Understanding morphological, physiological and biochemical characteristics of faba bean (*Vicia faba* L.) under drought condition

By

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I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Md Abdul Muktadir
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June, 2019
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>∆</td>
<td>Carbon isotope discrimination</td>
</tr>
<tr>
<td>A</td>
<td>Net photosynthesis rate</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminum</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BARI</td>
<td>Bangladesh Agricultural Research Institute</td>
</tr>
<tr>
<td>BN</td>
<td>Branch number plant$^{-1}$</td>
</tr>
<tr>
<td>BSFTA</td>
<td>Bistrimethylsilyltrifluoroacetamide</td>
</tr>
<tr>
<td>Ca</td>
<td>Atmospheric carbon concentration</td>
</tr>
<tr>
<td>Ci</td>
<td>Intercellular carbon concentrations</td>
</tr>
<tr>
<td>CT</td>
<td>Canopy Temperature</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variance</td>
</tr>
<tr>
<td>DMRT</td>
<td>Duncan Multiple Range Test</td>
</tr>
<tr>
<td>DW</td>
<td>Dry Weight</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh Weight</td>
</tr>
<tr>
<td>G x E</td>
<td>Genotype x Environment</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GC-QQQ</td>
<td>Gas chromatography triple quadrupole</td>
</tr>
<tr>
<td>GDD</td>
<td>Growing Degree Days</td>
</tr>
<tr>
<td>gm</td>
<td>Mesophyll conductance</td>
</tr>
<tr>
<td>gₙ</td>
<td>Stomatal conductance</td>
</tr>
<tr>
<td>HI</td>
<td>Harvest Index</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IRMS</td>
<td>Isotope Ratio Mass Spectrometry</td>
</tr>
<tr>
<td>IRT</td>
<td>Infrared thermometer</td>
</tr>
<tr>
<td>LA</td>
<td>Leaf Area</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LRN</td>
<td>Lateral Root Number</td>
</tr>
<tr>
<td>LW</td>
<td>Leaf Width</td>
</tr>
<tr>
<td>MCW</td>
<td>Methanol-Chloroform Water</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NDVI</td>
<td>Normalized Difference Vegetation Index</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>PA</td>
<td>Polyamine</td>
</tr>
<tr>
<td>PBA</td>
<td>Pulse Breeding Australia</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEPC</td>
<td>Phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative Trait Loci</td>
</tr>
<tr>
<td>RCBD</td>
<td>Randomized Complete Block Design</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>REML</td>
<td>Residual Maximum Likelihood</td>
</tr>
<tr>
<td>RLD</td>
<td>Root Length Density</td>
</tr>
<tr>
<td>RWC</td>
<td>Relative Water Content</td>
</tr>
<tr>
<td>SH</td>
<td>Shoot Height</td>
</tr>
<tr>
<td>TE</td>
<td>Transpiration Efficiency</td>
</tr>
<tr>
<td>TMCS</td>
<td>Trimethylchlorosilane</td>
</tr>
<tr>
<td>TRL</td>
<td>Taproot length</td>
</tr>
<tr>
<td>TW</td>
<td>Turgid Weight</td>
</tr>
<tr>
<td>WD</td>
<td>Water Deficit</td>
</tr>
<tr>
<td>WUE</td>
<td>Water Use Efficiency</td>
</tr>
<tr>
<td>WW</td>
<td>Well Watered</td>
</tr>
<tr>
<td>ψ</td>
<td>Leaf water potential</td>
</tr>
<tr>
<td>ψp</td>
<td>Turgor potential</td>
</tr>
<tr>
<td>ψs</td>
<td>Osmotic potential</td>
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Summary

Drought stress is one of the most important limiting factors for the sustainable production of faba bean (*Vicia faba* L). However, limited information has been gathered regarding the underlying mechanisms of drought tolerance of this crop. This study covered the characterization of physiological, biochemical and morphological traits/markers for use in enhancing the drought tolerance of faba bean by assessing key traits under the field and controlled environments. The investigations utilised a range of plant tissues, i.e. leaf, flower, root and grain tissue’s response under water deficit conditions. Leaf-level carbon isotope discrimination was found as a suitable candidate trait to identify drought tolerant genotype as well as predict grain yield under field conditions. Large genotypic variation (16.84‰ ~ 21.96‰) among studied genotypes made it a potential selection tool for breeding programs. Among yield contributing characters of faba bean in field conditions, plant height along with 100 seed weight were the most important contributors towards grain yield (0.60**). Shorter pod filling duration was also shown to have great importance for yield production under conditions of drought.

Hydroponic assay showed great promise as a tool for root phenotyping and plant selection at the seedling stage. The results from hydroponic assays were confirmed through a subsequent sand culture study and suggested as an effective screening platform as genotypes had a similar response under both conditions. Leaf chemistry of faba bean genotypes showed the presence of increased *myo*-inositol and sucrose in irrigated treatments for all five genotypes over drought stress. Accumulation of carbohydrates in response to drought stress suggests them as an efficient trait to be used in the breeding program as well as assessing plant health in the natural system. Comparison of water use efficiency prediction through leaf-level carbon isotope abundance and gas exchange suggested the former would be a suitable tool as a drought tolerance screening tool. The relatively small differences in carbon isotope abundance between leaf and grain tissue suggest that abundances in either tissue are appropriate for inclusion in breeding programs. Reproductive function leading to pod formation during stress was primarily governed by the pistil. Reciprocal crosses between water deficit and well-watered plants showed a greater number of pod formation when the pollen bearing plant was stressed than the pistil bearing plant. No pods were formed in both plants under drought, but few pods were formed when the pollen from stressed plant were used to pollinate the plants in well-watered conditions. Finally, the grain quality of faba bean tends to be resilient and not affected by water deficit. This was examined through quantification of essential amino acids and major mineral
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Chapter 1
General introduction

Grain legumes are a critical component of sustainable cropping systems worldwide due to their inherent capacity to fix nitrogen and provide nutritious food and feed. Grain legumes cover approximately 27% of the world’s food production area and provide 33% of protein requirements (Graham and Vance, 2003). Rising global temperatures and more erratic climatic conditions threaten to the sustainable food production system; with legumes being severely affected (Daryanto et al., 2017). Grain legumes are usually cultivated under rainfed conditions where yield variability is high mainly due to seasonal fluctuation of weather, especially rainfall and temperature (Daryanto et al., 2015).

Among the grain legumes, faba bean (*Vicia faba* L) is suitable to grow in a rotation due to its high nitrogen fixation capacity, i.e. is 160 kg ha⁻¹ (Hoffmann et al., 2007) and high (24 to 35%) seed protein content (Crépon et al., 2010). In Australia, faba bean is an important crop for its export potential primarily to the Middle-Eastern countries. Despite the rotational benefits and feeding value of faba bean production, the area under cultivation is decreasing due to its high sensitivity to abiotic stresses, especially water deficit (Foyer et al., 2016). Development of genotypes adapted to water deficit conditions through genetic improvement is an important strategy to address this challenge. Programs aimed at the genetic improvement of the drought resistance of this crop are hampered due to lack of high throughput screening methodologies for this crop. Whilst genotypic variation in response to drought has been observed in faba bean, understanding the physiological, morphological and biochemical basis of drought tolerance is essential for the development of cultivars adapted to drought conditions.

Targeting yield improvement under conditions of water-limitation, a series of experiments were carried out both in the field and under control environments to investigate morphological adaptations, physiological characteristics and biochemical patterns that are elicited by water
deficit in contrasting faba bean genotypes. Overall, the main objective of the thesis was to identify traits attributable towards drought tolerance in faba bean breeding along with better nutrition. The specific objectives were

i) to investigate genotypic differences in carbon isotope abundance that are present in studied faba bean population for the development of drought resistant cultivars

ii) to study the influence of environment, especially water deficit on the acclimation process of contrasting faba bean genotypes in northern NSW growing environment

iii) to investigate the relative changes in concentration and quantification of metabolites under water deficit conditions

iv) to identify biochemical indicators from leaf and grain tissue of contrasting faba bean genotypes along with testing whether water deficit hamper the nutritional quality of those genotypes

v) to develop a rapid and reproducible screening protocol at seedling stage for drought resistant faba bean breeding program.

This thesis is composed of five research chapter preceded by a literature review to highlight the context and main areas of research that I propose are most profitable to pursue in improving faba bean production. Each research chapter is written in a publication format.

Specifically, each chapter appears as follows:

**Chapter 2: Drought resistance faba bean breeding: Physiological and biochemical insights**

This chapter is a literature review focusing on current knowledge of biochemical and physiological markers for legumes improvement that can be incorporated into drought tolerance faba bean breeding programs. Through a detailed literature review, I determine the
most important biochemical and physiological traits and their contribution towards drought
tolerance. Part of this chapter is published as a book chapter which detail can be found in
author(s) attribution statement.

Chapter 3: Diversity in carbon isotope discrimination of field grown faba bean and its
association for yield prediction

Through a field study at northern NSW, this chapter determines the interrelationship between
leaf-level carbon isotope discrimination, grain yield and drought tolerance among 96 faba bean
accessions from a different origin. Five genotypes were selected based on their isotopic value
for subsequent experiments.

Chapter 4: Phenology, morphology and physiology of selected faba bean genotypes
grown under irrigated and rainfed condition in Northern NSW

In this study, in a phenotypic basis, I determined the effect of water deficit on five faba bean
genotypes, possessing contrasting carbon isotope discrimination values, over two growing
seasons 2015 and 2016 in both rainfed and irrigated trials at Narrabri, northern NSW.

Chapter 5: Carbon isotope discrimination and soluble metabolites reflect physiological
status among contrasting faba bean genotypes in response to water deficit

This study investigates the suitability of using naturally occurring carbon isotopes in bulk leaf
material to predict water use efficiency (WUE) as an alternate measure of plant gas exchange
on five faba bean genotypes grown in rainfed and irrigated trial. I also assessed the most
abundant carbohydrates and sugar alcohols along with plant water status and biomass
accumulation from those faba bean genotypes in the field.
**Chapter 6: Hydroponics as a tool for screening drought tolerance in faba bean**

This study develops a suitable seedling root-based screening protocol to enhance both the speed of selection and to assess below ground traits. I test a rapid screening technique of material grown in a hydroponic system for tolerance to root dehydration and standardise against genotypes grown in sand culture. A set of 96 faba bean genotypes (details in chapter 3) were evaluated under hydroponic conditions by periodic removal from the nutrient solution. This experiment was carried out at greenhouse facility of plant breeding division, Bangladesh Agricultural Research Institute, Bangladesh.

**Chapter 7: Effect of water deficit on the physiological, biochemical and reproductive biology of contrasting faba bean genotypes**

In my final study, I quantified carbohydrates, amino acids, mineral nutrients and the abundance of naturally occurring carbon isotopes in leaf and grain of faba bean genotypes grown under well-watered and water deficit conditions. I also conducted experiments to identify the impact of water deficit on reproductive biology, i.e. pollen viability, pollen germination and pistil function towards pod formation of contrasting faba bean genotypes. The experiment was carried out at the Centre for Carbon, Water and Food, University of Sydney.

Overall, my thesis seeks to enhance our capacity to screen for advantageous traits to enhance the production, resilience and nutritional quality of *V. faba*. 


Chapter 2
Physiological and biochemical basis of faba bean breeding for drought resistance – a review

Abstract

Grain legumes are commonly used for food and feed all over the world and are the main source of protein for over 1 billion people worldwide, but their production is at risk from climate change. Water stress (water deficit) and heat stress both significantly reduce the yield of grain legumes and faba bean is considered particularly susceptible. Genetic improvement of faba bean for drought tolerance (water deficit tolerance) by conventional methods and molecular breeding is time-consuming and laborious since it depends mainly on selection and adaptation in multiple sites. Lack of high throughput screening methodology and low heritability of advantageous traits under environmental stress challenged breeding progress. Alternatively, selection based on secondary characters in a controlled environment followed by field trials is successful in some crops, including faba bean. In general, measured features related to drought tolerance are shoot and root morphology, stomatal characteristics, osmotic adjustment and the efficiency of water use. Here we focused on current knowledge of biochemical and physiological markers for legumes improvement that can be incorporated into drought tolerance faba bean breeding programs.

2.1 Introduction

Among the cultivated legume crops, faba bean (Vicia faba L.) ranked sixth regarding world production with a value of 4.3 million tons from 2.3 million ha in 2015 (FAOSTAT, 2017). Dry bean (Phaseolus vulgaris L.) ranked first next to pea (Pisum sativum L.), chickpea (Cicer arietinum L.), cowpea (Vigna unguiculata (L.) Walp) and lentil (Lens culinaris Medik.). Faba bean was one of the primitive domesticated crops in the Middle East (Caracuta et al., 2015)
and spread from there eastward to central Asia, India and China, westward to North Africa and Europe, and in historical times to Australia and the Americas (Lawes et al., 1983). While the wild antecedent of faba bean is not known (Emshwiller et al., 2015), carbonised remains dating back to 14000 B.P. are viewed as examples of it (Caracuta et al., 2016). Faba bean arrived in Australia in 1788 (Worgan, 1788) but remained a minor crop until the 1980s. The importance of faba bean in Australia is well understood, and the country is the largest exporter of faba bean globally and ranked fourth for production after China, Ethiopia and the United Kingdom (FAOSTAT, 2017).

Grain legumes have substantial advantages in cereal-based cropping systems in breaking the life cycles of certain diseases, host-specific nematodes and weed species (Watson et al., 2017). Like other grain legumes, faba bean with its symbiotic rhizobacteria fixes atmospheric nitrogen in a wide range of conditions. Biological nitrogen fixation provides about 80% of the plant’s nitrogen needs (Somerville, 2002), reaching 160 kg ha\(^{-1}\) (Hoffmann et al., 2007; Horst et al., 2007), and about half of the crop’s nitrogen content is left in the field after grain harvest (Baddeley et al., 2013). Hence, it is considered important for both its contribution to residual nitrogen in crop rotation (Watson et al., 2017) and its potential in green manuring (Baddeley et al., 2017).

Faba bean seeds have high protein content and a good source of energy and fibre (Duc, 1997). Average protein content is 29% (Feedipedia, 2018), ranging 24 to 35% (Crépon et al., 2010) which count it as most protein-rich of starchy legumes. Mature beans are cooked into a stew or paste in many countries and fed to livestock in many others. Fresh beans are a popular vegetable in the UK, France and Spain. Whole-crop silage can be made and is a way of rescuing value
from the crop if growing conditions such as exceptionally wet autumn prevent harvest of dry beans.

Among cultivated grain legumes, faba bean is thought to be sensitive to water deficit (Khan et al., 2010). Yield in irrigated agriculture is significantly higher than in rainfed systems (Oweis, 2005) although the majority of its cultivation lies outside irrigated areas. Yield based meta-analysis data from 1980 to 2014 indicated that faba bean yield was reduced by 40% following a 65% reduction in water availability, and the loss in yield depends on cultivar and other environmental conditions (Daryanto et al., 2015). Improving drought tolerance is vital to improve performance and stability of yield, but efficient selection protocol and mixed breeding system of this crop are the big challenges to address the drought effect (Stoddard et al., 2006; Drayner, 1956). Field phenotyping for drought response is expensive and time-consuming and is often unrepeatable because of variation in the timing of onset, length and severity of the drought (Khan et al., 2007). Average outcrossing is about 33%, depending on cultivar and environmental conditions (Stoddard and Bond, 1987) and if cross-pollination occurs before the stress occurred, it will reduce the effectiveness of selection for the drought response (Palmer et al., 2009). On the other hand, controlled conditions screening based on secondary physiological characteristics of roots and shoots, including stomatal conductance (gs), mesophyll conductance (gm), relative water content (RWC), water potential, osmotic potential, root morphology, root plasticity and isotopic carbon discrimination can provide generally reliable and repeatable information for selection. Metabolic profiling, which provides insight into how changes in metabolite concentrations are influenced by changes in the environment, also holds great promise.
2.2 Yield response to drought

Faba bean is prone to water deficit from seedling to maturity (Khan et al., 2010; Khan et al., 2007), but in Mediterranean-type climates, where it is sown in autumn and harvested in spring as temperature increases and rainfall declines, faba bean often experiences so-called “terminal” drought (Amede et al., 2003). Adapted material escapes terminal drought by early maturity, completing its life cycle before a drought occurs. Besides terminal drought faba bean, often experiences transient drought in subtropical and temperate environments. Transient drought produces shorter stature as well as few reproductive nodes in faba bean (Gnanasambandam et al., 2012). In contrast, to escape from terminal drought, there is no comparable and simple strategy to deal with unpredictable, intermittent or transient drought. Maintaining higher RWC under water deficit, which demonstrates effective stomatal regulation and stomatal function (Khazaei et al., 2013b), root morphology, plasticity and function (Belachew et al., 2018), epicuticular wax and osmotic adjustment (Khan et al., 2010), play vital roles in management of transient drought.

Although faba originates from a semi-arid region, drought tolerance is not common. Due to its relatively shallow root system (Manschadi et al., 1998; Mwanamwenge et al., 1998), faba bean unable to extract water from deep soil causes moisture stress during the reproductive phase when the topsoil dries. Genetic resources that can adjust with different moisture level exist in faba bean (Abdelmula et al., 1999; Amede et al., 1999; Grzesiak et al., 1997; Khan et al., 2007; Link et al., 1999; Ricciardi et al., 2001) indicating that there is potential to breed for moisture stressed environments. Link et al. (1999) selected drought tolerant cultivars and breeding lines from the Mediterranean region where water distribution is irregular and moisture levels moderate (~500 mm rainfall). Maalouf et al. (2015) also found different tolerance level against
water deficit in his study. The existence of wide genotypic variation makes it possible to develop drought tolerant faba bean genotype through classical breeding approach.

Plant developmental stage along with the magnitude of water deficit determines yield loss of faba bean. The most susceptible stage to developmental inhibition has been variously described as flowering (El Nadi, 1969), early podding (Mwanamwenge et al., 1999) and pod setting (Xia, 1994), but all of these indicate the early reproductive phase, as is generally agreed (Al-Suhaibani, 2009; Khan et al., 2007). Moderate drought stress had a negative effect on the pod number per plant but no effect on seed size and seed number pod⁻¹ (Adisarwanto and Knight, 1997; El Nadi, 1969). The extent of drought stress determines whether the plant had partial damage or complete death.

Seed quality is also affected by water deficit. Genotypic background along with environmental factors i.e. drought and heat are the major factors that regulate seed protein content (Rharrabti et al., 2001). Winter faba beans had slightly higher protein concentration than spring beans, which may be interpreted as indicating that water deficit tended to increase protein content (Duc et al., 1999). Faba bean crops grown in water deficit condition had improved protein content (Alghamdi, 2009) and sulphur content (Schumacher et al., 2011) than those grown with adequate water. In drought-prone environments reduced protein biosynthesis is due to reducing N fixation and partitioning (Singh, 2007). On the other hand, Smith (2018) reported that drought caused yield reduction but did not alter nutritional quality (calcium, iron, potassium, magnesium, phosphorus, sulphur and zinc) and amino acids concentration in common bean. Generally, seed minerals were not impacted by drought may be due to their immobile nature. High N fixation observed in faba bean under optimal condition whereas drought considerably impaired N fixation (Kabbadj et al., 2017; Neugschwandtner et al., 2015). Under water deficit,
modification of the bacteroid environment causes the decline in N fixation (Guerin et al., 1990) and reduced nodule numbers were also observed when exposed to drought (Sangakkara et al., 1996).

2.3 Physiological attributes related to drought tolerance

Phenotypic selection for drought tolerance based on secondary traits is well established (Boyer, 1982; Lafitte et al., 2003; Richards, 2006). Drought tolerance trait selection was found to be more meaningful when it was conducted on a large population (Wery et al., 1994). A number of physiological traits including water use efficiency (WUE), (Amede et al., 1999), stomatal features (Bond et al., 1994; Khazaei et al., 2013a; Ricciardi, 1989) leaf temperature and carbon isotope discrimination($\Delta$), (Khan et al., 2007) were found to be suitable in faba bean breeding program.

Drought stress interacts with low temperatures, soil salinity and Al$^{3+}$ toxicity. Salinity and drought affecting the osmotic equilibrium in a similar way (Mahajan and Tuteja, 2005) while Al$^{3+}$ toxicity hampers root development (Belachew and Stoddard, 2017) and cold affects the movement of water within roots and the ability of the plant to form symbiosis (Aroca et al., 2007). Among legumes, Al-drought interaction has been studied in common bean (Yang et al., 2012) and soybean ($Glycine$ $max$ (L.) Merr. (Goldman et al., 1989), and the toxicity exacerbated the effect of drought mainly due to its inhibition of root growth. On the other hand, drought was reported to ameliorate Al$^{3+}$ injury by reducing the uptake of the toxic ion. Al-drought interaction has a synergistic effect on crops, rendering them unable to recover once they are exposed to these stresses. Salinity coupled with drought reduced symbiotic nitrogen fixation, which may be due to its effect on the supply of photosynthates or oxygen to the nodule and bacteroids (Drevon et al., 2015). Crosstalk in stress signaling and gene expression has been
reviewed in drought and cold stresses responses (Shinozaki et al., 2003) and cold and salinity responses (Mahajan and Tuteja, 2005). In faba bean, little genetic connection between drought and freezing tolerance was observed (Ali et al., 2016).

