one-half volume) while stirring. The solution was stored in refrigerator.

Sodium dodecyl sulfate: (SDS) 5% (w/v)

NaOH, 0.8 M

Folin-Ciocalteu phenol reagent: 2 N (Sigma Chemical Co.,)

Sodium deoxycholate: (DOC) 0.15% (w/v)

Trichloroacetic acid: 72% (w/v)

Bovine serum albumin: (BSA) 0.5 mg/ml (Sigma Chemical Co.,)

Reagent A: 1 part CTC reagent with 2 parts 5% SDS and 1 part 0.8 M NaOH were mixed together. The reagent was prepared fresh every two weeks.

Reagent B: 1 part 2N Folin-Ciocalteu phenol reagent was mixed with 5 parts nanopure (NP) water.The solution was stored in an amber bottle.

Appendix 7.2

Visual scoring of leaf damage at the end of pre-treatment with 30°C and 35°C

Genotype	Score due to the pre-treatment at:	
	30°C	35°C
Fiord	0	4
Icarus	0	3
Acc278	0	1
Acc286	0	1
Acc287	0	1
Acc482	1	1
Acc524	0	4
Acc527	0	2
Acc610	1	1
Acc611	0	3
Acc617	1	7
Acc649	0	4
Acc664	0	3
Acc722	0	9
Acc735	0	1
Acc766	0	7
Acc820	0	1
Acc868	0	8
Acc973	1	5
Acc974	0	2
Acc979	0	4

Score	Comment
0	No leaf burning
1	10% leaf burning
2	20% leaf burning
3	30% leaf burning
4	40% leaf burning
5	50% leaf burning
6	60% leaf burning
7	70% leaf burning
8	80% leaf burning
9	90% leaf burning
10	100% leaf burning

Appendix 7.3

Protocol developed to quantify RI_{EC} of faba bean genotypes

The technique of measuring ATT involved pre-treating the seedlings at 30°C in dark for 24 hours in an enclosed growth cabinet. The seedlings were covered with large plastic bags, which were sealed around the tope of the pots to create high humidity. After this, leaf samples consisting of ten leaf discs (0.72 cm^2) were collected in an airtight tube from each replication. They were gently rinsed at least four times with NP water to remove electrolytes adhering to the cut edges. The tubes were capped and samples heat shocked at 45°C for 45minutes in a pre-heated constant temperature water bath. 10 ml of NP water was added to the samples before incubating at 10°C for 24 hours. Long incubation ensures complete diffusion of electrolytes from the leaf samples while low temperature prevents tissue deterioration. After incubation, the samples were equilibrated with the room temperature, tubes gently shaken, and initial EC of the fluid media was measured. Leaf samples were autoclaved at 120 kPa pressure for 20 minutes in the same fluid for a complete kill and cooled to room temperature. During autoclaving, the caps were removed and tubes were covered with aluminium foil to prevent boiling over of the fluid. The tubes were shaken by hand, final EC of the fluid was measured, and RI_{EC} calculated. RI_{EC}% represented the extent of injury caused to the samples and therefore was inversely proportional to the ATT of a genotype.

Appendix 7.4

Protocol developed to radiolabel and separate HSP of faba bean genotypes on single dimension SDS-PAGE

Leaf samples were obtained when 3-4 fully opened leaves appeared. They were collected in 4 ml scintillation vials containing 50 μl unlabelled incubation buffer. To each of the sample was added 10 μCi of the label in 100 μl buffer and the samples were vacuumed for 5 minutes. The control samples were incubated at room temperature and the treatment samples heat shocked at 45°C for 2 hours. Total protein was extracted, pelleted and label incorporation was measured after the completion of the treatment. Crude protein extract was used to separate HSP by SDS-PAGE. The lanes were loaded with equal amounts of radioactivity (100, 000 DPM/lane). Standard protein markers were run with the crude protein extracts. The gels were autoradiographed for 10 days at –70°C and the HSP bands were visualised.