### 2.4 Root traits

Compared with the above-ground traits, relatively limited studies have been performed on below-ground traits. Root is a complex trait, involves clumsy measurement systems and maintaining a high level of precision is difficult in field-level screening (Kashiwagi et al., 2006). Among root architectural features, morphology and plasticity are the two general types of variation that exist. Variations in root morphological characteristics have a significant influence on the capacity and efficacy of a plant to search for and absorb moisture and nutrients from the soil. Deep-rooted soybean genotypes had higher water-absorption capacity from deep horizons of soil lead to higher yield potential (Fenta et al., 2014). Moreover, the alteration of root architecture (plasticity) follows several mechanisms under stress and nutrient availability. For example, the root/shoot biomass ratio increased in *Lupinus albus* and *L. mutabilis* during water deficit (Carvalho et al., 2004). Changes in root density of *L. angustifolius* genotypes in response to phosphorus availability have also been observed (Chen et al., 2013). Faba bean genotypes having extensive and prolific root characteristics usually exhibit drought tolerance (Grzesiak et al., 1997). Variability in root characteristics and root-based phenotyping for drought tolerance also found in cowpea (Matsui and Singh, 2003), chickpea (Chen et al., 2017; Kashiwagi et al., 2006; Ramamoorthy et al., 2017), lentil (*Lens spp.*, Mohsenzadeh Rabani, 2018; Singh et al., 2013), and common bean (Abenavoli et al., 2016, Burridge et al., 2016).

Under water deficit condition, Husain et al. (1990) examined increased root growth in per unit area during the flowering stage. Manschadi et al. (1998) described root length density along
with dry root weight is considerably low under drought condition. Belachew et al. (2018) have identified root phenotypic markers related with drought avoidance characteristics in young faba bean plants in GROWSCREEN-Rhizo boxes (GROWSCREEN-Rhizo is a phenotyping robotic tool measuring the root and shoot growth (see Nagel et al. (2012). Accordingly, two accessions (IG 70622 and IG 11320) exhibiting deeper root system and higher root area coverage showed drought avoidance traits by maintaining their primary and tertiary root lengths relatively well. The same observation was stated by Muktadir et al. (2017) while screening drought adapted genotypes under hydroponic system screening. The growth of the tap root in faba bean is strongly influential over total rooting depth; however, it appears unable to acquire water for growth, even if the root surface has access to it. At shallow depths, lateral root growth is important to access surface soil nutrition and moisture and is thought to be more influential over plant health. Selection on the basis of a rapid growth of taproot and expansion of lateral root system to maintain RLD and dry root weight may be effective in drought resistant breeding program. Measurement includes root mass, rooting volume, root length and root/shoot ratio.

2.5 Shoot related traits

Under stress conditions, selection for higher yield is not always meaningful as yield is controlled by many QTLs (quantitative trait loci). To select genotypes for drought tolerance, the breeding process should be focused on the contributing characters rather than yield itself. Contributing characters need to be causal and shoot-related traits play vital role in maintaining water status when grown in water deficit conditions.
2.5.1 Leaf/Canopy temperature

The temperature of the exterior of the canopy is related to the vapour pressure deficit and directly influences the amount of transpiration that leads to cooling by evaporation. Direct and easy measurement of the canopy temperature (CT) without disturbing the crop can be completed by using an infrared thermometer (IRT). Blum et al. (1982) introduced CT as a selection trait for dehydration tolerance. During water deficit, closed stomata causes a decrease in transpiration which ultimately leads to an increase in leaf temperature (O'Neill et al., 2006). In the field conditions, maintaining lower CT is generally linked with improving absorption capacity of a plant under stress condition (Blum, 2011). CT can be used as a substitute selection trait to screen faba bean under control environment facility (Khan et al., 2010; Khan et al., 2007). In a large faba bean germplasm collected from contrasting moisture regions around the globe revealed that leaflet and canopy temperatures were the most informative measurements distinguishing germplasm from wet and dry origins (Khazaei, 2014a; Khazaei, 2014b). CT also found to be a prominent trait for soybean (McKinney et al., 1989), chickpea (Devasirvatham and Tan, 2018; Kashiwagi et al., 2008; Zaman-Allah et al., 2011), cowpea (Hall, 2012) and lentil (Biju et al., 2018). The preference of this trait is due to its noninvasive nature and early evaluation is possible in a large number of genotypes. Thermal infrared imaging of crop canopies is also applicable to study stomatal characteristics.

2.5.2 Leaf water relations

During water deficit conditions plant may maintain water potential by the production of cellular metabolites/osmotica- the maintenance of sustaining turgor pressure, which stomata sense also depends on the accumulation of these compounds. Plant species and their developmental stage are the determinants of metabolites type and functions. Relatively few candidate molecules exist that can function as cellular osmotica as they must be compatible with cellular function
at high concentrations. These include selected molecules within the organic acid, inorganic ions, polyols, polyamines, and carbohydrate groups of compounds. In chickpea, carbohydrates and polyols contributed more than 50% to the osmotic pool (Amede et al., 2003) and in peas ranging from 10-46% (Sánchez et al., 2004). More broadly, osmotic potential ($\psi_s$) and turgor potential ($\psi_p$), which are the two main components of leaf water potential ($\psi$), found an effective selection trait for drought tolerant screening. Generally, leaf $\psi$ is considered a major component of leaf-level water deficit (Hsiao, 1973) and it can ultimately drive interpretations of plant water status (Pantuwan et al., 2002; Turner, 1982). Sustaining leaf $\psi$ under stress involves a number of mechanisms related to root and shoot, considered to be connected to drought avoidance (Levitt, 1980). Higher leaf turgor in response to water deficit is one of the adaptation strategies for many plant species (Gebeyehu, 2006; Jongdee et al., 2002). Osmotic adjustment driven drought adaptation also observed in chickpea (Basu et al., 2007; Turner et al., 2007a), pea (Sánchez et al., 1998) and lentil (Leport et al., 2003; Leport, 1998).

### 2.5.3 Relative Water Content (RWC)

RWC is a robust and simply assessable selection criterion which can describe plant water status to metabolism irrespective of plant parts and species. It can be expressed as water content of tissue in normal condition compare with hydrated condition (Lawlor and Cornic, 2002). During water deficit condition, RWC plays an important role by preserving water (stomatal features, leaf area reduction and leaves dropping) or maximise water absorption (root plasticity). Sinclair and Ludlow (1985) found RWC superior over water potential to assess plant water status. Khazaei et al. (2013a) stated RWC as one of the most important traits differed between wet- and dry-adapted faba bean accessions under non-stress conditions. As RWC is composed of relative change in cell turgor and $\psi_s$, so it relates both solute concentrations and cell wall rigidity (Kaiser, 1987). RWC can efficiently identify drought tolerant genotypes based on their
plant water status in faba bean (Khan et al., 2007; Khazaei, 2014a; Siddiqui et al., 2015),
common bean (França et al., 2000) and chickpea (Rahbarian et al., 2011; Talebi et al., 2013).
So, it can be said genotypes that can sustain higher RWC under water deficit environment
would be suitable for drought tolerance faba bean breeding programs.

2.5.4 Stomatal Conductance

Drought avoidance involves maintenance of gas exchange during drought conditions which
cause either higher stomatal conductance, $g_s$ (low stomata sensitivity to drought) or stomatal
closure to restrict water loss. There is a trade-off between gas exchange and stomata closing
during stress which ultimately affects photosynthesis and then carbon assimilation. Stomatal
conductance has been proposed as an effective selection tool and, when measured on multiple
plants in a canopy, is equally effective as CT (Condon et al., 2007). The measurement of $g_s$
(unit: mol m$^{-2}$ s$^{-1}$) depends on several stomatal features. In faba bean stomatal characteristics
are assessed through microscopy (Grzesiak et al., 1997; Khazaei et al., 2013b) but due to the
rapid response against stress average stomatal opening size is hard to measure. Leaf porometer
(e.g., Delta-T Devices: AP4) or portable photosynthesis machines (e.g., LICOR-6400) quantify
$g_s$ but may not reflect variability over longer timeframes. $g_s$ measurement is highly influenced
by environmental conditions (high G × E interaction), time of the day and plant developmental
stage. Leport (1998) studied on different grain legumes namely, white lupin (*L. albus*),
chickpea, faba bean, field pea, grass pea (*Lathyrus sativus*), and lentil under water deficit
conditions and found all the species responded similarly i.e., $g_s$ remarkably reduced during
drought in early stage but at the later stage after pod filling it had little or no effect. Faba bean
genotypes were having higher stomatal density, yielded low and showed low drought
tolerance, while genotypes having low stomatal density perform better in stress condition
(Ricciardi, 1989). In a relatively small set of faba bean germplasm $g_s$ found to be a most vital
trait (Khan et al., 2007; Nerkar et al., 1981). A considerable variation for $g_s$ in 402 faba bean accessions under non-stress conditions, indicates the potential use of this trait (Khazaei et al., 2013b). The gas exchange measurement along with stomatal morphology characteristics have been genetically mapped in faba bean. The QTLs governing stomatal morphology, $g_s$ and CT were all co-located in faba bean chromosome 2 (Khazaei, 2014b).

2.5.5 **Carbon isotope discrimination**

Carbon isotopes discrimination ($\Delta$) is proposed extensively to envisage plant physiological status especially WUE. It is extensively used to evaluate transpiration efficiency (TE) in cereals and recently to legumes; common bean (Lockhart et al., 2016; Smith et al., 2016; Zacharisen et al., 1999) soybean, (Clay et al., 2003); chickpea, (Khan et al., 2004), cowpea (Hall et al., 1990) and lentil (Turner et al., 2007b). Its potential as a drought-adapted faba bean accession with high WUE is described by Khazaei et al. (2018) where cv. Mélodie had lower $\Delta$ value compared to drought susceptible lines, e.g., Aurora/1. There has been some controversy found regarding the relationships between $\Delta$ and TE. Turner et al. (2007b) also argued about $\Delta$ and pointed TE was not significantly correlated with $\Delta$ in three grain legumes, i.e., lentil, narrow-leaved lupin (*L. angustifolius*) and chickpea. Carbon isotopes discrimination may not give actual indication sometimes to predict WUE, because generally $\Delta$ measured from leaf tissue which has limitation against short-term environmental changes (Bowling et al., 2008). Carbon isotopes discrimination-based screening methods is expensive and required a sophisticated instrument, e.g. isotope ratio mass spectrometry (IRMS).

2.6 **Metabolomics for Legume breeding**

Nowadays metabolic products act as a biomarker in crop improvement due to its strong linkage with environmental characteristics (Steinfath et al., 2010). So far more than 200,000
metabolites have been identified in plants which are responsible for a diverse set of functions. Quantification and qualification of these composite characters into subsets of metabolism can offer a unique view of the chemical and physiological response mechanisms induced by plants (Caretto et al., 2015). Identification and quantification of low molecular weights metabolites required special analytical and separation techniques. The separation techniques involve gas chromatography (GC) (Weckwerth, 2011), liquid chromatography (LC) (Scherling et al., 2010; Weckwerth, 2011), and high-performance liquid chromatography (HPLC) (Soga, 2007). These methods can characterise a vast range of metabolites at extraordinary scales and accuracy. Recently nuclear magnetic resonance (NMR) create great attention for the researcher to do metabolites profiling (Ward et al., 2007).

Metabolic profiling in grain legumes is rare and seemingly absent for faba bean. Zhang et al. (2012) studied metabolic variations between symbionts in legumes M. truncatula through an untargeted quantitative mass spectrometry-based (MS) method. Short-term water deficit effects on overall growth through metabolic changes were studied by NMR based metabolic profiling (Silvente et al., 2012). Plant produces many primary and secondary metabolites during stress. Two different opinions can define this process: osmotic adjustment where plant regulates solute synthesis to decrease osmotic potential under adverse situation (Turner, 2018) and solutes accumulation which happens due to reductions in interruption or fluctuations in solute transport (Serraj and Sinclair, 2002). Generally, osmotica are easily synthesised from readily accessible antecedents and preferably, transformed to metabolically active and movable compounds. Overall, pre-existing metabolomics resources for legume research will be benefited faba bean improvement. However, speedy progress in the application of these tools may uncover new solutes/biochemical markers for stress tolerance in faba bean breeding.
2.7 Metabolites accumulation in legumes in response to drought and their use as a biomarker

2.7.1 Amino Acids

Amino acids and other nitrogen-based molecules accumulation against stress response are one of the most important biochemical signals for almost all plants. They are the essential metabolites that accumulate during stresses in different legumes including model legume *M. truncatula* to soybean (Charlton et al., 2008; Dias et al., 2015; Hernández et al., 2009; Muscolo et al., 2015; Watson et al., 2015). This accumulates enhanced protein breakdown which is triggered by stress. Moreover, higher amino acid concentration helps a plant to survive under stress (Krasensky and Jonak, 2012).

Proline is found to be the most prevalent amino acids found in plant tissues under stress conditions (drought, cold, salinity). Singh et al. (1972) demonstrated a correlation between drought and increased free proline accumulation in tolerant barley cultivar compare to a more drought susceptible genotype. Similar correlation was found in other crops (Aspinall, 1981). However, the article also illustrated a poor correlation between drought and proline accumulation (Hayat et al., 2012). Among legumes, *Lotus* showed a positive association and soybean had increased concentrations during reproductive stage only (Sanchez et al., 2012; Silvente et al., 2012). Again, during water deficit, proline accumulation was observed in the pre-flowering stage of peanut (*Arachis hypogea*) (Zhang et al., 2017). So, it means proline accumulation not only rely on water status but also on the growth stage of the plant. Short-term stress can be identified through proline quantification. Venekamp et al. (1989) applied water deficit for one day in the seedling stage and ended up with a statement that water deficit-induced proline accumulation. Proline concentration in faba bean observed with the increase
of stress intensity and variation in proline concentration in genotypic level is low under optimal condition (Ammar et al., 2015; Kabbadj et al., 2017; Migdadi et al., 2016; Siddiqui et al., 2015). However exogenous proline application often decreased stomatal opening under drought which had a positive impact on drought tolerance mechanism (Rai and Sharma, 1991; Rajagopal, 1981). Proline accumulation indicates plant physiological status i.e. whether it stressed or not but unable to describe tolerance level in faba bean.

Besides proline accumulation, other amino acids are also observed in plants (Rai, 2002). In lentil methionine, isoleucine, valine, arginine, and histidine increased under drought stress (Muscolo et al., 2015). Drought stress-mediated GABA (gamma-aminobutyric acid) synthesis was observed in Arabidopsis, soybean and common bean (Bouche and Fromm, 2004). Abiotic stress enhance cytosolic Ca\textsuperscript{2+} levels which ultimately promote GABA synthesis, but till now no evidence found where GABA stimulates stomata opening or closing through this ion transporter function (Ramesh et al., 2017).

### 2.7.2 Polyamines

Besides amino acids, another plant response to abiotic stresses is polyamines (PA) synthesis, but the fact between PA accumulation and fortification remains vague. Three commonly found polyamines are putrescine, spermidine, and spermine. Usually stress tolerant accessions contain a higher amount of PA than sensitive accessions (Hatmi et al., 2014; Juzoń et al., 2017). Nevertheless, all three PA did not accumulate at the same time but rather one type of PA showed a stronger response. For example, under PEG osmotic stress conditions lentil cotyledon and root exhibit increased putrescine and spermidine while cadaverine presence was non-significant (Muscolo et al., 2015). Drought-adapted yellow lupin accessions produce a high amount of PAs compared to moderate adapted genotypes. Drought stress also increased
the concentrations of spermidine and spermine whereas putrescine was absent (Juzoń et al., 2017). Beside leaves, seeds commonly exhibit opposite trends where leaves accumulate more PAs and in seeds reduce content. Nayyar et al. (2005b) observed more PAs accumulation in chickpeas compared to soybean under water deficit conditions. In relay with legumes, 18 rice (Oryza sativa L.) cultivars showed significant higher spermine levels (Do et al., 2014). With this information, it can be said that PA accumulation does not depend on a single factor rather multiple events like as plant species, stress types and level, the physiological status of the studied tissues are involved. With the help of existence PA dynamics of abiotic stress can unveil contradictory results of PA accumulation. Stress tolerance of a given species often correlates with PA pool size which can answer some underlying question about the importance of PAs in assisting protection against stresses. In faba bean, spermine, cadaverine and total polyamine increase parallel with salinity level whereas putrescine and spermidine levels decreased (Sadak and Abdelhamid, 2015). In faba bean, PAs accumulation against drought has not been studied to date.

2.7.3 Organic Acids

Organic acid plays an important role in plants metabolism as an early source of photosynthesis and precursors of many other compounds. Organic acids support stomata regulation, ion equilibrium, ammonium fabrication and nutrients absorption (Ryan et al., 2001). Among profiled metabolite classes estimated from soybean leaf, organic acids occupy around 20% (Benkeblia et al., 2007). Nitrogen fixation in nodule is impacted by malic acid concentration as this organic acid regulates bacteroid respiration (Arrese-Igor, 1999). The abundance of organic acids also depends on types of organ. Organic acids were less in lentil root compared to cotyledon (Muscolo et al., 2015). Sassi et al. (2010) observed a reduced amount of organic acids in dry bean leaves while faced osmotic stress. Amount of succinate was found double in
drought treated soybean leaves. Reduced amount of organic acid in sensitive genotypes suggested metabolic flexibility, plant shifted from growth to survival (Silvente et al., 2012). Organic acids function against stress in faba bean has not yet been demonstrated but it can be a great area of interest in coming days.

2.7.4 Carbohydrates

Carbohydrates are often referred to as appropriate osmotica and one of the main components of osmoregulation in many plant species (Morgan, 1984). The metabolic profile resulting from the drought of a model and forage legume Lotus genus showed the accumulation of sugars, fructose, galactose, glucose and maltose, while arabitol, ununitol and galactitol are the most abundant polyols (Sanchez et al., 2012). In soybean leaves, about 30% of the metabolites identified were soluble sugars and sugar alcohols which are quite important for plant adaptation against stresses, especially during water deficit (Benkeblia et al., 2007). The close association among carbohydrates and their involvement in primary metabolism suggested a molecular modification may be possible to improve stress tolerance through synthesis and degradation of these compounds (Rajam et al., 1998). However, few accept the hypothesis that “Osmotic adjustment arises from the accumulation of solute due to the persistence of photosynthesis after cessation of leaf growth” (Turner and Jones, 1980).

Among the dominant carbohydrates, trehalose, the non-reducing disaccharide accumulates in large quantities (Bianchi et al., 1993; Drennan et al., 1993). A high amount of trehalose can stabilise proteins and membranes (Paul et al., 2008). It has recently been outlined that rhizobium inoculation can also increase the dehydration tolerance of legumes through trehalose biosynthesis (López et al., 2006; López et al., 2008; Räsänen et al., 2004; Zacarias et al., 2004). Moreover, increased trehalose concentrations found to be significant to contribute towards
drought tolerance (Iordachescu and Imai, 2008). Polyhydric alcohols or polyols is one of the major group of metabolites reviewed by Merchant and Richter (2011). The quantity of polyols found in plant tissues is found to be sensitive to environmental conditions (Merchant et al. 2006a; Monson et al. 2006). Further, Dumschott et al. (2018) characterised D- pinitiol as influenced by developmental stage. D-Pinitol is present in most legumes at some level under optimal conditions, but triggered during water and salinity stress (Ford, 1984). There remains a gap in understanding conditions leading to pinitol accumulation as well as its molecular control.

2.8 Conclusions

Improvement of global faba bean production will impact the food supply across many nations. Tools to improve faba bean production will incorporate both management activities and improved genotypes. Adoption of these tools must balance the necessity for broad-scale characterisation and cost with the precision required for appropriate detection at the plant scale. Some of the traits, like canopy temperature can be used efficiently for large volume accessions, while for example carbon isotope discrimination largely limited in suitability to a low number of accessions. Beside these, metabolite-based biomarkers and bio-indicators can easily supplement the selection process in a breeding program. Identification and quantification of a given primary and/or secondary metabolites against specific stress could become valuable decision making tools for a breeder if their associations are of sufficient strength. The available metabolomic resources from other legumes should be explored to understand molecular insight of stresses. The larger genome size of faba bean makes next-generation sequencing technologies a bit cumbersome for the crop. Finally, no single trait and approach are adequate to improve yield against the most complex trait namely drought, rather a combination of characteristics and methods are required.
Chapter 3
Diversity in carbon isotope discrimination of field grown faba bean and its association for yield prediction

Abstract
The use of carbon isotopes to assess plant physiological status is common but few studies extend such correlations to grain yields. A field study was performed to examine the suitability of carbon isotope discrimination ($\Delta$) isolated from leaves as an early prediction of grain yield in faba bean (*Vicia faba* L.) genotypes under late sown growing environments. The interrelationships between $\Delta$, grain yield and drought score were evaluated on 96 faba bean genotypes from diverse origin. Carbon isotope discrimination (based on leaf level organic matter) and drought score were highly correlated in our experiment ($R^2=0.90$). Among the tested accessions, locally developed breeding lines (Australian breeding lines) had better correlation for $\Delta$ vs yield and drought score vs yield compared to the exotic germplasm. Considerable genotypic variation was found for $\Delta$ among studied genotypes, which varied over 16.84 ‰ to 21.95 ‰. In our study, the variation of $\Delta$ was not significantly correlated with grain yield in general but showed a positive correlation for Australian breeding lines. These results suggested $\Delta$ can be a quick option to select tolerant genotypes for a large number of locally bred faba bean accessions, but not for exotic germplasm that is not adapted to conditions experienced in this region.

3.1 Introduction

A changing and increasingly variable climate is likely to negatively influence the production of widely grown crops, specifically those with rotations that occupy seasonal transitions. Cool season grain legumes such as faba bean often occupy sites/seasons subjected to both frost and drought stress (also termed here water deficit) in their life cycle due to its considerably long growing period (almost six months). Among cultivated grain legumes, faba bean is highly
sensitive towards water deficit (see for example Khan et al. (2010). In Australia, both the yield and production of faba bean depend on suitable climatic conditions and may not follow a regular pattern (Figure 3.1). Daryanto et al. (2015) demonstrated through a meta-analysis from 1980 to 2014, yield reduction was highest in faba bean (40%) under severe water scarcity (i.e., > 65% water reduction). In many cases, water limitation is closely coupled with heat and during flowering to pod development may cause significant yield reductions. Drought tolerant faba bean genotypes have been found in few studies (Abdelmula et al., 1999; Amede et al., 1999; Grzesiak et al., 1997; Khan et al., 2007; Link et al., 1999; Ricciardi et al., 2001) suggesting breeding prospect for the adaptation and yield improvement of this crop in dry environments.

**Figure 3:1** Pattern of faba bean production in Australia from 1999-2013 (Source: FAOSTAT, 2017)
A high throughput, cost-effective and non-invasive tool for the rapid and reliable evaluation of growth and yield under field conditions is of critical importance to plant breeder across a range of natural systems. Carbon isotope discrimination at the leaf level has been proposed as a method for evaluating WUE in C₃ plants and as an accurate technique for screening plants with higher tolerance under water deficit conditions. Carbon isotope abundance is extensively used to assess transpiration efficiency (TE) in cereals and recently in legumes such as common bean (Lockhart et al., 2016; Smith et al., 2016; Zacharisen et al., 1999) soybean (Clay et al., 2003), chickpea (Khan et al., 2004), cowpea (Hall et al., 1990) and faba bean (Khan et al., 2007). Drought tolerance can be predicted through Δ measurement as a surrogate for WUE or more directly by leaf-level gas exchange. However, Δ has several advantages over gas exchange. Measurements of Δ reflect WUE across the period of time in which the pool of C was incorporated into the plant tissue; therefore, Δ is time integrated and not sensitive to short term (hour-day) variation in WUE. In contrast, WUE measured by gas exchange provides instantaneous values of photosynthesis and conductance rates and therefore may not be representative of overall WUE across the scope of diurnal and seasonal conditions. Besides, the measurement of leaf level Δ is less time and labour intensive compared to whole plant WUE measurements in field conditions despite requiring off-site analysis. With these properties in mind, the potential for Δ to act as a selection tool for plant improvement programs requires further investigation. Therefore, the aims of this work were:

i) to investigate whether genotypic differences in carbon isotope abundance are present in faba bean which may be exploited to the development of suitable cultivars; ii) to determine whether a relationship exists between Δ and grain yield under water deficit to identify contrasting genotypes for future breeding programs, and iii) to compare the values of Δ against drought score evaluations with the aim of assigning the value of Δ or drought score for the selection of drought-tolerant faba bean genotypes.
3.2 Methodology

3.2.1 Field Trial

This experiment was carried out at the I.A Watson Grains Research Centre, Narrabri, NSW-2390, Australia (30° 27' S, 149° 80' E), from May to November 2014. The soil of the experimental trials soil was vertosols according to Isbell (1996), and pH was classified as moderately alkaline, with a value of 8.2. Rainfall is summer dominant with an annual average of 547 mm and general crop growing condition for this region is rainfed. Daily maximum and minimum temperature along with accumulated rainfall data were recorded through a data logger which was placed 200 meter away from field trial. Further details can be found here: http://ozforecast.com.au/cgi-bin/weatherstation.cgi?station=11250

3.2.2 Weather conditions

On average, temperatures varied between 2 °C and 30 °C, (Figure 3.2) throughout the growing season. The lowest temperature was observed -6 °C in July and highest in November 37.5 °C.
Figure 3:2 In-season maximum and minimum temperature (°C) and rainfall (mm) over three sowing date in *Vicia faba* field trials conducted in 2014 at I. A. Watson Grain Research Station, Narrabri

3.2.3 Plant materials and trial design

Ninety-six diverse faba bean genotypes were collected from faba bean improvement program at I. A. Watson Grain Research Centre, Narrabri. The set of germplasm included commercial cultivars (5), exotic germplasm (16) and breeding lines (75) from the national faba bean breeding programs in Australia. Germplasm list was presented in Appendix 3.1. Seed sowing was completed on the 26th of May, 2014 with a view to exposing them to drought where regular seeding for this region is the first week of May (Abebe et al., 1998; Singh et al., 1997). The experiment was laid out in a RCBD (Randomized Complete Block Design) fashion with two replicates. The plot was comprised with 10 m long two rows where row to row distance was 50 cm. The plant density was approximately 20-25 plants m$^{-2}$.

3.2.4 Crop management

The experiment was sown in a field which had wheat in the previous crop season, ploughed and leveled. Seeds were pre-treated with commercially available faba bean inoculant
(Rhizobium strain, group G WSM 1455). Inoculation was achieved by suspending a mesh bag containing peat inoculant in a water tank and pumping the water/inoculant suspension into the furrow following seed placement. Sowing was carried out using a four-row planter with 50 cm inter-row spacing. Pre-emergence and post-seeding weedicides namely, Spinnaker 700WG (Imazethapyr 700 g kg⁻¹) and Terbyne 750 WG (Teerbuthylazine 750g/kg) were applied at a recommended rate to prevent the establishment of a range of grass and broadleaf weeds. No mineral fertilizer was applied in the trial. A continuous buffer blocks of PBA Warda were planted on the outside of the trial. 30 mm of water was applied by overhead lateral move irrigator on 10th September and 30th September, 2014. Grain yield was weighed after harvesting trial plots by using a mechanical harvester (HALDRUP C-65).

### 3.2.5 Data recording

A visual score of drought (0-5; 0: no wilting, 1: 20% wilted plot; 2: 40% wilted plot; 3: 60% wilted plot; 4: 80% wilted plot; 5: More than 80% wilted plot) was adopted from (Chapman et al., 1990). Scoring was completed during the grain filling stage when 50% plant per plot had at least three filled pods. Genotypes had similar phenology, scored on the same day between 8.00 am to 11.00 am. Harvesting was completed by mechanical harvester upon maturity. Plot yield data was calculated into kg ha⁻¹.

### 3.2.6 Carbon isotope analysis

Leaf samples were collected on 22nd October 2014 on a clear sunny day between 3.00 pm to 5.00 pm. From each plot, a second fully expanded leaf from the top was collected with a sharp razor and immediately placed into a 2 ml microtube tube in an icebox to reduce metabolism. Within 2 hours, leaf samples were placed in an oven at 65 °C for 48 h to completely dry. An oscillating matrix mill was used to grind the dried leaf sample. Approximately 3 mg of ground
leaf was then placed into silver capsules (IVA Analysentechnik, Meerbusch, Germany) and then analysed according to the protocol outlined in Smith (2018). The precision for the standard materials was between 0.04 ‰ and 0.08 ‰.

3.3 Statistical analysis

Correlations were determined between yield, drought score and Δ (calculation of Pearson correlation coefficients). Genotypes were separated according to the origin (Exotic and Australian breeding line). A correlation was performed firstly on full germplasm set and later the genotypic group of origin. Regression coefficients were calculated with the Sigma Plot 11.0 software package. The broad sense heritability (h² B) = $\delta^2 g / \delta^2 p$, the phenotypic variance ($\delta^2 p$) = $\delta^2 g + \delta^2 e$, where $\delta^2 g$ = the variance of genetic effect, $\delta^2 e$ = the environmental/error variance.

3.4 Results

The germplasm set in this study comprised with Australian breeding lines and some un-adapted lines from different origin around globe. Due to huge diversity in their grain yield we didn’t perform any structured analysis rather we focused on arithmetic mean of yield. Our primary target was to extract the diversity information based on carbon isotope discrimination from this germplasm set.
3.4.1 Genotypic variability in Δ and grain yield

Variation among accessions was significant for grain yield, drought score and Δ (P < 0.01) for all. The average grain yield ranged from 237 to 1992 kg ha\(^{-1}\) (Table 3.1). The lowest grain yield was observed in ATC65276 (237 kg ha\(^{-1}\)) - an exotic germplasm. Among the Australian breeding lines, the highest yield was 1992 kg ha\(^{-1}\) produced through genotype 11NF007e-6 (Table 3.1). Almost half of the studied genotypes produce grain yield ≥ 1000 kg ha\(^{-1}\) (Figure.3). There were significant difference among genotypes in grain yield (P < 0.01). Drought score varied from 0-5 for Australian breeding and exotic lines. Two genotypes, one from the exotic origin and another from an Australian breeding line AC0805#4912 and 11NF008b-15 showed a higher level of tolerance against drought and scored zero. Out of 96 genotypes, 12 genotypes including one exotic line showed high sensitivity towards drought with a score of five. Drought response among genotypes varied significantly (P < 0.01). Leaf level Δ varied from 16.84‰ to 21.95‰ which was the average value for C\(_3\) leaves (~20‰) (Mihoub et al., 2016). The lowest value for Δ (16.84‰) was observed in IX564c/1-7 genotype and highest (21.95‰) was in Ac0805#4912. Mean Δ for exotic lines was higher than the Australian breeding line (Table 3.1). Values of Δ across genotypes were highly significant (P < 0.01). Though we did not show intrinsic WUE in our experiment, we calculated from Δ and found WUE was highly variable among genotypes 89.22-179.75μmol CO\(_2\) mol\(^{-1}\) H\(_2\)O (data not presented).

Table 3:1 Descriptive statistics of 96 faba bean genotypes grown in Narrabri, 2014

<table>
<thead>
<tr>
<th>Traits</th>
<th>Australian breeding lines</th>
<th>Exotic lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min.</td>
<td>Max.</td>
</tr>
<tr>
<td>Drought Score</td>
<td>0.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Carbon isotope</td>
<td>16.84</td>
<td>20.07</td>
</tr>
<tr>
<td>Yield (kg ha(^{-1}))</td>
<td>237</td>
<td>1992</td>
</tr>
</tbody>
</table>
Figure 3.3 Variability in grain yield (red circles) and leaf carbon isotopes (Δ) values (blue bars) of 96 diverse faba bean genotypes grown at Narrabri, 2014. Data presented were the arithmetic means of two replications.

3.4.2 Correlation among traits

To understand the contribution of different traits towards drought tolerance, correlations were calculated between drought score and different characteristics. Relationships illustrated the superiority of Australian breeding lines over exotic germplasm. There was a poor correlation ($R^2=0.23$) between grain yield and Δ that were observed among examined genotypes (Figure 3.4). However, when these accessions were separated into two groups, i.e. exotic germplasm and Australian breeding lines, a better correlation was observed among Australian breeding lines and grain yield ($R^2=0.46$), whereas with exotic lines had a weak correlation ($R^2=0.13$). Moreover, Δ was significantly correlated with yield for all genotypes ($P < 0.01$) and Australian breeding lines ($P < 0.001$).
Figure 3:4 Correlation between grain yield (kg ha\(^{-1}\)) and carbon isotope discrimination for the 96 faba bean genotypes at Narrabri, NSW 2014. A. Correlation between the yield of all germplasm and drought. B. Correlation between the yield of all exotic lines and drought. C. Correlation between the yield of Australian breeding lines and drought.

The degree of yield reduction increased with drought intensity. A weak correlation (R\(^2\) = 0.18) was observed across the whole germplasm with drought score. However, a higher correlation (R\(^2\) = 0.50; P < 0.01) was found in the Australian breeding lines with drought score (Figure 3.5C).
Figure 3:5 Correlation between grain yield (kg ha\(^{-1}\)) and drought score for the 96 faba bean genotypes at Narrabri, NSW 2014. A. Correlation between the yield of all germplasm and drought. B. Correlation between the yield of all exotic lines and drought. C. Correlation between the yield of Australian breeding lines and drought.

Correlation between drought score and \(\Delta\) was found highly significant (\(R^2 = 0.89; P < 0.01\)) (Figure 3.6A). The interrelationship between Australian breeding lines and drought score was also significant (\(R^2 = 0.95; P < 0.01\)) (Figure 3.6B) with the same patterns observed for exotic lines (Figure 3.6B).
Figure 3.6 Relationship between Δ and drought score for the 96 faba bean genotypes at Narrabri, NSW 2014. A. Correlation between Δ of all genotypes and drought. B. Correlation between Δ of all exotic lines and drought. C. Correlation between Δ of Australian breeding lines and drought. ** and *** represents significant differences where P-value was < 0.01 and < 0.001 respectively.
3.4.3 Heritability estimates for grain yield and carbon isotope

Overall heritability was high for both grain yield and carbon isotope which has a range from 65.13 to 93.88 (Table 3.2). Heritability was first estimated for all genotypes and later for Australian breeding lines as well as lines from exotic origin. Heritability was low in exotic lines compare to Australian breeding lines. Carbon isotope showed stable and high heritability for both Australian breeding and lines from exotic origin.

**Table 3:2** Heritability estimates for 96 faba bean genotypes under late sown condition at narrabri, NSW.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Yield</th>
<th>Carbon isotope discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>EMS</td>
</tr>
<tr>
<td>All genotypes</td>
<td>180071</td>
<td>24520</td>
</tr>
<tr>
<td>Australian breeding lines</td>
<td>283560</td>
<td>17351</td>
</tr>
<tr>
<td>Exotic origin</td>
<td>150275</td>
<td>52304</td>
</tr>
</tbody>
</table>

3.5 Discussion

3.5.1 Use of diversity in carbon isotope discrimination for plant improvement

One of the principal requirements for a successful breeding program is diversity in germplasm. A desirable trait must show high heritability, wide genetic variation and a good correlation with yield (Araus et al., 2002). In our study, we observed the genotypic effect on Δ is highly significant and varied from 16.84‰ ~ 21.96‰ (Table 3.1, Figure 3.1). Stomatal conductance and photosynthetic capacity are highly influential for the variation in Δ. Therefore, variation in Δ is a collective form of genotypic variability in stomatal conductance and the intensity of terminal drought. Variability for Δ on a large population has not been previously shown for faba bean but demonstrated for chickpea (Krishnamurthy et al., 2013). The Δ value found in this experiment is comparable with a previous study conducted by Khan et al. (2007) on faba bean where Δ was varied over 20.1‰ ~ 23.3‰ among four genotypes. Considerable variation
among adaptive traits is desirable for breeding programs but heritability must be examined before that trait may be used as a selection criterion. All the tested genotypes showed high heritability for both carbon isotope and grain yield. Australian breeding lines evolved with higher heritability than exotic lines. In this field study the broad sense heritability estimation was similar (88%-90%) to cowpea reported by Hall et. al (1990). The high heritability recorded for Australian breeding lines may be associated with the breeding environment where these genotypes have been developed. The high heritability of Δ and its strong genetic correlation with grain yield indicates that Δ is a suitable selection criterion in faba bean breeding programs. WUE, calculated from isotopic discrimination values can also give a performance indicator of the genotypes under drought stress that has been demonstrated in legumes and cereals (Polania et al., 2016; Rebetzke et al., 2002). This variation can be utilised to identify water-efficient faba bean genotypes under field conditions. Turner et al. (2007b) showed no such relationship between three grain legumes under well-watered conditions.

Similarly, experiments conducted during the wet season had, expectedly, no such correlation in cowpea (Austin et al., 1990). These results highlight that selection based on Δ is influenced by weather and soil moisture; therefore, they must be taken into account in designing appropriate selection trials. Genotypes with low discrimination values have relatively high water use efficiency and higher photosynthetic capacity; hence this does not always translate to yield for faba bean because as an indeterminate crop its phenology is quite complex. A further complication is that due to the ‘isohydric’ approach employed by faba bean during water deficit, the reductions in stomatal conductance inhibits carbon fixation and consequently yield but Δ will not be influenced. However, having higher WUE and higher grain yield would be desirable selection criteria under water-limited environments.
3.5.2 Grain yield prediction through Carbon isotope discrimination

Within breeding strategies to improve drought tolerance, misconceptions often arise among yield potential, WUE and drought tolerance (Blum, 2011). The inconsistent association between Δ and yield is a critical issue for using Δ in drought tolerance or WUE breeding programs. This inconsistency is primarily driven by the soil, moisture, environment and has been established through a range of study, i.e. chickpea (Krishnamurthy et al., 2013), canola (Luckett and Cowley, 2011), safflower (Mihoub et al., 2016) and cowpea (Ngugi et al., 1996). Our study demonstrated a significant correlation between Δ and grain yield among the full germplasm (Figure 3.4A) as well as for Australian breeding lines (Figure 3.4B) but not for the lines from the exotic origin (Figure 3.4C). Krishnamurthy et al. (2013) also demonstrated similar findings where Δ of adapted accessions were associated with grain yield. Accessions demonstrating higher Δ (i.e. low WUE) produced lower grain yield. This statement is true for most of the genotypes studied in our trial but two genotypes 11NF02c-11 (1560 Kg ha⁻¹) and 11NF03a-10 (1620 Kg ha⁻¹) had higher grain yield despite their high Δ value, i.e. low WUE. These genotypes can be valuable to investigate further, what other properties enabled them to produce higher grain yield despite their low WUE. In general, the yield was quite variable regardless of the Δ value in our study (Figure 3.3). Our target is to select genotypes that can produce higher grain yield and efficiently use stored moisture. Combined, these observations suggest that under environments commonly limited by the availability of water, Δ acts as a suitable selection tool for projected yield in adapted genotypes. While this is a promising observation taken from a large population, several caveats (heritability, seasonal variation) must be considered before adopting a broad scale application of Δ as a selection tool for faba bean breeding program.
3.5.3 Variation in drought score and its relationship with grain yield

Drought score has been extensively used as a selection criterion in a breeding program (Chang, 1974; Singh et al., 2013; Singh et al., 1997). The degree of screening efficiency largely depends on the intensity of drought along with crops developmental stage. We completed scoring for drought tolerance during the early podding stage as faba bean was found to be most sensitive to drought during this stage (Mwanamwenge et al. 1999). Drought score was completed based on per cent wilted plant in a plot basis, which ultimately reflects the biomass accumulation of a plot and biomass contributed to harvest index as well as yield. As expected, genotypes having high drought score had the lowest grain yield. Our findings align with a study with Nikolić et al. (2012) where the yield of maize genotypes negatively correlated with drought score. In our experiment, yield had significant negative correlation with drought score for Australian breeding lines but non-significant for full germplasm set (Figures 3.5A & 3.5C). The similar findings observed for chickpea where drought score in the late sowing condition was highly correlated with grain yield (Singh et al., 1997). The non-significant relationship between exotic lines and drought score may define by their distinct indeterminate nature. A high rainfall (after drought scoring) helps to produce flowers and subsequently pod up to the end of exotic lines life cycle. The late rainfall helped most of the genotypes to recover from drought and produced yield. Finally, it can be said selection for drought tolerance based on grain yield need to be conducted under true water deficit conditions (using rainout shelter) to get accurate results and selection at vegetative stage need to be supplemented by total plant number of the experimental plot.

3.5.4 Efficacy of Δ a selection tool

In C3 plants, low Δ values of leaves and seeds were primarily linked with drought tolerance (Monneveux et al., 2005), and consequently to high WUE (Farquhar and Richards, 1984).
Numerous studies have been completed where $\Delta$ is used to identifying drought tolerant genotypes in different crops namely sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*) faba bean and maize (*Zea mays*) (Adiredjo et al., 2014; Brito et al., 2014; Khan et al., 2007; Monneveux et al., 2007). In most of these cases, researchers have correlated yield with the sensitivity of genotypes under different moisture level. In contrast, here we compared the traditional visual assessment for drought scoring (percentage of wilted plant per plot) with $\Delta$. The significant higher correlation between drought score and $\Delta$ may be due to the phenology of the crop and scoring time (drought scoring and leaf collection for $\Delta$ analysis were done on the same day). It has previously been shown that the time and frequency of the scoring influenced the correlation between yield and drought score in chickpea (Singh et al., 1997). Therefore, it is recommended that further investigations involving repeated scoring throughout developmental stages need to be completed to ascertain the influence of developmental stage on the correlation between $\Delta$ and drought score. The similar observation was also demonstrated in rice where visual scoring was significantly correlated with leaf $\Delta$ in seedling stage against salt stress (Shaheen and Hood-Nowotny, 2005). To have an understanding of diversity in $\Delta$ and their determinant factors, it is sensible to collect samples from different growth stages as well as different tissues.

### 3.6 Conclusion

Significant variation in $\Delta$ is observed among our studied genotypes from different environmental origins. The documentation of this diversity will assist in the design of future experiments seeking improvements in WUE and prediction of grain yield. Development of superior genotypes having high WUE and grain yield is required to increase production. To this end, genotypes 11NF026c-11 and 11NF003a-1 showing higher grain yield, but low WUE in this experiment might be interesting to researches to understand underlying mechanisms.
possessing opposite qualities. To the best of our knowledge, this is the first experiment to assess the association between Δ and grain yield in a large number of faba bean accessions under field environments. A higher correlation among Δ and grain yield on Australian breeding lines suggest the potential of this trait as a selection criterion under field trials. We also showed a higher correlation among the drought score and Δ, which needs to be confirmed by sequential measurements under controlled conditions.
Chapter 4
Phenology, morphology and physiology of selected faba bean genotypes grown under irrigated and rainfed conditions in Northern NSW

Abstract
Faba bean (Vicia faba L) has wide genetic variation depending on the trait of interest. Crop phenology needs to be matched with the environmental condition to improve adaptation and maximise yield potential. Few studies have been carried out to understand faba bean phenology especially to examine effect for different sowing dates and for the impact of water deficit. Here we examined five faba bean genotypes for their grain yield and component characters based on phenology, morphology and physiology. Our objectives were to identify adaptation traits and suitable phenology that contributed to grain yield. Faba bean genotypes, AC0805#4912, 11NF008b-15, 11NF010c-4, 11NF020a-1 and PBA Warda possessing contrasting carbon isotope discrimination value were evaluated two growing seasons 2015 and 2016 in both rainfed and irrigated trial. Due to adequate rainfall, no treatment effect was observed for all studied characters except maturity, plant height and pods plant\(^{-1}\). To reach maturity, required thermal time was almost similar for all genotypes but to reach each phenological stage; it differed. Yield variation was observed for season to season and genotype. Genotype AC0805#4912 had the lowest grain yield despite its highest branch plant and the highest number of pods plant\(^{-1}\). Physiological characters (NDVI and canopy temperature) measured in this study only showed genotypic effect. Selection based on plant height, leaf area, seed weight and harvest index would increase the selection efficiency in faba bean breeding program.

4.1 Introduction

Grain legumes are an essential component of the cropping system due to their nitrogen fixation capability. Among cultivated legumes, Faba bean (Vicia faba L) can fix around 160 kg ha\(^{-1}\)
nitrogen in a growing season, half of which is left in the field for the following crop (Baddeley et al., 2013; Hoffmann et al., 2007). The high nitrogen fixation is correlated with its higher above ground biomass production which makes it favourable to growers. Changing climatic conditions, i.e. unprecedented rainfall and high/low temperature make faba bean cultivation vulnerable in coming years. The acreage of faba bean declined over last 50 years but interestingly global yield (t ha⁻¹) increased (Foyer et al., 2016) meaning that presently faba bean cultivation is restricted to favourable climatic conditions. The adaptation of faba bean in wide-ranging environmental conditions, make it suitable for farmers’ options but systematic approaches are needed to develop variety which has adaptability in adverse climatic condition (Köpke and Nemecek, 2010).

Faba bean seed is used as food and feed due to their high protein content. Average protein content is 29% (Feedipedia, 2018), ranging from 24 to 35% (Crépon et al., 2010) making it the most protein-rich grain of starchy legumes. Its cultivation in a rotational cropping system helps to reduce soil-borne disease incidence in cereals (Landry et al., 2016). In Australia, faba bean is widely grown as a rotational crop with wheat. Australia is the largest exporter of faba bean globally and ranked fourth for production after China, Ethiopia and the United Kingdom (FAOSTAT, 2017).

Faba bean is found to be the most drought-prone crop among cultivated legumes, and moisture stress caused almost 50 % yield loss depending on the crop developmental stage (Khan et al., 2010). Breeding programs aiming to improve drought tolerance are primarily confined to the selection of high yielding genotypes under moisture stress environments. However, selection of genotypes solely based on grain yield may not be effective as the incidence and severity of drought changes season to season. Usually, plants modify their morpho-physiological traits such as reduced stomatal activity, leaf drooping etc. to cope with moisture stress. Selection of drought tolerant genotypes based on physiological characteristics had some progress in this
crop (Khan et al., 2007; Khazaei et al., 2013). Thus, selection for drought tolerance, supplemented with physiological traits can contribute to an effective breeding program.

Several morphological and physiological characters were studied to select drought tolerant genotypes in faba bean (Ali, 2015; Siddiqui et al., 2015). However, most of them are from either the Mediterranean or continental environment with a cool summer. Due to the plasticity of a character or environmental sensitivity, it is judicious to understand the underlying mechanisms that regulate phenology of a plant under moisture stress. This could pave the way for dissecting adaptation strategies of faba bean at different moisture levels. Therefore, on top of yield potential of different genotypes, it is necessary to know the growth habit, morphological and physiological characteristics of that specific genotype (Agegnehu et al., 2006).

Regardless of economic significance and the importance of sustainable farming, the interrelationship between phenology, physiology and growth characteristics of faba bean grown under moisture stress is poorly understood. This study was undertaken to study the influence of environment, especially moisture effect on the acclimation process of contrasting faba bean genotypes in northern NSW growing environment. These were achieved through documenting phenological development, morphological traits and yield components analysis of selected faba bean genotypes in a rainfed and irrigated trial over two seasons.
4.2 Methodology

4.2.1 Plant materials and trial design

The experiment was carried out over two growing seasons, i.e. in 2015 (11th May to 25th October) and 2016 (7th May to 29th October) at the I. A. Watson Grains Research Centre at Narrabri, New South Wales (30°16'27.0"S 149°48'33.5"E). This experiment was designed to test four contrasting faba bean genotypes (AC0805#4912, 11NF020a-1, 11NF010c-4, and 11NF008b-15) and PBA Warda as a popular variety suitable for northern NSW growing region. These four genotypes were selected from 96 diverse genotypes having diverse carbon isotope (Δ) value from our initial screening experiment (Chapter 3) which was conducted at the same location in 2014. Each genotype was sown in five replicates of individual plot comprised of four rows of 10 m long and 50 cm apart. The plant density was adjusted approximately 20 plants m⁻². Two treatments were applied as irrigated and rainfed. Irrigated and rainfed blocks were sown side by side but separated by a buffer plot of PBA Warda variety (48 m X 10 m). Daily maximum and minimum temperature along with accumulated rainfall data were recorded through a data logger placed near field trial. Further details can be found here: http://ozforecast.com.au/cgi-bin/weatherstation.cgi?station=11250

4.2.2 Experimental field management

The experiment was sown in a field which had wheat in the previous crop season, ploughed and leveled. Seeds were pre-treated with commercially available faba bean inoculant (Rhizobium strain, group G WSM 1455). Inoculation was achieved by suspending a mesh bag containing peat inoculant in a water tank and pumping the water/inoculant suspension into the furrow following seed placement. Sowing was carried out using a four-row planter with 50 cm inter-row spacing. Pre-emergence and post-seeding weedicides namely, Spinnaker 700WG
(Imazethapyr 700 g kg\(^{-1}\)) and Terbyne 750 WG (Teerbuthylazine 750g kg\(^{-1}\)) were applied at a recommended rate to prevent the establishment of a range of grass and broadleaf weeds. No mineral fertilizer was applied in the trial. Karate Zeon® (250 g L\(^{-1}\) lambda-cyhalothrin) at the rate of 36 ml ha\(^{-1}\) were applied to control insect pest as well as aphids which were regular farm practice during 50% flowering. Mancozeb (750 g kg\(^{-1}\)) was sprayed before the canopy closure (1.7 kg ha\(^{-1}\)) to control rust and chocolate spot. In 2015, irrigated plots received two irrigations, 35 mm each at early flowering and late podding stage on top of in-season rainfall of 180.6 mm. The rainfed trial received only 180.6 mm rainfall throughout the growing season. In 2016, rainfed plots received 447 mm rainfall over the season. The irrigated plots received a total 472 mm during this period which is 25 mm more than the rainfed plot. Grain yield was weighed after harvesting trial plots by using a mechanical harvester (HALDRUP C-65).

4.2.3 Data recording

Morphological, physiological and phenological data were recorded from flowering to maturity. Unless stated otherwise, all the physiological and morphological parameters were recorded at three pod stage, i.e. when 50% plants of a plot had at least three developing pods.

Leaf area measurement was adopted from Peksen (2007) with the formula

\[ LA = 0.919 + 0.682LW \]

Where \( LA \) = leaf area \((\text{cm}^2)\), \( L \) = leaflet length \((\text{cm})\), \( W \)= Leaflet width \((\text{cm})\)

![Leaf Measurement Diagram](image)

**Figure 4:1.** Measured parts of the faba bean leaflet to determine the leaflet length \((L)\) and width \((W)\). Source (Peksen, 2007).
Plant height was measured using a ruler from the base of the stem at ground level to the tallest growing tip from 10 randomly selected plants per plot and tagged them for final harvest. The number of branches plant$^{-1}$ was counted manually. Pods plant$^{-1}$ and harvest index (HI) of those ten tagged plants were harvested at maturity in an individual mesh bag and let them dry in a shed. Pods plant$^{-1}$ was achieved by counting the pods and average from those representative plants. Harvest index was measured with the formula:

$$\text{HI (\%)} = \left(\frac{\text{Grain yield}}{\text{Biological yield}}\right) \times 100.$$ 

Harvesting of whole plot was completed by mechanical harvester upon maturity. Plot yield data were converted into kg ha$^{-1}$.

Physiological data, i.e. Normalised Difference Vegetation Index (NDVI) measurements were completed using a GreenseekerTM machine on a clear sunny day between 10.00 am to 12.00 pm. Canopy temperature (°C) was measured with a FlukeTM infrared temperature gun on the same day of the NDVI measurement.

Data on four phenological stages, i.e. first flowering, flowering to pod initiation, pod filling and maturity were initially recorded as the number of days and later converted to growing degree days (GDD). Days to first flowering was recorded when the plot had at least one fully opened flower. Days to pod initiation and pod filling duration were recorded as days required initiating pod from flower and duration to develop full pod with filled seed. Five flowers per replicate were tagged to monitor pod initiation and pod filling duration. Days to maturity was recorded when at least 75% plant of the plots turned to physiological maturity.
GDD to reach each phenological stage was calculated through the following equation:

$$GDD = \left( \frac{T_{\text{max}} + T_{\text{min}}}{2} - T_{\text{base}} \right)$$

Where, $T_{\text{max}}$ and $T_{\text{min}}$ are the maximum and the minimum daily temperatures ($^\circ$C), $T_{\text{base}}$ was the base temperature equal to 0 $^\circ$C, if the minimum temperature went down below 0 $^\circ$C it was tailored as 0 $^\circ$C.

4.2.4 Weather

The weather conditions for both seasons at the experimental site was summer rainfall dominant. Water supply during the vegetative growth period was high and rainfall was evenly distributed throughout the growing period. In 2015 rainfed trial received 180.6 (mm) rain and irrigated trial 251 (mm) whilst in 2016 rainfed trial received 447 (mm) and irrigated trial received extra 25 (mm) at pod filling. Monthly distribution of precipitation along with average maximum and minimum air temperature is presented in Figure 4.2. In 2015 growing season 16 days had minimum temperature < 0 $^\circ$C (0 to -2 $^\circ$C) and the crop was in vegetative to the early podding stage and five days had maximum temperature > 30 $^\circ$C (30 to 35 $^\circ$C) and the crop was in early maturity stage. In 2016, seven days had minimum temperature < 0 $^\circ$C (0 to -1.3 $^\circ$C) and the crop was in vegetative to an early podding stage and five days had maximum temperature >30 $^\circ$C (30 to 32 $^\circ$C) and the crop was in early maturity stage.
Figure 4.2 In-season monthly rainfall (mm), average maximum and average minimum temperature (°C) for faba bean genotypes grown at Narrabri in 2015 (Left panel) and 2016 (Right panel).

4.3 Statistical analysis

GenStat 18th Edition (VSN International, Hemel Hempstead, UK) was used to perform all analyses. A linear mixed model (Residual Maximum Likelihood or REML) was used to estimate variance components and their interactions. Data for the year 2015 and 2016 were combined and Bartlett’s test for homogeneity for yield was performed. For the combined analysis seasons, treatments and genotypes were considered as fixed effects and replicate within each genotype considered random effects. Pearson correlation among traits along with a level of significance was performed by XLSTAT 2018.

4.4 Results

4.4.1 Phenology and morphology

The studied genotypes showed variation among different phenological stages regarding thermal requirements to reach each phenological stage (Figure 4.3). Thermal time for first flowering varied from 601 to 717 GDD with an average of 654 GDD. Genotype AC0805#4912
had the highest (717) GDD for its first flowering and PBA Warda had the lowest (601) GDD. From the first flower to pod initiation thermal time was more or less similar for all studied genotypes with a range from 65-93 GDD. In this phenological stage, genotype 11NF008b-15 had the highest (93) GDD and 11NF020a-1 had the lowest (65) GDD to develop pod from its first flower. For the duration of pod filling, PBA Warda had the highest (248) and AC0805#4912 had the lowest (180) GDD for pod filling stage which was opposite from first flowering stage. From pod filling to maturity, 11NF010c-4 had the lowest GDD requirement, i.e. 189 GDD and the highest for PBA Warda (271). Overall, the highest accumulated thermal time from germination to physiological maturity was for genotype AC0805#4912 which was 1222 GDD and the lowest (1155 GDD) was for 11NF010c-4.

![Figure 4:3](image)

**Figure 4:3** Growing degree days (GDD) for the phenological stage of five faba bean genotypes grown under rainfed and irrigated conditions. Data were mean values from the 2015 and 2016 seasons and there was no treatment effect observed in those seasons.

It was found that rainfall was higher in 2016 growing period compared to 2015 (Figure 4.2); as a result, all the studied morphological traits had higher mean value in 2016. Genotype AC0805#4912 had the highest days to 50% flowering and maturity time whilst PBA Warda
had the earliest (Table 4.1) with a range from 70-86 days and 147-165 days respectively. Days to 50% flowering had significant (P < 0.05) genotypic effect but had no significant treatment effect. Days to maturity had both genotypic and treatment effect was significant (P < 0.01). Leaf area was found highest in PBA Warda (8.7 cm²) and the lowest (4.6 cm²) in AC0805#4912. It was also significant for genotype and genotype-season interaction (P < 0.01) but non-significant for treatment, season as well as season-treatment interaction. The highest plant height (108 cm) was observed in 11NF020a-1 and the lowest (63 cm) was in genotype AC0805#4912. Plant height was significant for genotype (P < 0.01), season, as well as treatment (P < 0.05). Plant height was also significant for genotype-season interaction (P < 0.05)

4.4.2 Physiological parameters

Normalised Difference Vegetation Index (NDVI) was found to be significant (P < 0.05 and) among genotypes. The overall NDVI ranged from 0.70 to 0.72. The highest (0.72) NDVI was found in genotypes AC0805#4912 and PBA Warda whilst lowest (0.70) in 11NF008b-15. NDVI was non-significant for treatment, season and all the interactions. Canopy temperature which may reflect plant water status under stress conditions was also found to be non-significant for treatment, season and all the interactions but found significant (P < 0.01) among genotypes. Canopy temperature was lowest (21 °C) for the genotype 11NF010c-4 and highest (22 °C) for 11NF080b-15 (Table 4.1). There were no two way and three-way interactions observed for NDVI and canopy temperature.

4.4.3 Grain yield and yield components

Genotype AC0805#4912 had the highest number of branches plant⁻¹ (15) and the lowest (4) was in 11NF010c-4 and PBA Warda. The number of branches plant⁻¹ was significant for
genotype and the genotype-season interaction ($P < 0.01$) but non-significant for treatment, season as well as season-treatment interaction. The highest number (132) of pods plant$^{-1}$ was found in AC0805#4912 whilst the lowest (35) in 11NF010c-4. The number of pods plant$^{-1}$ was significant for genotype ($P < 0.01$), season, treatment and for season-treatment interaction ($P < 0.05$). No three-way interaction was observed for any morphological traits.

The average 100 seed weight ranged from 27- 65 g. The lowest was found for genotype AC0805#4912 and the highest for 11NF010c-4. There was significant ($P < 0.01$) difference found in seed weight for genotype, season and genotype-season interaction. Overall, HI was found lowest in season 2015 compared to 2016 (Table 4.1). Harvest index was ranging from 36-42 %. The highest (42%) HI was observed in genotype 11NF010c-4 and 11NF020a-1, and the lowest (36%) in AC0805#4912. Harvest index was also found significant ($P < 0.01$) for genotype and season only. The lowest grain yield was found in genotype AC0805#4912 and highest was in PBA Warda with a range from 1574-2228 Kg ha$^{-1}$ (Table 4.1). There was a significant difference in grain yield for seasons and 2016 had higher grain yield (Table 4.1). Genotype also found significant ($P < 0.01$) for grain yield. There were significant ($P < 0.05$) two-way interactions between season and genotype that were also observed for grain yield. Genotype was the dominant determinant in the interaction whilst season also had an impact (Table 4.1). There were no three-way interactions observed for any yield-constructing trait.
Table 4:1 Analysis of variance of measured morphological and physiological traits of faba bean genotypes grown under irrigated and rainfed condition over two years (2015 and 2016) in Narrabri, northern NSW.

<table>
<thead>
<tr>
<th>Season</th>
<th>Days to 50% flower</th>
<th>Days to maturit</th>
<th>Number of branch plant$^{-1}$</th>
<th>Leaf area (cm$^2$)</th>
<th>Plant Height (cm)</th>
<th>Number of Pods plant$^{-1}$</th>
<th>100 seed weight (g)</th>
<th>Harvest index (%)</th>
<th>Yield (Kg ha$^{-1}$)</th>
<th>Normalised difference vegetation index -</th>
<th>Canopy temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td>76</td>
<td>151</td>
<td>6</td>
<td>7.8</td>
<td>94</td>
<td>62</td>
<td>53</td>
<td>43</td>
<td>1875</td>
<td>0.71</td>
<td>22</td>
</tr>
<tr>
<td>2016</td>
<td>77</td>
<td>157</td>
<td>7</td>
<td>8.1</td>
<td>96</td>
<td>66</td>
<td>55</td>
<td>39</td>
<td>2142</td>
<td>0.72</td>
<td>23</td>
</tr>
</tbody>
</table>

Genotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Days to maturit</th>
<th>Number of branch plant$^{-1}$</th>
<th>Leaf area (cm$^2$)</th>
<th>Plant Height (cm)</th>
<th>Number of Pods plant$^{-1}$</th>
<th>100 seed weight (g)</th>
<th>Harvest index (%)</th>
<th>Yield (Kg ha$^{-1}$)</th>
<th>Normalised difference vegetation index -</th>
<th>Canopy temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC0805#491</td>
<td>86d</td>
<td>165e</td>
<td>15b</td>
<td>4.6a</td>
<td>63a</td>
<td>132c</td>
<td>27a</td>
<td>36a</td>
<td>1574a</td>
<td>0.72b</td>
</tr>
<tr>
<td>11NF008b-</td>
<td>73b</td>
<td>150b</td>
<td>5a</td>
<td>8.2b</td>
<td>103c</td>
<td>45b</td>
<td>63c</td>
<td>40b</td>
<td>1995b</td>
<td>0.70a</td>
</tr>
<tr>
<td>11NF010c-</td>
<td>73b</td>
<td>152c</td>
<td>4a</td>
<td>8.2b</td>
<td>105c</td>
<td>35a</td>
<td>65c</td>
<td>42b</td>
<td>2121bc</td>
<td>0.71ab</td>
</tr>
<tr>
<td>11NF020a-</td>
<td>78c</td>
<td>158d</td>
<td>5a</td>
<td>8.6b</td>
<td>108c</td>
<td>53b</td>
<td>56b</td>
<td>42b</td>
<td>2050bc</td>
<td>0.71ab</td>
</tr>
<tr>
<td>PBA Warda</td>
<td>70a</td>
<td>147a</td>
<td>4a</td>
<td>8.7b</td>
<td>97b</td>
<td>48b</td>
<td>58b</td>
<td>41b</td>
<td>2228c</td>
<td>0.72b</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.6</td>
<td>1.1</td>
<td>25.3</td>
<td>11.1</td>
<td>4.8</td>
<td>17.6</td>
<td>4.6</td>
<td>10.0</td>
<td>9.6</td>
<td>3.6</td>
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<td>F test (G)</td>
<td>**</td>
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<td>**</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>ns</td>
</tr>
<tr>
<td>Season (S)</td>
<td>*</td>
<td>**</td>
<td>ns</td>
<td>*</td>
<td>*</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Treatment</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
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<tr>
<td>S*T</td>
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<td>ns</td>
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<tr>
<td>S*G</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>**</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>T*G</td>
<td>ns</td>
<td>**</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>S<em>T</em>G</td>
<td>ns</td>
<td>ns</td>
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<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

* And ** represents significant at P < 0.05 and P < 0.01, ns stands for non-significant. Means with different letters within a column represents a significant difference at P < 0.05 by DMRT test (Duncan, 1955)
4.4.4 Correlations among traits

Pearson correlation of coefficient analysis was conducted to identify the relationship between different studied traits and is presented in Table 4.2. The analysis was performed with the combined data measured from the 2015 and 2016 seasons. All the studied traits were significantly correlated with grain yield except NDVI. Grain yield had significant positive correlations with plant height (0.60**), leaf area (0.55**), canopy temperature (0.20*), 100 seed weight (0.60**) and harvest index (0.35**). On the other hand, days to 50% flowering (-0.63**), days to maturity (-0.44**), number of branch plant$^{-1}$(-0.56**) and number of pods plant$^{-1}$ (-0.62**) were negatively correlated with grain yield. Harvest index was also significantly correlated with most of the traits but not with NDVI, canopy temperature and maturity. HI had significant positive correlation with plant height (0.44**), leaf area (0.31**) and 100 seed weight (0.54**) whilst significant negative correlation with days to 50% flowering (-0.31**), number of branch plant$^{-1}$(-0.43**) and number of pods plant$^{-1}$ (-0.34**). Hundred seed weight had a significant positive correlation with plant height (0.86**) and leaf area (0.71**) whilst negative with flowering (-0.83**), maturity (-0.68**), branch number plant$^{-1}$(-0.86*) and pods plant$^{-1}$(-0.91**).
Table 4.2 Pearson correlation between morphological, physiological and yield component traits of faba bean genotypes grown under irrigated and rainfed condition over two years (2015 and 2016) in Narrabri, northern NSW.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Days to 50% flower</th>
<th>Days to maturity</th>
<th>Plant height</th>
<th>Leaf area</th>
<th>Number of branch plant(^1)</th>
<th>Normalised difference vegetation index – NDVI</th>
<th>Canopy temperature</th>
<th>Number of Pods plant(^{-1})</th>
<th>100 seed weight</th>
<th>Harvest index</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to 50% flower</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days to maturity</td>
<td>0.87**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant height</td>
<td>-0.74**</td>
<td>-0.64**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf area</td>
<td>-0.67**</td>
<td>-0.67**</td>
<td>0.80**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of branch plant(^{-1})</td>
<td>0.80**</td>
<td>0.68**</td>
<td>-0.83**</td>
<td>-0.67**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normalised difference vegetation index - NDVI</td>
<td>0.08</td>
<td>0.10</td>
<td>-0.25*</td>
<td>0.30</td>
<td>0.11</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canopy temperature</td>
<td>-0.12</td>
<td>-0.28*</td>
<td>0.17</td>
<td>0.24*</td>
<td>-0.17</td>
<td>-0.10</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of Pods plant(^{-1})</td>
<td>0.83**</td>
<td>0.70**</td>
<td>-0.86*</td>
<td>-0.74**</td>
<td>0.85**</td>
<td>0.22*</td>
<td>-0.15</td>
<td>1</td>
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<td></td>
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<tr>
<td>100 seed weight</td>
<td>-0.83**</td>
<td>-0.68**</td>
<td>0.86**</td>
<td>0.71**</td>
<td>-0.86**</td>
<td>-0.15</td>
<td>0.09</td>
<td>-0.91**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harvest index</td>
<td>-0.31*</td>
<td>-0.12</td>
<td>0.44**</td>
<td>0.31*</td>
<td>-0.43**</td>
<td>-0.03</td>
<td>-0.01</td>
<td>-0.34**</td>
<td>0.54**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Yield</td>
<td>-0.63**</td>
<td>-0.44**</td>
<td>0.60**</td>
<td>0.55**</td>
<td>-0.56**</td>
<td>-0.11</td>
<td>0.20*</td>
<td>-0.62**</td>
<td>0.60**</td>
<td>0.35*</td>
<td>1</td>
</tr>
</tbody>
</table>

* And ** represents significant at \( P < 0.05 \) and \( P < 0.01 \)
4.5 Discussion

Globally, most grain legumes including faba bean are predominantly cultivated under rainfed conditions. In rainfed production systems, achieving potential yield depends on the maximum use of precipitation and it is necessary that crop phenology match the growing seasonal conditions. Being an indeterminate crop, faba bean phenology and morphology is quite complicated because under favorable environments vegetative and reproductive growth is simultaneous.

Crop phenology is one of the critical factors that can influence faba bean yield and production. Understanding the target environmental conditions of specific growing seasons can help to choose the crop variety that can match with the specific environment. Thus, one of the drought adaptation strategies, drought escape has been practiced as an agronomic drought adaption strategy in faba bean breeding by selecting for early flowering where earliness escaped the heat and moisture stress leading to higher grain yield (Mwanamwenge et al., 1998). Faba bean growth stage may define with different phenological stages including vegetative development, first flowering, pod initiation, pod development and maturity. In both growing seasons, our crop did not face significant drought stress and, early flowering and early maturing genotype PBA Warda produced the highest grain yield.

Moreover, our examined genotypes were indeterminate/semi determinate and the timing of maturity was similar due to high temperature in the terminal stage of crop restricting vegetative growth. Usually, early maturing crops have a yield penalty due to reduced assimilation. However, in our study PBA Warda voids this assumption. One of the reasons, why PBA Warda was the best performing variety in this region might be due to its capacity to convert assimilates to sink efficiently. This was also supported by our correlation study where early flowering and
early maturing traits produced higher grain yield (Table 4.2). Siddique et al. (1999) opined early phenology as an important adaptation trait for grain legumes cultivated in Australia. A similar result also described by Manning (2017) for northern NSW growing condition where the higher yield of faba bean was correlated with early maturing genotype IX148f.

Another way of mitigating the effects of drought is to curtail the critical stage, i.e. reproductive stage when water is not a limiting factor. We found genotype ACO805#4912 had the shortest pod filling period and this genotype was found to be drought tolerant in our earlier field study (Chapter 3). Williams and Saxena (1991) also found chickpea yield under stress was maintained through a shorter period of pod filling. However, it was suggested by Etemadi et al. (2018) that partitioning of assimilates to seed should be considered whilst counting this trait for breeding purposes. Thus, selecting for appropriate phenology to suit the growing conditions will increase production. Whilst AC0805#4912 was found drought tolerant in our earlier studies, it was the lowest yielder in this study suggesting drought tolerance alone will not give high yield.

The number of branches plant$^{-1}$ is an important character to produce higher grain yield through higher biomass production (Loss and Siddique, 1997). Branch plant$^{-1}$ in faba bean is regulated either genetically (Mwanamwenge et al., 1998) or by environmental conditions like water deficit (Hegab et al., 2014). In our experiment, variation in branch plant$^{-1}$ was mainly genotype dependent because plant density was maintained, i.e. each plot had the almost equal number of plants. Genotype AC0805#4912 had the highest branch plant$^{-1}$ but they produced the lowest grain yield. Hence, secondary branches of this genotype were not as productive as the main branch (field observation, data not shown). In our correlation study, branch plant$^{-1}$ was also negatively correlated with yield. The possibilities of lower grain yield in genotype AC0805#4912 were, AC0805#4912 was from exotic origin and yield contributing characters,
i.e. leaf area, 100 seed weight, HI etc. were extremely low for this genotype compare to other studied genotypes. Interaction with seasons and treatments for this character was not significant in our study. Migdadi et al. (2016) also demonstrated similar findings in faba bean trial under water deficit where branch plant\textsuperscript{1} was not significant for genotype-treatment interaction. Ultimately, breeders need to be critical in adopting this trait as a selection criterion in drought stress breeding as it may not confer yield gains across all environments.

The leaf area is one of the controlling factors for canopy interception and photosynthate accumulation of a crop. In faba bean, yield from a specific node is correlated (0.67 to 0.94) to leaf area (Ishag, 1973) and during drought stress plants exhibited reduced leaf area (Husain et al., 1990). In the current studies, genotypes with high leaf area, such as PBA Warda produced highest grain yield as expected (Table 4.1). Higher leaf area produces more assimilates which turn to economic yield. Concurrently, correlation coefficient results also showed a positive correlation between leaf area and grain yield. Our results conform with other studies in faba bean by Osman et al. (2013) where leaf area significantly regulate grain yield. Plant height for faba bean has, perhaps unsurprisingly, been shown to be correlated with total biomass production (Musallam et al., 2004). Faba bean plant having more pods in the upper node will facilitate mechanical harvesting and the economic seed was usually harvested from six to thirteen nodes (Manning, 2017). Thus, higher plant height is a desirable character in general. Our correlation study also found plant height as an important trait and it was positively correlated with grain yield. Similar observations were also described by Ammar et al. (2015) in a diversity study on faba bean. We found plant height was significant in regarding genotype, treatment and season indicating the genetic as well as environmental sensitivity of the trait. Hegab et al. (2014) found plant height sensitive to irrigation while Abou-Taleb (2002) found day/ night temperature during vegetative growth.
Generally, the components of faba bean yield are determined by the number pods plant$^{-1}$, seed weight and the number of seed pod$^{-1}$. Ishag (1973) found the number of pods plant$^{-1}$ as the most important determinant for dry matter production in faba bean. In our experiment, we observed the highest number of pods in genotype AC0805#4912 but it was not the highest yielding among studied genotypes. The correlation study also showed significant negative relationships among grain yield and number of pods plant$^{-1}$. One of the possibilities, perhaps due to resource limitations, if there are more pods plants will not be able to supply enough assimilates required for seed development. Li and Yang (2014) also found a non-significant relationship between pods plant$^{-1}$ and grain yield, which is explained through a trade-off between pod size and the number of pods. The Pearson correlation study found 100 seed weight was significant and positive towards final yield which is similar to Ammar et al. (2015) findings on faba bean. Lower 100 seed weight often benefited farmers by lower seeding rate while using faba bean as a cover crop (Etemadi et al., 2018). Both pods plant$^{-1}$ and 100 seed weight were found to be significant for genotypic and seasonal variation. The reasons for seasonal variation may be the effect of an extended photoperiod in 2016. Under favourable conditions, faba bean distributed assimilates in a more balanced way which is an agreement with Kulig et al. (2014).

NDVI is a non-invasive indication of plant health through green biomass estimation. NDVI was the only character in our study which had no significant correlation with grain yield. The reason may be the timing of measurement and NDVI measurement changed temporally. Timing and frequency of NDVI measurement can significantly improve yield prediction which aligns with Guenette & Hernandez-Ramirez (2018). Canopy temperature characterises the water content of the leaf as stomatal features directs it. Canopy temperature in our study significantly correlated with grain yield but there were no significant interactions between season and treatment observed which indicates in both seasons plants had available moisture in the soil. In a large faba bean germplasm collected from contrasting moisture regions around
the globe, the analysis revealed that leaflet and canopy temperatures were the most informative measurements distinguishing germplasm from wet and dry origins (Khazaei, 2014a; Khazaei, 2014b).

As an indeterminate crop, faba bean had competition between vegetative and reproductive stages. This competition profoundly influenced faba bean yield and HI. In the current study, genotype AC0805#4912 had the lowest HI which was actually due to its higher biomass and longer vegetative stage. De Costa et al. (1997) illustrated faba bean HI was reduced in excessive moisture as it enhanced more vegetative growth. The overall decrease in HI in 2016 may be for the same reason. The HI of this study was within a range of (36-42%) which is similar to other studies of faba bean (Agung and McDonald, 1998; Gezahegn et al., 2016). Correlation results also demonstrated a significant positive relation between HI and grain yield which is an agreement with Loss and Siddique (1997). Grain yield of faba bean depends on the efficient dry matter partitioning, and the prevailing stress conditions often regulate this partitioning. If the stress occurred during the reproductive stage, it restricts translocation of assimilates to sink which reduce final grain yield. In the present study, as expected grain yield had significant variation in genotypic level and at the seasonal level. Although supplemental irrigation did not affect, seasonal differences in yield showed the moisture had a significant effect on yield. 2016 produced the highest yield and it was the wettest year. Overall, 14% higher grain yield observed in 2016, was due to adequate moisture and longer photoperiod which was also demonstrated in other studies on faba bean where weather condition (rainfall) significantly influenced grain yield (Kulig et al., 2014; Mwanamwenge et al., 1998).

4.6 Conclusion

Though faba bean is sensitive to drought and heat stress, the unprecedented climatic conditions for both years did not elicit any treatment effects. As a result, this study provides the relative
growth characteristics of selected genotypes. In general, all the studied genotypes had similar phenology except AC0805#4912. From our earlier field study AC0805#4912 was found drought tolerant and therefore its morphological characters, i.e. high branch number and low 100 seed weight would be traits of interest. Besides, the lower pod filling duration can indicate rapid pod development. For trait identification, most of the traits either positively or negatively correlated with grain yield in our study and we recommend selection based on plant height, leaf area, seed weight and harvest index. A further investigation is recommended to assess the changes in phenology and yield components at different moisture level.
Chapter 5
Carbon isotope discrimination and soluble metabolites reflect physiological status among contrasting faba bean genotypes in response to water deficit

Abstract
Identification and validation of biomarkers and bioindicators to select drought tolerance genotypes under field conditions are paramount to plant breeding programs. However, the co-occurrence of different abiotic stresses (drought, heat, radiation etc.) makes it difficult to develop generalised protocols to monitor the physiological health of the plant system. In our study, we assessed most abundant carbohydrates and sugar alcohols in five faba bean genotypes under field conditions and the abundance of naturally occurring carbon isotopes in bulk leaf material to predict water use efficiency (WUE). We also assessed plant water status and biomass accumulation. Metabolite accumulation significantly differed under rainfed compared to irrigated conditions. Among the accumulated sugars, inter-specific variation in glucose was most prevalent and found higher concentration in rainfed trial. myo-Inositol accumulation also followed that of glucose accumulation that is rainfed trial had higher amount compared to irrigated trial. Among the contrasting genotype, AC0805#4912 had the highest harvest index (HI) and grain yield. Overall, irrigated trial exploit higher HI and grain yield. WUE calculated from carbon isotope abundance was consistently offset with measured WUE from measurements of leaf gas exchange. All genotypes demonstrated significant relationships between predicted and measured WUE (P< 0.05) except PBA Warda. Thus, bulk leaf-level carbon isotope abundance to calculate WUE may be used as an effective and accurate selection criterion for improving WUE in faba bean breeding programs in field condition.
5.1 Introduction

Among abiotic stresses, drought is most influential on faba bean yield and production (Daryanto et al., 2015). However, systematic research can advance yield improvement for water-limited cultivation (Hsiao et al., 2007). Novel traits that indicate plant water status can inform selection programs to identify superior lines for both high yield and increased resilience of yield. In water-limited environments, WUE is found to be one of the most important traits for yield improvement across a range of crops (reviewed by Blessing et al. (2018). The primary driver of plant productivity is the accessible moisture content in the soil. Most commonly, plants deploy a range of approaches from reduced stomatal conductance (gs) to the accumulation of metabolites to maintain tissue hydration through either passive or active osmotic adjustment (Iannucci et al., 2002; Serraj and Sinclair, 2002). Genotypes that can assimilate carbon at low gs are often considered to be drought tolerant based on the assumption that sustained growth will equally maintain yield. This assumption gives little regard to the resilience of yield and conservative growth strategies under the effects of water limitation. Thus, WUE is suggested as an essential determinant of plant productivity under drought conditions and is the focus of targeted screening tools for many crops (Kashiwagi et al., 2013; Loss et al., 1997; Xu and Hsiao, 2004) to identify genotypes that can produce sustainable yield during drought rapidly.

Generally, WUE is the ratio of yield and the amount of transpired water to produce that yield. Instantaneous WUE can be measured at a leaf through the gas exchange by dividing photosynthesis (A) to gs (Chaves and Oliveira, 2004) and intrinsic WUE can be estimated via isotopic fractionation models (Farquhar et al., 1982a) offering advantages in both cost and time integration. Generally, WUE involves measurement of gas exchange at a single point whilst
periodic measurement throughout the day is also suggested (Cernusak et al., 2005; Smith et al., 2016) which may often beyond the capabilities of researchers studying large populations.

Carbon isotope discrimination ($\Delta$) frequently used as a method for evaluating WUE for screening plants with higher tolerance under water deficit conditions (Cernusak et al., 2009). Application of $\Delta$ to measure WUE over time is simple and rapid (Farquhar et al, 1982a). In higher plants, $\Delta$ is obtained from a pool of carbon fixed over the period of time that the sampled tissue develops. Such an approach not only reflects WUE over a longer, more meaningful period, it is also less time consuming. Alternatively, for example, the total amount of water used by a crop (neutron probe measurement), combined with yield measurements is required to estimate whole plant level WUE which is time consuming and laborious. For this reason, $\Delta$ measurement has become one of the selection traits for drought screening in many crops (von Caemmerer et al., 2014). $\Delta$ can, therefore, be an alternate option for rapid plant screening.

Accumulation of chemical entities due to stresses (often termed “stress metabolites) may give an indication of plant responses against specific stress (Weckwerth, 2011). However, most of the metabolomic studies conducted under control conditions involve water, heat, light stress either singularly or in a combination to identify/quantify metabolites accumulating under these stresses. Among the major metabolic groups, carbohydrates and sugar alcohols play a vital to alleviate adverse effects of stress through osmolytic and osmo-protective mechanisms (Bohnert and Shen, 1998); Loescher (1987). Regardless of these advantages, limited studies have been conducted so far with no previous field study characterising metabolite accumulation in faba bean.
Despite this, yields of faba bean are increasing globally, despite reductions in the harvested area over the last 50 years (Foyer et al., 2016). Unstable yield, especially due to abiotic factors, makes faba bean a ‘risky’ crop among cultivated grain legumes. Genotypic differences for drought responses exist, but the underlying mechanisms are yet to be understood (Khazaei et al., 2013a; Link et al., 1999). Genetic advancement for this crop requires the identification of physiological and biochemical responses due to water deficit in field condition for use in plant improvement programs.

Faba bean genotypes were selected for contrasting Δ value in an earlier field trial (Chapter 3). Here, we subsequently investigated the suitability of Δ isolated from leaves as the alternate of plant gas exchange measurement in five diverse faba bean genotypes under irrigated and rainfed conditions. Quantification of metabolites is also determined to investigate the relative changes in concentration under water deficit conditions. We further compared WUE from field-based gas exchange to that of calculated from leaf level Δ. These parameters are presented on a background of biomass accumulation and yield under rainfed and irrigated conditions.

My hypotheses were i) rainfed condition will induce a reduction in stomatal conductance across all faba bean genotypes with a proportional, but lower reduction in tolerant genotype. ii) genotypes differ in reductions of gs and A. iii) rainfed condition will induce a change in the concentration of soluble metabolites and iv) WUE from gas exchange and calculated from leaf level Δ found similar in irrigated and rainfed conditions.
5.2 Methodology

5.2.1 Plant materials and trial design

This experiment was designed to test four contrasting faba bean genotypes (AC0805#4912, 11NF020a-1, 11NF010e-4, and 11NF008b-15) and PBA Warda, a popular variety suitable for northern NSW growing region. This experiment was carried out at the I.A Watson Grains Research Centre, Narrabri, NSW-2390, Australia (30° 27’ S, 149° 80’ E), during 2016 (7th May to 29th October). Four genotypes were selected from 96 diverse genotypes having a diverse Δ value from our initial screening experiment which was conducted at the same location in 2014. Each genotype was sown in four replicates of individual plots comprised of four rows of 10 m long and 50 cm apart. Two treatments were maintained as irrigated and rainfed. Irrigated and rainfed blocks were separated by a buffer plot of PBA Warda variety (48 m X 12 m).

5.2.2 Experimental field management

The experiment was sown in a field which had wheat in the previous crop season, ploughed and leveled. Seeds were pre-treated with commercially available faba bean inoculant (Rhizobium strain, group G WSM 1455). Inoculation was achieved by suspending a mesh bag containing peat inoculant in a water tank and pumping the water/inoculant suspension into the furrow following seed placement. Sowing was carried out using a four-row planter with 50 cm inter-row spacing. Pre-emergence and post-seeding weedicides namely, Spinnaker 700WG (Imazethapyr 700 g kg⁻¹) and Terbyne 750 WG (Teerbuthylazine 750g kg⁻¹) were applied at a recommended rate to prevent the establishment of a range of grass and broadleaf weeds. No mineral fertilizer was applied in the trial. Karate Zeon® (250 g L⁻¹ lambda-cyhalothrin) at the rate of 36 ml ha⁻¹ were applied to control insect pest as well as aphids which were regular farm practice during 50% flowering. Mancozeb (750 g kg⁻¹) was sprayed before the canopy closure
(1.7 kg ha\(^{-1}\)) to control rust and chocolate spot. The season was rainfall dominated and rainfed plots received 447 mm rainfall over the season. The irrigated plots received a total 472 mm during this period which is only 25 mm more than the rainfed plot. Grain yield was weighed after harvesting trial plots by using a mechanical harvester (HALDRUP C-65).

### 5.2.3 Gas exchange measurements

Four randomly selected plants from each plot were measured using a Walz GFS-3000 portable gas exchange system. Leaf-level photosynthesis was measured from 09:00 to 16:00 on the same leaf at least four times over the day. Leaf temperature, chamber CO\(_2\) concentration along with air humidity was set at ambient. Data were completed on two consecutive days where day one was allocated for irrigated and day two for rainfed trial. The average temperature was 24 °C and 21 °C, average relative humidity was 60.2 % and 62.3 % and average PAR was 1543 μmol m\(^{-2}\)s\(^{-1}\) and 1523 μmol m\(^{-2}\)s\(^{-1}\) on sampling day one and sampling day two respectively. After insertion of leaves into the gas exchange chamber, leaves were allowed to stabilise with chamber conditions until steady-state gas exchange rates were established. Leaves were chosen from the main stem (2nd/3rd fully expanded).

### 5.2.4 Leaf water content and biomass determination

Leaf water potential was determined from the same leaf, which was tagged for the gas exchange measurements. The leaflets were excised and transported (15 mins maximum) in a zip locked bag over ice to preserve water content for subsequent measurement with a Wescor PSYPRO. Relative water content (RWC) was determined from the adjacent leaf which was used to measure leaf water potential. Leaves were placed in distilled water and allowed to soak overnight at 4 °C. Subsequently, leaves were weighed immediately to have turgid weight. Finally, leaves were dried in an oven at 65 °C for 48 h to have a constant weight.
RWC was determined by:

\[ \text{RWC} (\%) = \left[ \frac{\text{FW-DW}}{\text{TW-DW}} \right] \times 100, \]

Where, FW- Fresh weight of leaves, TW- Turgid weight of leaves and DW- Oven dry weight of leaves.

At physiological maturity, one m² of each plot was harvested and placed in a polyethene mesh bag until dried to determine biological yield. After weighing the plants, they were threshed to determine grain yield. Finally, HI was measured according to with formula

\[ \text{HI} (\%) = \frac{\text{Grain yield}}{\text{Biological yield}} \times 100. \]

Harvesting of whole plot was completed by mechanical harvester upon maturity. Plot yield data were converted into kg ha⁻¹.

### 5.2.5 Tissue collection and carbon isotope analysis

Leaf samples for chemical and isotopic analysis were collected on the same day immediately after gas exchange measurement between 4.00 pm to 5.00 pm. The isotopic abundance measurement was completed on the same the tagged leaf which was used for gas exchange measurement. The leaf was cut with a sharp razor and immediately put into 2 ml Eppendorf tube and placed in an ice box to reduce metabolism. Within 2 hours, leaf samples were placed in an oven at 65 °C for 48 h to completely dry. An oscillating matrix mill was used to grind the dried leaf sample. Approximately 3 mg of ground leaf was then placed into silver capsules (IVA Analysentechnik, Meerbusch, Germany) and then analysed according to the protocol outlined in Smith (2018). The precision for the standard materials was between 0.04‰ and 0.08‰
5.2.6 Quantification of metabolites

Dried leaf samples were transferred to 2 ml microcentrifuge tubes and ground using an oscillating matrix mill. Methanol-Chloroform water extractions were then performed according to Merchant et al. (2006a). Approximately 40 mg of ground leaf samples were weighed into 2 ml screw cap tubes and the exact weight for each sample was recorded. For the methanol-chloroform-water solution (MCW, 12:5:3 by volume), the water fraction of the extraction solution contained 0.1% pentaerythritol (98+%, Alfa Aesar, Haverhill, MA, USA) as an internal standard. 1 ml of the solution was added to each sample and incubated at 70 °C for 30 minutes, then allowed to cool for 5 minutes before centrifuging for 2 minutes at 10,000 g. Next, 800 μl of supernatant was pipetted into clean 2 ml pop cap microcentrifuge tubes. 500 μl of MilliQ water and 200 μl of chloroform was added to 800 μl of supernatant and mixed thoroughly with the vortex. Samples were then centrifuged for 2 minutes at 10,000 g and then left to stand for 10 minutes. Next, 700 μl of the top aqueous phase was removed and added to a clean 1.7 ml microcentrifuge tube. Samples were then shaken at room temperature for 2 hours and centrifuged for 2 minutes at 10,000 g. Finally, 400 μl of supernatant was removed and pipetted into a clean, labeled 2 ml microcentrifuge tube and stored at -80 °C until samples could be run on the gas chromatography triple quadrupole mass spectrometer (GC-QQQ).

5.2.7 Derivatisation and GC-QQQ protocol

In order to analyse non-polar analytes using gas chromatography triple quadrupole mass spectrometer, samples had to be derivatised according to Merchant et al., 2006b. First, 50 μl of thawed sample extractions were transferred to 2 ml Snap Seal™ (Sigma- Aldrich, St. Louis, MO, USA) vials and the liquid was evaporated using a speed vacuum concentrator (Scanspeed 40, Labogene, Lyngby, Denmark). Once liquid had been dried down, vials were capped. Next, 400 μl anhydrous, 98% pyridine (Sigma-Aldrich, St. Louis, MO, USA) was injected into each
sample vial using a syringe. This was followed by 50 μl of a 1:10 mix of trimethylchlorosilane (TMCS): bistrimethylsilyl trifluoroacetamide (BSFTA) injected slowly, directly into the pyridine.

Special safety care was taken with derivatising agents and air was always removed from vial using the syringe after injection in order to relieve pressure. Samples and standards were then loaded onto the GC-QQQ with in line heat incubation of 75 °C for 1 hour. The separation and quantification of target metabolites was completed using an Agilent 6890A gas chromatograph with QQQ 7000 mass selective detector on scan mode from 50-500 AMU (70 eV) (Agilent Technologies, Santa Clara CA, USA) according to the protocol detailed in Merchant et al. (2006b). The samples were injected with a 20:1 split injection onto a HP-5 column (0.25 mm i.d., 30 m, 0.25 μm film thickness; Agilent Technologies, Santa Clara CA, USA), with helium carrier gas at 1 ml per minute. The temperature program had an initial oven temperature set of 60°C for 2 minutes, ramping up to 220 °C at 10 °C min-1 for 5 minutes, then to 300 °C at 10 °C min-1 for 5 minutes. Peaks were integrated and compound data was extracted using Agilent MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA). Scanned data for a mixed standard was extracted to determine the most abundant or best extracted ion peak for each compound. Metabolite concentrations are reported as mg g⁻¹ dry weight sample material.

5.2.8 Modelling of carbon isotope abundance

Predicted isotope values in plant components were calculated using the equation (Farquhar et al., 1989):

\[ \Delta = a + (b-a) \left( \frac{C}{C_a} \right) d, \]
Where “a” is fractionation caused by gaseous diffusion through the stomata (4.4‰), “b” is the effective fractionation caused by carboxylating enzymes (approximately 27‰), “Ci” is the internal CO₂ concentration (calculated by Walz GFS-300 software), “Ca” is atmospheric CO₂ concentration (set to ambient), and “d” is a term combining fractionation during photorespiration, dark respiration, and dissolution and diffusion from the gaseous phase to the chloroplasts. For relationships with measured δ¹³C, assimilation weighted Ci was calculated (Cernusak et al., 2005) to account for proportional changes in the contribution of photosynthesis to δ¹³C across the course of the light period. This was achieved by the formula:

\[
\sum_{i=1}^{A} c_i = \frac{A_1}{\sum A} \cdot \frac{A_2}{\sum A} \cdot \frac{An}{\sum A} \cdot Ci_n
\]

Where A₁ and Ci₁ were the first observed A and Ci respectively in the light period. The Δ was then calculated as the difference between the isotopic composition of atmospheric CO₂ (δ¹³C air≈−8‰) as carbon source, and that of the plant organic matter (δ¹³C plant) as photosynthetic product using Δ (‰) = (δ¹³C air − δ¹³C plant)/1+ plant.

### 5.2.9 Water use efficiency measurement

Intrinsic water use efficiency was calculated through the formula outlined by Osmond et al. (1980):

\[
WUE = \frac{A}{g_s}
\]

Where A is net photosynthesis (μmol m⁻²s⁻¹ CO₂) and gₘ is stomatal conductance (mmol m⁻²s⁻¹ H₂O). Modelled WUE was calculated from the formula outlined by Seibt et al. (2008), originally developed by Farquhar and Richards (1984)

\[
WUE = \frac{Ca}{1.6} \left( \frac{b-\Delta}{b-a} \right)
\]
Where the value “1.6” is the ratio of the diffusion rate between CO₂ and H₂O across the stomatal cavity, and “b” is the effective fractionation caused by carboxylating enzymes RuBisCO and PEP carboxylase (approximately 27‰).

5.3 Statistical analysis

The effects of treatments on water content and carbohydrates concentration were examined by analysis of variance (ANOVA) using GenStat 18th Edition (VSN International, Hemel Hempstead, UK). Duncan multiple range tests were used to determine genotype and treatment effect. Regression analysis with best fitting line and significance level (P values) for the relationship of WUE calculated using GraphPad Prism7 software (GraphPad Software, San Diego, CA, USA).

5.4 Results

5.4.1 Effect on leaf water potential

Leaf water potentials were higher (less negative) in irrigated plots compared to rainfed plots for all genotypes at the pod filling stage though they were non-significant (Figure 5.1). Genotype had a significant effect in this trial. Among genotypes, 11NF010c-4 had the lowest water potential (most negative) and AC0805#4912 had the highest (least negative). No other genotypes except 11NF020a-1 differed according to treatment.
**Figure 5:1** Predawn leaf water potentials (MPa) of contrasting faba bean genotypes at the three-pod stage grown under field condition (irrigated and rainfed trial) at Narrabri, Australia. Error bars were calculated from four replicates. Letters represents a significant difference ($P < 0.05$) among genotypes calculated through DMRT. Asterisks (*) represent significance between irrigated and rainfed treatments.

### 5.4.2 Effect on relative water content

Relative water content was not significantly different between treatments. However, a significant variation existed among genotypes. The highest RWC was observed in genotype AC0805#4912 which was counted as drought tolerant genotype in our earlier study (Chapter 3) and the lowest was in 11NF010c-4.
Figure 5.2 Relative water content (%) of contrasting faba bean genotypes grown under field conditions (irrigated and rainfed trial) at Narrabri, Australia. RWC was determined at three pod stage. Error bar was calculated from four replicates. Letters represent a significant difference (P < 0.05) among genotypes calculated through DMRT. Asterisks (*) represent significance between irrigated and rainfed treatments.

5.4.3 Gas exchange measurement in irrigated and rainfed trial

Treatment effect to photosynthetic rates were absent but showed similar patterns among genotypes and across the course of the day (Figure 5.3). Irrigated plots had a slight increase in net photosynthetic rate compare to rain-fed plots. Notably, genotype 11NF010c-4 showed a lower photosynthetic rate across the day for both irrigated and rainfed trial whereas AC0805#4912 had the highest one for both trials over the time period of data recording.
Figure 5:3 Net photosynthetic rate (A, \( \mu \text{mol m}^{-2} \text{ ms}^{-1} \)) among five contrasting genotypes under rainfed and irrigated trials. Data were recorded four times over the day. Error bars were calculated from four replicates. The measurement was done at the three pod stage; plant age was between 120-128 days.

Genotypic differences in g_s were significant (P < 0.05) among genotypes for irrigated and rainfed plots (Figure 5.4). Average g_s were higher in the irrigated trial compare to the rain-fed one. The highest g_s was observed in genotype AC0805#4912 whereas the lowest was found in 11NF010c-4 across the day. During mid-day, a sharp decline in g_s was observed for both trials.
Figure 5:4 Stomatal conductance (gs, mmol m$^{-2}$ ms$^{-1}$) among contrasting faba bean genotypes under rainfed and irrigated conditions. Data were recorded four times over the day. Error bars were calculated from four replicates. Measurements were taken at the three pod stage; plant age was between 120-128 days.

The ratio of ci/ca translated the same observation as that of the net photosynthetic rate. Average ci/ca is slightly higher in the irrigated trial compared to rainfed one (Figure 5.5). Among genotypes, AC0805#4912 had higher for both trials whereas 11NF008b-15 had the lowest value for both trials.
Figure 5.5 Ratio between intercellular carbon concentrations (Ci) to atmospheric carbon concentration (Ca) measured among five contrasting faba bean genotypes under rainfed and irrigated conditions. Data were recorded four times over the day. Error bar was calculated from four replicates. Measurements were completed at the three pod stage; plant age was between 120-128 days.

5.4.4 Concentrations of major sugars in faba bean grown under rainfed and irrigated trial

Significant changes were found in most of the identified carbohydrates in both trials except fructose (Figure 5.6). Generally, soluble sugar concentrations were higher in the rain-fed trial compared to irrigated. Fructose concentration for genotypes PBA Warda, 11NF008b-15 and 11NF020a-1 showed an opposite trend: higher in irrigated trial compare to the rainfed trial. Genotype 11NF008b-15 had the lowest fructose concentration than all other studied genotypes. The treatment effect was found to be significant for genotypes 11NF008b-15 and 11NF010c-4 (Figure 5.4A). The concentration of glucose was found to be significant for both genotype and treatments. The highest concentration was observed in genotypes 11NF010c-4 and lowest in PBA Warda (Figure 5.4B). myo - Inositol accumulation also followed that of glucose accumulation that is rainfed trial had higher amount compared to irrigated trial and found to be significant. Genotype AC0805#4912 had significantly differed from other genotypes and had
the highest concentration among studied genotypes. (Figure 5.4C). Among the identified metabolites, sucrose was found to be the most abundant metabolite and was also observed in increased amounts in the rainfed trial for all genotypes. Drought sensitive genotype 11NF010c-4 had significantly lower concentrations of sucrose compared to all other studied genotypes with the highest concentration observed in PBA Warda (Figure 5.4D).
Figure 5:6 Average metabolite concentrations (mg g\(^{-1}\) leaf material) in contrasting faba bean genotypes under rainfed and irrigated conditions. Error bar was calculated from four replicates. Different letters represent a significant difference (P < 0.05) among genotypes calculated through DMRT. Asterisks (*) represents significance between irrigated and rainfed treatments.

5.4.5 Measured vs predicted WUE

Water use efficiency measured using gas exchange and WUE calculated from the bulk leaf-level carbon isotope abundance was similar for both rainfed and irrigated trials. WUE calculation based on modelled assimilation weighted Ci is higher than calculated from bulk leaf \(\Delta\). Both the predicted and measured WUE did not clearly define the treatment effects. Genotypes from both drought tolerant and sensitive group (from Chapter 3) act similar to the
modelled methods. All relationships except for PBA Warda found significant (P < 0.05).

**Figure 5:7** Predicted water use efficiency (WUE) vs measured water use efficiency has grown under irrigated and rainfed condition of contrasting faba bean genotypes. Water use efficiency was calculated according to the formula Farquhar and Richards (1984) and Seibt et al. (2008). The measurement was completed at the three pod stage; plant age was between 120-128 days.
5.4.6 Yield and biomass production under rainfed and irrigated condition

Significant differences in HI were observed among genotypes. Treatments also elicited a significant influence on each of the genotypes AC0805#4912 and 11NF020a-1. Highest HI was found for the genotype AC0805#4912 and lowest for 11NF010c-4 and PBA Warda. Generally, HI was higher in the irrigated trial except for genotypes 11NF020a-1 and PBA Warda.

![Harvest index (%) of contrasting faba bean genotypes grown under field condition (irrigated and rainfed trial) at, Narrabri, Australia.](image)

**Figure 5:8** Harvest index (HI) (%) of contrasting faba bean genotypes grown under field condition (irrigated and rainfed trial) at Narrabri, Australia. Error bars were calculated from four replicates. Different letters represent a significant difference (P < 0.05) among genotypes calculated through DMRT. Asterisks (*) represents significance between irrigated and rainfed treatments.

Significant genotypic variation for grain yield was observed among tested genotypes. PBA Warda had the highest grain yield and 11NF010c-4 had the lowest. Irrigated plots produced higher yield compared to rainfed, but they were not statistically significant. Only genotype AC0805#4912 had significantly responded against treatment, producing more yield under
irrigation. It was also observed that rainfed plot produced more yield for genotype 11NF020a-1 though they were non-significant (Figure 5.9).

![Graph showing yield of faba bean genotypes](image)

**Figure 5.9** Yield (Kg ha\(^{-1}\)) of contrasting faba bean genotypes grown under field conditions (irrigated and rainfed trial) at Narrabri, Australia. Error bars were calculated from four replicates. Letters represent a significant difference (P < 0.05) among genotypes calculated through DMRT. Asterisks (*) represent significance between irrigated and rainfed treatments.

### 5.5 Discussion

The accumulation of metabolites and changes in the amount of naturally occurring carbon isotopes represent two of the primary mechanisms by which plants may acclimate to changes in water availability. Quantification of metabolites across genotypes may be useful to establish biochemical markers as they are linked with phenotypic expression (Steinfath et al., 2010). In this study, we found glucose, myo-inositol and sucrose accumulation is significantly decreased under irrigated conditions compared to rainfed. Further, we identified that carbon isotope abundance among faba bean genotypes reflects a daily integral of WUE, with the incorporation
of a relatively consistent offset to that measured by leaf-level gas exchange. Combined, these results illustrate the potential for biochemical and isotope analysis to determine novel pre-breeding traits to improve faba bean production under water-limited conditions.

5.5.1 Physiological responses and biomass production among faba bean genotypes

Physiological responses to the effects of drought are complex and highly variable. Faba bean lines in this study showed considerable changes in water relation traits, i.e. water potential and RWC, among genotypes under rainfed conditions. Our chosen genotypes showed significantly different responses to that observed in our previous field study (Chapter 3). Genotype AC0805#4912 and 11NF008b-15 exhibited increased drought tolerant response whereas genotype 11NF010c-4 and 11NF020a-1 responded sensitively towards drought in our field study (Chapter 3). The treatment effect was not significant for RWC. Treatment effect on water potential was also absent except for the genotype 11NF020a-1 (Figure 5.1).

One of the earliest plant defense mechanisms during water deficit is stomatal closure (Reynolds-Henne et al., 2010). The sensitivity of $g_s$ to leaf water loss for 11NF020a-1 is an indication of this genotype being sensitive to water deficit. Stomatal response to water deficit generally involves stomatal closure to reduce water loss. However, this closure also controls CO₂ movement, ultimately influencing carboxylation and plant metabolism (Buckley, 2005). Complete stomatal closure was not observed in our rainfed trial throughout the day indicating that this investigation was suitable for the study of plant physiological responses within the boundaries of physiological function. A sharp decline was observed in $g_s$ in the middle of the day which is a commonly observed phenomenon for stomata due to the higher temperature and as well as water deficit. Under field conditions, gas exchange measurement is influenced by light intensity, heat and/or relative humidity (Chaves et al., 2003). Higher photosynthetic rate
observed in the early morning for both trials indicated the presence of enough moisture in the soil to supply rehydration during the dark period.

Furthermore, instability of gas exchange measurement may happen due to adaptive characteristics, such as alteration in water potential (Farooq et al., 2009) to alleviate the drought effect under field conditions. It can be said from our study, selection solely based on physiological measurement may not be reproducible in the natural system due to temporal heterogeneity. More integrative approaches like an isotopic abundance of different plant organs can make it more meaningful to study plant performance under field conditions.

Though the irrigated trial received one extra irrigation of 25 mm on top of the rain, no significant difference was observed in grain yield (Figure 5.9) except for the genotype AC0805#4912. Genotypes from the drought tolerant group performed well with the irrigated condition as well. Treatment response to biomass production was not significant for genotype except AC0805#4912 and 11NF020a-1 (Figure 5.8) where the irrigated trial had higher biomass compared to rainfed. Indeterminate growth of faba bean makes it sensible for the genotypes to produce higher biomass with the presence of adequate moisture. Moreover, in field conditions, water holding capacity and high soil volume contribute to sustaining yield in the rain-fed trial.

5.5.2 Biochemical responses among faba bean genotypes

Identification and quantification of metabolites from field grown samples provided meaningful insight into plant behaviour in the cropping system. Leaf sample integrity may be compromised under field based conditions due to sampling strategy, or the interpretation of results in response to prevalent relative humidity, light, temperature and soil nutrient in both the short and long term (Poorter and Nagel, 2000). Sugar alcohol accumulation found to be an important
trait under environmental fluctuations (Loescher, 1987; Merchant and Richter, 2011). In addition, the distribution of these compounds also follows taxonomical patterns of plants (Bieleski and Briggs, 2005; Merchant et al., 2007) correlating with increasing aridity and water deficit. Despite considerable advantages of sugar alcohol measurement, a few studies were conducted in field conditions (Streeter et al., 2001).

In this study glucose, *myo*-inositol and sucrose increased significantly in irrigated treatments for all studied genotypes despite only a slight increase of moisture level in the irrigated trial. The reasons behind the significant alteration of metabolites may be co-occurrence of stress as well as short term response towards stress. Under field conditions, the co-occurrence of stress (drought-heat) is frequent and often misleads individual effect. Plants response against two different abiotic stress is not unique and are difficult to separate from one to another as the plant mimics the condition (Mittler, 2006). On the other hand, short term influence of light, humidity and moisture can change biochemical pattern (Poorter and Nagel, 2000). Sugar accumulation (glucose, sucrose and maltose) in Arabidopsis also cannot be extrapolated to the field (Rizhsky et al., 2004). This suggests that, although our water deficit was relatively mild, these changes may be influenced by other environmental effects. The accumulation of sugar in our study may also support what is observed among other sugar alcohol accumulating species where accumulation functioning as primary metabolism derived carbon sink; hence alleviated sugar mediated suppression of photosynthesis under stress (Dumschott et al., 2018). Future studies should target both the influence of individual stresses and plant developmental stages on metabolite accumulation in this crop and genetic variation of these processes.
5.5.3 Predictions of WUE based on the isotopic abundance

Stable carbon isotope (δ\(^{13}\)C) of leaf material to predict water use efficiency is well established for a wide array of plant species (Farquhar and Richards, 1984; Seibt et al., 2008). However, increased precision of WUE calculation has been observed through the use of compound specific isotope abundances of metabolite (Lockhart et al., 2016; Smith et al., 2016). Carbon isotope discrimination fluctuates with water availability primarily due to the effect of stomatal aperture influencing diffusional fractionation. Therefore, it can be used to predict spatial and temporal carbon-water relationships (Seibt et al., 2008). As expected, there was no treatment effect in gas exchange parameters. The main advantage of Δ to assess WUE over gas exchange was that it is integrative across time (days to weeks) and therefore may not reflect short-term environmental changes (minutes to hours).

Predicted WUE was highly correlated with the measured WUE values for all genotypes in our study except PBA Wara (Figure 5.7). The offset between predicted and measured on the full carbon isotope model may be attributed to the parameterization of the model (such as the fractionation of isotopes attributable to phosphoenolpyruvate carboxylase, PEPC) and the inclusion of fractionation events that are currently not contained within the model (see for example parameter': (Farquhar and Sharkey, 1982b). A number of established implications and indirect parameters counted within the model may be an issue while interpreting outcome (Seibt et al., 2008). One of the possibilities is not to incorporate post photosynthetic fractionation in the model and the difficulties in parameterising processes governing post-photosynthetic fractionation (Cernusak et al., 2009). Similarly, RuBisCO and PEP fractionation are hard to measure (Farquhar et al. 1989). The efficacy to predict WUE governs by the increased understanding of the process related to the model. For the use of Δ in screening applications such as those suggested here, parameterisation of post photosynthetic fractionation
events is critical, but also likely to be species specific due to differences in biochemical fraction during metabolite synthesis. An improved understanding in this area helps to develop cost-effective and reproducible tools to study plant carbon-water relationship.

5.6 Conclusion

The leaf level soluble chemistry grown under field conditions provides an overall indication of plant performance. While controlled environment conditions facilitate individual trait identification, field conditions elicit a number of plant responses. The consistent offset between predicted and measured WUE from that of gas exchange to modelled WUE based on $\Delta$ indicates considerable promise for this technique.
Chapter 6
Hydroponics as a tool for screening drought tolerance in faba bean

Abstract

Drought is an important abiotic factor that contributes to yield loss in faba bean (*Vicia faba* L). Plant characteristics for drought tolerance coupled with efficient screening protocols underpin effective breeding programs. Here we test a rapid screening technique against material grown in a hydroponic system for tolerance to root dehydration. A set of 96 faba bean genotypes from different origins were evaluated under hydroponic conditions by periodic removal from the nutrient solution. A further investigation was conducted in sand culture to compare with the hydroponic approach. Root traits differed between treatments and significant correlation was observed among measured root traits for both hydroponics and sand culture. Root to shoot mass ratio (RMR) and drought score (DS) found to be the most important traits under the seedling stage to screen drought tolerant genotypes. Principal component analysis (PCA) demonstrated the contribution of PC1 and PC2 were 64.08 and 63.08 % towards total variation during well-watered (WW) and water deficit (WD) environments, respectively. PCA and mean comparison of root data identified five tolerant genotypes AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18, 11NF011a-3 and five sensitive genotypes 11NF010c-4, 11NF020a-1, AC1869, 11NF021a-12 and 11NF007a-4. Results also suggested that hydroponics is a simple and reliable phenotyping tool to identify drought tolerant faba bean genotypes. The potential of these genotypes could be used for root trait phenotyping assisted breeding with improved adaptation to dry environments.

6.1 Introduction

Among cultivated grain legumes, faba bean ranked as sixth for production volume and is found over a wide-ranging set of environmental conditions (Redden et al., 2014). Its use for both food
and feed is widely accepted and also found as protein-rich starchy legumes containing 24 to 34%
protein of seed dry matter (Crépon et al., 2010). Its high biomass has also been contributed
to soil health improvement in cereal dominant production systems (Ruisi et al., 2017).
Sustainable faba bean production is challenged by changing climatic conditions with drought
determined to be the most contributing edaphic factor (Farooq et al., 2016). Though faba bean
has mostly been grown under rainfed conditions, irrigated farming increases yield significantly
to that of rainfed systems (Oweis, 2005). Considering cultivated grain legumes, faba bean
found most drought responsive (for review see: (Khan et al., 2010). Yield reduction due to
drought was recorded as much as 50% (Mwanamwenge et al., 1999) with an overall average
of 40% (Daryanto et al., 2015) across a range of environments. However, the crop showed field
level drought adaptation (Abdelmula et al., 1999; Ammar et al., 2015) and diversity for drought
tolerance (Belachew et al., 2018; Khazaei et al., 2013a). Thus, there are potential options for
further improvement in faba bean if traits related to drought tolerance can be identified.

Mechanisms conferring drought adaptation in faba bean are not fully characterised. For
example, degrees of osmotic adjustment, stomatal regulation of water use and other factors
contributing to isohydric behaviour are not fully understood. Sustainable yield under drought
conditions is of utmost need in coming years but a few challenges, like effective screening
protocols make the progress slow (Stoddard et al., 2006). Understanding the specific role of
traits is essential for developing rapid, efficient and reproducible phenotyping platform that
applicable in drought tolerance breeding programs.

Among different stress adaptation strategies, root architectural characters of a plant influence
on survival under stress conditions. Under changing environments, roots can modify to absorb
water and nutrients alternatively known as plasticity (Chen et al., 2015). Limited studies have
been completed on roots due to their complexity and cumbersome methodologies, particularly in field-based screening (Kashiwagi et al., 2006). In legume crops, root associated traits are considered to be highly important to sustain growth under dry environments (Vadez et al., 2008). Root based phenotyping research especially in the seedling root study conducted on a wide range of legumes: cowpea (Matsui and Singh, 2003), chickpea (Kashiwagi et al., 2005) lentil (Singh et al., 2013) and recently in faba bean (Belachew et al., 2018).

High throughput, cost-effective, non-invasive tools for the rapid and reliable phenotyping is of critical importance to plant breeder across a range of natural and agricultural systems. Phenotyping of plants under controlled environments allow the imposition of uniform stress conditions (Tuberosa, 2012). Several investigators have developed and applied a number of methods based on soil and hydroponics to study at the seedling stage on the basis of physiological/biochemical or morphological traits to identify desired genotypes. Standardised protocols are needed to produce reproducible results for investigations seeking to determine drought stress tolerance in faba bean. Limited studies were carried out on the consequences of water deficit particularly regarding root characteristics in faba bean (Belachew et al., 2018; Grzesiak et al., 1997; Manschadi et al., 1998). It is therefore a recognised need to identify genotypes with high levels of drought tolerance through a detailed examination of diversity in root related traits of faba bean. We considered the following objectives for this study. a) To develop a seedling root based screening protocol of faba bean under hydroponic conditions and b) to identify genotypes with different drought tolerance level based on their root traits and drought score that can serve in future breeding.
6.2 Methodology

6.2.1 Plant materials and growth conditions

Ninety-six diverse faba bean genotypes that were used in our initial field trial at Narrabri. The set of germplasm included commercial cultivars (5), exotic lines (16) and a breeding population (75), were used in this study. Details of germplasm list and pedigree were given in (Appendix 3.1). The experiment was carried out at Bangladesh Agricultural Research Institute (BARI), Bangladesh (24° 22′ 8.84″ N, 88° 39′ 42.3″ E). The temperature of the greenhouse was maintained at 25 ± 2 °C for 14 h, and 15 ± 2 °C for ten h for light and without light period respectively. Light intensity and relative humidity were fixed at 550-560 μmol m⁻²s⁻¹ and 60% ± 5% and respectively. An automatic light source was illuminated if natural light went down below 400 PAR. Studies were conducted in two independent culture conditions, i.e. hydroponic and sand.

6.2.2 Hydroponic culture

Seeds were soaked in a conical flask containing 0.05% HgCl₂ (3 - 4 min) followed by distilled water washing three times and seeded them in an autoclaved quartz granule filled tray. After sowing, the tray was covered with a black polyethylene sheet for four days and subsequently removed to ease germination. Eight days old seedlings were transferred in opaque pots, (18 x 21 cm in diameter and height) containing the nutrient solution (Figure 6.1). After removal of the endosperm, sponges were used to wrap seedlings firmly through the lid of opaque pots (Figure 6.1B). Each lid had six holes, where a single plant was placed in each hole (Figure 6.1). Half-strength Hoagland solution was used for seedling growth and development (Hoagland and Arnon, 1950) Recipe for the solution given in (Table 6.1). The experiment had three
replications and each pot contained six seedlings were treated as a single replicate. Nutrient solution was replaced in every four days until harvest. Plants were allowed to grow two more weeks followed by drought treatments that were applied by removing the plant from the solution and placing them in open air for 5 hours (10.00 am - 15.00 pm) and returning to the nutrient solution. The treatment period was seven days. WW plants were retained in hydroponic solution until their harvest. At four weeks age, seedlings were harvested for measurement.

Figure 6:1 Faba bean seedlings treated under hydroponic conditions. A. Diversified root development among genotypes. B. Seedlings were placed in an opaque pot after removal of endosperm. C. Control seedlings received continuous water supply throughout the treatment period.
Table 6:1 Preparation of half-strength Hoagland solution for plant nutrition during water deficit screening methodology on *Vicia faba* L.

<table>
<thead>
<tr>
<th>Name of Chemicals</th>
<th>Nutrient solution concentration (mmol/L)</th>
<th>Weight of reagent (g/10L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO$_3$)$_2$.4H$_2$O</td>
<td>2</td>
<td>944.6</td>
</tr>
<tr>
<td>K$_2$SO$_4$</td>
<td>0.75</td>
<td>261.39</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.65</td>
<td>324.12</td>
</tr>
<tr>
<td>KCl</td>
<td>0.1</td>
<td>14.91</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.25</td>
<td>68.046</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.001</td>
<td>0.618</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>0.001</td>
<td>1.690</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>0.0001</td>
<td>0.25</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.001</td>
<td>2.875</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$.H$_2$O</td>
<td>0.000005</td>
<td>0.062</td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>0.2</td>
<td>146.82</td>
</tr>
</tbody>
</table>

6.2.3 Sand culture screening

Five genotypes from each tolerant and sensitive group based on rooting traits were grown in seven L plastic pots filled with autoclaved sand. The medium was soaked with an equal amount of nutrient used for hydroponic conditions. At germination, three healthy seedlings were grown for three weeks. Each pot was considered as a single replicate and experiment was conducted with three replications for both genotype and treatment. Pots were irrigated every day to supply adequate moisture for growth and establishment of the seedlings. After three weeks, WD treatment imposed by ceasing irrigation for one week in half of the pots. Another half treated as control by maintaining soil water content at 80% field capacity. At harvesting (four weeks) seedlings were removed carefully by washing each pot with continuous water flow and
immediately placed into zip lock bags to reduce transpiration loss and transported to the lab for further measurement.

6.2.4 Data recording

Shoot height (SH), branch number per plant (BN), taproot length (TRL) and lateral root number (LRN) were counted manually. Fresh shoot and root weight were measured gravimetrically. Fresh root and shoot dried at 60 °C (72 h) in an electric oven to weigh Root-to-shoot mass ratio (RMR). Drought scoring was completed on a scale of 0-4 adopted from Singh et al. (2013).

6.3 Statistical Analysis

Descriptive statistics and Analysis of variance (ANOVA) were performed by GenStat 18th Edition. Means for each rooting trait were separated by Duncan's Multiple Range Test (Alpha 5% and 1%) (Duncan, 1955). Principal component analysis and Pearson correlations among traits were done by XLSTAT 2018.

6.4 Results

6.4.1 Variation in growth parameters under hydroponic conditions

Effect of treatment was observed in most of the shoot and root related parameters i.e. BN, SH, TRL, LRN, FSW, DSW, FRW, DRW and DS studied under hydroponic conditions (Table 6.2). Genotypes also exhibited variation in rooting pattern as well as shoot related traits. WD condition decreased all traits except RMR compared to WW (Table 6.2). Among root traits, the Maximum reduction was observed in TRL (62%) with the other three (LRN, FRW and DRW) reduced by around 40% compared to the WW treatment (Table 6.2). Mean RMR in WD
treatment was 0.35 whereas, in WW treatment, it was 0.32. Genotypes from different origin responded differently against WD treatment and drought score was varied from ‘0 – 4’. Genotype AC0805#4912 (from Yemen) showed maximum drought tolerance with a score of 0 and genotype 11NF020a-1 bred at Narrabri showed the least drought tolerance with a score ‘4’. As control genotypes received adequate water, no drought symptoms were observed. Among the shoot parameters, FSW had a maximum reduction (43%) in WD treatment and the lowest reduction was in BN (only 4%). Most of the accessions from exotic origin were sensitive to drought stress except genotype "74,"i.e. AC0805 #4912.

Genotype AC0805#4912 demonstrated drought tolerance in our field study also (Chapter 3). The first visible symptom of WD treatment, i.e. wilting commenced after two hours of air exposure and most of the sensitive genotypes wilted within five hours. Within the first three days of treatment, no major symptoms of drought, like yellowing of leaves were observed. Differences among the genotypes became prominent with the progression of water deficit. Complete seedling death was observed on day five in the drought sensitive lines. Root characteristics mean comparison enabled us to identify accessions that are tolerant under WD conditions. Based on the drought score, RMR and a combined PCA analysis (Figure 6.2) we determined five genotypes from two groups that are drought tolerant and drought sensitive.
**Table 6.2** Descriptive statistics of root traits in 96 faba bean genotypes grown in a hydroponic phenotyping platform under well-watered and water deficit condition at BARI, Bangladesh

<table>
<thead>
<tr>
<th>Trait</th>
<th>Description (Unit)</th>
<th>Well-Watered</th>
<th>% Reduction</th>
<th>Water Deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Min.</td>
<td>Max.</td>
<td>Mean</td>
</tr>
<tr>
<td>BN</td>
<td>Branch number</td>
<td>2</td>
<td>7</td>
<td>5.08</td>
</tr>
<tr>
<td>SH</td>
<td>Shoot height (cm)</td>
<td>12.5</td>
<td>54.2</td>
<td>36.09</td>
</tr>
<tr>
<td>TRL</td>
<td>Taproot length (cm)</td>
<td>25.5</td>
<td>65.1</td>
<td>52.01</td>
</tr>
<tr>
<td>LRN</td>
<td>Lateral root number</td>
<td>22</td>
<td>63</td>
<td>40</td>
</tr>
<tr>
<td>FSW</td>
<td>Fresh shoot weight</td>
<td>18.9</td>
<td>46.7</td>
<td>35.91</td>
</tr>
<tr>
<td>DSW</td>
<td>Dry shoot weight (g)</td>
<td>2.09</td>
<td>6.24</td>
<td>3.56</td>
</tr>
<tr>
<td>FRW</td>
<td>Fresh root weight (g)</td>
<td>6.25</td>
<td>38.8</td>
<td>21.87</td>
</tr>
<tr>
<td>DRW</td>
<td>Dry root weight (g)</td>
<td>0.41</td>
<td>2.95</td>
<td>1.78</td>
</tr>
<tr>
<td>RMR</td>
<td>Root-to-shoot dry</td>
<td>0.25</td>
<td>0.52</td>
<td>0.32</td>
</tr>
<tr>
<td>DS</td>
<td>Drought Score</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Genotypes AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18, and 11NF011a-3 under the tolerant group while genotypes 11NF0010c-4, 11NF02020a-1, AC1869, 11NF01a-12 and 11NF007a-4 under sensitive group (Table 6. 3). The tolerant genotypes had a drought score of zero (0) on the other hand; the sensitive group had drought score of four (4). All of the studied parameter except BN and SH followed a decreasing pattern in the sensitive group compare to the tolerant group and they are statistically significant (Table 6. 3)..
Table 6.3 Morphological parameters (mean value) of 10 selected genotypes from drought tolerant and drought sensitive groups based on their RMR and DS score grown under the hydroponic condition at BARI, Bangladesh.

<table>
<thead>
<tr>
<th>Entr</th>
<th>BN</th>
<th>SH</th>
<th>TRL (cm)</th>
<th>LRN</th>
<th>FSW (g)</th>
<th>DSW (g)</th>
<th>FRW (g)</th>
<th>DRW (g)</th>
<th>RMR</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td><strong>Tolerant</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>4.67bcd</td>
<td>35.6 vwxxy</td>
<td>31.51K</td>
<td>50.33LMN</td>
<td>34.48GHJ</td>
<td>4.55opq</td>
<td>17.37KLM</td>
<td>1.64W</td>
<td>0.360mn</td>
<td>0.00a</td>
</tr>
<tr>
<td>93</td>
<td>5.00bcd</td>
<td>34.23 stuv</td>
<td>21.47lmno</td>
<td>37.67pqr</td>
<td>37.00NOP</td>
<td>4.37pqr</td>
<td>17.70LM</td>
<td>1.80XY</td>
<td>0.412stu</td>
<td>0.00a</td>
</tr>
<tr>
<td>60</td>
<td>5.33bcd</td>
<td>28.05 efgh</td>
<td>22.92vwxxy</td>
<td>43.00ABCD</td>
<td>33.33EFG</td>
<td>4.51opq</td>
<td>16.49HIJK</td>
<td>1.83Y</td>
<td>0.406stu</td>
<td>0.00a</td>
</tr>
<tr>
<td>71</td>
<td>5.50cdef</td>
<td>33.68 rstu</td>
<td>21.00klmn</td>
<td>40.67xyzA</td>
<td>34.54GHJ</td>
<td>4.38pqr</td>
<td>16.33GHIJ</td>
<td>1.80XY</td>
<td>0.411stu</td>
<td>0.00a</td>
</tr>
<tr>
<td>84</td>
<td>5.67def</td>
<td>34.38 stuv</td>
<td>22.62tuvw</td>
<td>40.83xyzA</td>
<td>35.47JKL</td>
<td>4.54opq</td>
<td>16.33GHIJ</td>
<td>1.74X</td>
<td>0.383pqr</td>
<td>0.66a</td>
</tr>
<tr>
<td><strong>Sensitive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.33bcd</td>
<td>35.48vwxy</td>
<td>19.99ghij</td>
<td>33.67ijkl</td>
<td>30.89vwxy</td>
<td>3.36abc</td>
<td>10.38efgh</td>
<td>1.04uvw</td>
<td>0.310hijk</td>
<td>4.00i</td>
</tr>
<tr>
<td>22</td>
<td>6.00f</td>
<td>30.85ghij</td>
<td>20.27ghij</td>
<td>22.00ab</td>
<td>29.12qrst</td>
<td>3.50cdef</td>
<td>10.45efgh</td>
<td>1.21DEF</td>
<td>0.346km</td>
<td>4.00i</td>
</tr>
<tr>
<td>23</td>
<td>5.50cdef</td>
<td>38.30DEF</td>
<td>21.76lmno</td>
<td>22.28ab</td>
<td>20.42bcde</td>
<td>3.59stuv</td>
<td>10.06defg</td>
<td>1.09wxyz</td>
<td>0.304fgh</td>
<td>4.00i</td>
</tr>
<tr>
<td>30</td>
<td>4.67bcd</td>
<td>34.87uuvwxyz</td>
<td>18.23efgh</td>
<td>29.50defg</td>
<td>28.11lmno</td>
<td>3.49cdef</td>
<td>10.56fghij</td>
<td>1.15ABC</td>
<td>0.330ijk</td>
<td>4.00i</td>
</tr>
<tr>
<td>82</td>
<td>5.00bcd</td>
<td>37.55CDE</td>
<td>24.19EFG</td>
<td>20.33a</td>
<td>26.00ghij</td>
<td>3.45bcd</td>
<td>15.01zAB</td>
<td>1.12ABC</td>
<td>0.325ghi</td>
<td>4.00i</td>
</tr>
</tbody>
</table>

Means followed by different letters within a column are significantly different at P < 0.05 by DMRT test (Duncan, 1955)

AC0805#4912 = 74, 11NF008b-15 = 93, IX564c/1-7 = 77, 11NF026c-18 = 71, 11NF011a-3 = 84
11NF021a-12 = 2, 11NF010c-4 = 22, 11NF020a-1 = 23, AC1869 = 30, 11NF007a-4 = 41

BN= Branch number plant\(^1\), SH= Shoot height, TRL= Taproot length, LRN= Lateral root number, FSW= Fresh shoot weight, FRW= Fresh root weight, DSW= Dry shoot weight, DRW= Dry root weight, RMR= Root-to-shoot mass ratio, DS= Drought score.
Table 6.4 Analyses of Variance for shoots and root traits at seedling stage of 96 faba bean genotypes subjected to well-watered and water deficit grown under the hydroponic condition

<table>
<thead>
<tr>
<th>Effect</th>
<th>df</th>
<th>BN</th>
<th>SH</th>
<th>TRL</th>
<th>LRN</th>
<th>FSW</th>
<th>DSW</th>
<th>FRW</th>
<th>DRW</th>
<th>RMR</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Replication</td>
<td>2</td>
<td>0.33</td>
<td>513.70</td>
<td>223.99</td>
<td>1095.06</td>
<td>608.42</td>
<td>0.57</td>
<td>214.81</td>
<td>0.40</td>
<td>0.002</td>
<td>0.07</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>4.5</td>
<td>167.90*</td>
<td>43.81**</td>
<td>337.35**</td>
<td>198.34**</td>
<td>0.05**</td>
<td>53.50**</td>
<td>2.35**</td>
<td>0.01**</td>
<td>1.7**</td>
</tr>
<tr>
<td>Genotype</td>
<td>95</td>
<td>1.29</td>
<td>2466.11**</td>
<td>1378.42**</td>
<td>5519.25**</td>
<td>2901.48**</td>
<td>3.48**</td>
<td>904.93**</td>
<td>0.53**</td>
<td>0.03**</td>
<td>1091.25**</td>
</tr>
<tr>
<td>Geno x Treat</td>
<td>95</td>
<td>0.33ns</td>
<td>5.16**</td>
<td>3.2**</td>
<td>10.39**</td>
<td>5.90**</td>
<td>0.08**</td>
<td>3.2**</td>
<td>0.73**</td>
<td>0.01**</td>
<td>1.7**</td>
</tr>
<tr>
<td>Error</td>
<td>382</td>
<td>0.32</td>
<td>2.04</td>
<td>0.94</td>
<td>4.01</td>
<td>0.03</td>
<td>0.03</td>
<td>1.91</td>
<td>0.12</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td></td>
<td></td>
<td>11.3</td>
<td>4.2</td>
<td>4.5</td>
<td>6.6</td>
<td>5.5</td>
<td>4.1</td>
<td>8.6</td>
<td>3.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* and ** represents significant probability levels where P < 0.05 and < 0.01. ns = non-significant.

BN= Branch number plant\(^{-1}\), SH= Shoot height, TRL= Taproot length, LRN= Lateral root number, FSW= Fresh shoot weight, FRW= Fresh root weight, DSW= Dry shoot weight, DRW= Dry root weight, RMR= Root-to-shoot mass ratio, DS= Drought score.

Water deficit, genotypes and interaction effect on different root parameters was studied. Water deficit significantly (P < 0.01) reduces all root parameters along with shoot height (P < 0.05). ANOVA also revealed significant genotypic differences were observed among all studied parameters excluding BN (P < 0.05). Genotype-treatment interaction was also significant (P < 0.01) for all traits except BN (Table 6.4).
6.4.2 Principal component analysis (PCA)

PCA was completed to identify promising genotypes by dividing the total variance into different component variables. PCA derived factor loading, eigen value and variability of the examined parameters were presented in Table 6.5. A biplot graph from PCA was also created (Figure 6.2). For the 96 genotypes, total variation can be explained by the two major components and it was valued 64.08% and 63.08 % for WW and WD environments, respectively. The first principal component was influenced by FRW, FSW, TRL and LRN and explained of 45.85 % of the total variation. These parameters had eigen values ≥ 0.50 (Table 6.5) and credited PCA value for genotypes grown under WW conditions. The second principal component was driven by RMR and DRW and calculated 18.23% variation from total variation. (Table 6.5).

Under WD condition, 35.95 % variation was explained by PC1 and dominated by FRW, FSW and LRN (described by eigen value ≥ 0.50). The second component demonstrated for 27.14% of the total variance and mainly dominated by RMR, DS and DRW (Table 6.5). Accessions AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18, and 11NF011a-3 were picked based on their PC1 and PC2 values and were found superior for WW condition (Figure 6.2B). On the other hand, genotypes 11NF010c-4, 11NF020a-1, AC1869, 11NF021a-12 and 11NF007a-4 selected as sensitive genotypes (Figure 6.2A).
Figure 6:2 PCA plot was showing the distribution of faba bean genotypes both well-watered and water deficit condition based on the first two components. Circle (red filled) represent selected lines.

AC0805#4912 = 74, 11NF008b-15 = 93, IX564c/1-7 = 77, 11NF026c-18 = 71, 11NF011a-3 = 84, 11NF021a-12 = 2, 11NF010c-4 = 22, 11NF020a-1 = 23, AC1869 = 30, 11NF007a-4 = 41
Table 6.5: Estimated factor loadings, eigen value and variability under well-watered and water deficit condition of 96 faba bean genotypes grown in a hydroponic phenotyping platform

<table>
<thead>
<tr>
<th>Parameters*</th>
<th>Estimated factor loadings for the 96 genotypes</th>
<th>Well-Watered</th>
<th>Water deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC1</td>
<td>PC2</td>
<td>PC1</td>
</tr>
<tr>
<td>BN</td>
<td>0.436</td>
<td>-0.236</td>
<td>0.235</td>
</tr>
<tr>
<td>SH</td>
<td>0.303</td>
<td>-0.379</td>
<td>0.398</td>
</tr>
<tr>
<td>TRL</td>
<td>0.511</td>
<td>-0.354</td>
<td>0.451</td>
</tr>
<tr>
<td>LRN</td>
<td>0.658</td>
<td>-0.150</td>
<td>0.705</td>
</tr>
<tr>
<td>FSW</td>
<td>0.805</td>
<td>-0.272</td>
<td>0.783</td>
</tr>
<tr>
<td>DSW</td>
<td>0.425</td>
<td>-0.317</td>
<td>0.200</td>
</tr>
<tr>
<td>FRW</td>
<td>0.785</td>
<td>-0.134</td>
<td>0.814</td>
</tr>
<tr>
<td>DRW</td>
<td>0.291</td>
<td><strong>0.689</strong></td>
<td>0.381</td>
</tr>
<tr>
<td>RMR</td>
<td>0.182</td>
<td><strong>0.791</strong></td>
<td>0.284</td>
</tr>
<tr>
<td>DS</td>
<td>-</td>
<td>-</td>
<td>0.024</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimated factor loadings for the 96 genotypes</th>
<th>Well-Watered</th>
<th>Water deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Eigenvalues ≥ 0.50 are in bold.

*BN= Branch number plant-1, SH= Shoot height, TRL= Taproot length, LRN= Lateral root number, FSW= Fresh shoot weight, FRW= Fresh root weight, DSW= Dry shoot weight, DRW= Dry root weight, RMR= Root-to-shoot mass ratio, DS= Drought score.

6.4.3 Sand culture method

Five drought tolerant (AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18, and 11NF011a-3) and five drought sensitive (11NF010c-4, 11NF20a-1, AC1869, 11NF021a-12 and 11NF007a-4) genotypes were grown in sand and supplemented with the same nutrient solution to confirm the hydroponic results. Drought score was similar to the hydroponic condition for sensitive group except the genotype “23”, 11NF020a-1 which showed some tolerance and scored ‘2’.

6.4.4 Correlation among growth parameters

Pearson correlation analysis was performed for both hydroponic and sand culture screening methods to identify relationships among the measured traits under both conditions. It showed
‘r’ values with a range of 0.003 - 0.968 (Table 6.6) for hydroponic and 0.023 - 0.975 for sand culture (Table 6.7). Correlation between the root related traits was highly significant for both culture conditions. The interrelationship of DS with all other characters except BN is highly significant. We also observed a highly positive correlation between RMR and DS in both growth conditions. TRL and LRN have also had a significant correlation with RMR and DS. Correlation study showed better relationships among the selected characters in hydroponic culture compare to sand culture (Table 6.6 and Table 6.7).
Table 6:6 Pearson correlation between growth parameters of 96 faba bean genotypes subjected to under hydroponic conditions. Values marked with ** and * represents significant at the 1% and 5% level respectively.

<table>
<thead>
<tr>
<th>Variables</th>
<th>BN</th>
<th>SH</th>
<th>TRL</th>
<th>LRN</th>
<th>FSW</th>
<th>DSW</th>
<th>FRW</th>
<th>DRW</th>
<th>RMR</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN</td>
<td>1</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SH</td>
<td>0.392*</td>
<td>1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRL</td>
<td>0.308**</td>
<td>0.092</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LRN</td>
<td>0.292**</td>
<td>0.142</td>
<td>0.385**</td>
<td>1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSW</td>
<td>0.505*</td>
<td>0.426**</td>
<td>0.250**</td>
<td>0.454**</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DSW</td>
<td>0.140</td>
<td>0.117</td>
<td>0.186</td>
<td>0.237**</td>
<td>0.412**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRW</td>
<td>0.375*</td>
<td>0.115</td>
<td>0.328**</td>
<td>0.608**</td>
<td>0.662**</td>
<td>0.401**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRW</td>
<td>0.258**</td>
<td>0.032</td>
<td>0.060**</td>
<td>0.291**</td>
<td>0.362**</td>
<td>0.265**</td>
<td>0.411**</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>RMR</td>
<td>0.230*</td>
<td>0.003</td>
<td>0.018**</td>
<td>0.242**</td>
<td>0.266**</td>
<td>0.223**</td>
<td>0.321**</td>
<td>0.968**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>0.495</td>
<td>0.751**</td>
<td>0.713**</td>
<td>0.702**</td>
<td>0.482**</td>
<td>0.651**</td>
<td>0.660**</td>
<td>0.824**</td>
<td>0.91**</td>
<td>1</td>
</tr>
</tbody>
</table>

BN= Branch number plant\(^1\), SH= Shoot height, TRL= Taproot length, LRN= Lateral root number, FSW= Fresh shoot weight, FRW= Fresh root weight, DSW= Dry shoot weight, DRW= Dry root weight, RMR= Root-to-shoot mass ratio, DS= Drought score.
Table 6:7 Pearson correlations between growth parameters of 10 selected faba bean genotypes subjected to water deficit grown under sand culture. Values marked with ** and * represents significant at the 1% and 5% level respectively

<table>
<thead>
<tr>
<th>Variables</th>
<th>BN</th>
<th>SH</th>
<th>TRL</th>
<th>LRN</th>
<th>FSW</th>
<th>DSW</th>
<th>FRW</th>
<th>DRW</th>
<th>RMR</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>SH</td>
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<td></td>
</tr>
<tr>
<td>TRL</td>
<td>0.476</td>
<td>0.369</td>
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</tr>
<tr>
<td>LRN</td>
<td>0.280**</td>
<td>0.367</td>
<td>0.591**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>FSW</td>
<td>0.614</td>
<td>0.216</td>
<td>0.174</td>
<td>0.774**</td>
<td>1</td>
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<tr>
<td>DSW</td>
<td>0.072</td>
<td>0.052</td>
<td>0.023</td>
<td>0.297</td>
<td>0.328**</td>
<td>1</td>
<td></td>
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</tr>
<tr>
<td>FRW</td>
<td>0.695**</td>
<td>0.189</td>
<td>0.380**</td>
<td>0.860**</td>
<td>0.772**</td>
<td>0.374**</td>
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<td></td>
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</tr>
<tr>
<td>DRW</td>
<td>0.181</td>
<td>0.401</td>
<td>0.538**</td>
<td>0.382**</td>
<td>0.438**</td>
<td>0.138</td>
<td>0.212**</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RMR</td>
<td>0.157</td>
<td>0.390</td>
<td>0.533**</td>
<td>0.310**</td>
<td>0.359**</td>
<td>0.103</td>
<td>0.105**</td>
<td>0.970**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>0.597</td>
<td>0.821**</td>
<td>0.624**</td>
<td>0.641**</td>
<td>0.419**</td>
<td>0.870**</td>
<td>0.762**</td>
<td>0.975**</td>
<td>0.965**</td>
<td>1</td>
</tr>
</tbody>
</table>

BN= Branch number plant\(^{-1}\), SH= Shoot height, TRL= Taproot length, LRN= Lateral root number, FSW= Fresh shoot weight, FRW= Fresh root weight, DSW= Dry shoot weight, DRW= Dry root weight, RMR= Root-to-shoot mass ratio, DS= Drought score.
6.5 Discussion

Identification and validation of root traits conferring adaptation during drought stress have created interest for plant breeders for over 100 years (Vines, 1888). A major challenge to this is a lack of root related genetic information, established reliable screening protocols and complex soil root interaction during stress (Belachew et al., 2018; Chen et al., 2015; Vadez et al., 2008). We investigated root related traits that contributed towards drought tolerance in large germplasm set of faba bean both in hydroponic and sand culture. The sand culture technique further confirmed results obtained from the hydroponic method.

Phenotypic data from hydroponic conditions exhibited large variation in most of the root and shoot related traits (Table 6.2). Faba bean accessions having low tolerance levels against drought under hydroponic condition were characterised by poor root vigour such as a reduction in TRL and LRN. Water deficit significantly reduced TRL and LRN in the hydroponic as well as sand culture in our study. In our study, we did not quantify total root length. However, we found TRL and LRN reduction was significant. LRN found to be the largest contributor towards the total root length in faba bean (Belachew et al., 2018). They found total root length reduction in stress condition was of 77%, which agreed with our results (62% reduction in TRL). A similar observation was also found in common bean where deep rooting and higher lateral root number was nominated for a drought tolerant ideotype (Polania et al., 2017). Lentil genotypes having longer roots and higher DRW were reported to be tolerant against terminal drought (Kumar et al., 2012). Deep rooting systems that is high volume and weight can help legume crops to survive during water deficit to access moisture from deep soil profile and at the same time having lateral roots increasing the chance to utilise surface soil moisture (Vadez et al., 2018).
et al., 2008). Combined, these traits can improve overall access to water throughout development promoting growth and yield production.

Plants maintain a close relationship between roots and shoot during all developmental stages. To sustain growth under stress conditions, roots have the primary response mechanism to maintain adequate nutrient and water supply. Inferior partitioning to root development compared to above ground components leads to poor grain yield under drought stress. Shoot-related traits, i.e. SH, FSW and DSW in our study displayed significant differences among genotypes at harvest (28 d after sowing except for BN). In our study, RMR is highly correlated with DS and had a relatively wide value 0.24 to 0.64 at four weeks after sowing. Generally higher value of RMR is linked with drought tolerance (Kashiwagi et al., 2005). Earlier studies showed plant distributed more energy to root than to shoot development to increase moisture acquisition and limit evaporation during stress (Palta and Gregory, 1997; Sharp and Davies, 1979). RMR values from previous studies on lentil and cowpea showed drought-tolerant lines had higher values than from sensitive ones; 0.57 to 0.66 (Idrissi et al., 2015) and 0.20 to 0.25 (Matsui and Singh, 2003) respectively. In our study, we also found a similar result where tolerant genotypes had RMR ranging from 0.36 to 0.41 and the sensitive group had 0.30 to 0.35. In a set of 89 faba bean genotypes from two distinct groups (wet set and dry set) also exhibits a significant variation among accessions ranging from 0.24 to 0.50 in RMR. However, the group-wise mean value was non-significant indicates RMR is a suitable trait for screening under stress condition and similar findings were demonstrated by Belachew et al., (2018).

Other measured growth-related traits (FSW, FRW, DSW and DRW) also decreased significantly in WD treatment and also showed a significant variation among genotypes. The capacity of moisture absorption under stress for common bean depends on the existence of a
deep taproot system, complemented with profuse later root (Gahoonia et al., 2006) traits also observed in chickpea (Ramamoorthy et al., 2017). These observations were found in the present study such that tolerant genotypes maintained higher FSW, FRW DSW and DRW (Table 6.3). A similar observation was carried out in a root diversity study under WD condition where higher DRW and DSW were determinants of drought tolerance (Belachew et al., 2018).

A significantly higher correlation for most of the studied parameters that are LRN, FSW, FRW, DRW, DSW, RMR and DS make these traits suitable for selection under both culture conditions. Positive correlations were also found between root area coverage (LRN and TRL) suggesting faba bean root system development followed an adaptable pattern in both direction, i.e. depth and breadth. We found RMR is highly correlated with DS and DRW. Thus, screening of genotypes for drought tolerance based on RMR is recommended. Similar findings were observed for lentil (Idrissi et al., 2015) and faba bean (Belachew et al., 2018) suggesting, RMR and DS are efficient parameters to study drought tolerance in faba bean.

The effectiveness of the hydroponic system over the soil to select drought tolerant accession is also studied. Plants were takeout from nutrient solution and exposed to air for five hours. Here we observed highly tolerant genotypes recovered quickly whereas sensitive genotype did not recover when they returned to nutrient solution after four hours of air exposure, indicating catastrophic damage to the hydraulic and biochemical infrastructure. As most of the root based screening method is based on destructive sampling, it is sometimes difficult to explain the contribution of a particular trait that sustains yield under stress conditions. Therefore, the hydroponic assay provides a superior alternative screening method as genotypes that are screened can be transplanted into the soil for subsequent investigations. The ‘r’ value among traits was higher in hydroponic condition compared to sand culture presenting the hydroponic
technique more suitable for screening. Moreover, the hydroponic system is less expensive and less laborious compared to field studies for a large number of accessions and results are reproducible as environmental conditions can be controlled.

6.6 Conclusions

Drought tolerance in faba bean is driven by a well-developed root system which likely enables plants to mine moisture during water deficit conditions. Five genotypes (AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18 and 11NF011a-3) were grouped as drought tolerant based on DS and high root area coverage under drought stress. Five more genotypes (11NF010c-4, 11NF020a-1, AC1869, 11NF021a-12 and 11NF007a-4) that were highly sensitive under drought stress were also identified. Thus, the genotypes mentioned above would be beneficial to develop new drought tolerant cultivars of faba bean. Finally, identification of drought-tolerant genotypes through the hydroponic platform would increase selection efficiency and lead to a new avenue for legume breeding.
Chapter 7
Effect of water deficit on the physiological, biochemical and reproductive biology of contrasting faba bean genotypes

Abstract

Drought stress (also termed water deficit) is one of the most important limiting factors for sustainable faba bean (*Vicia faba*) farming. A number of studies have been undertaken to identify different morphological and physiological indicators of water deficit (WD) tolerance in faba bean. However, very limited information is available on the WD impact on nutritional quality and reproductive biology of this crop. In our study, we compared carbohydrates, amino acids, mineral nutrients and the abundance of naturally occurring carbon isotopes ($\delta^{13}$C) in leaf and grain of faba bean genotypes grown under well-watered (WW) and WD conditions. This was demonstrated that $\delta^{13}$C reflects WD in leaf but not in grain. In general, nutritional quality (essential amino acids and grain mineral content) were not influenced by WD. Carbohydrate accumulation was found to be significant for WD treatment specifically by the presence of higher concentration myo-inositol in WD leaf tissue. Alternatively, sucrose concentration in grain tissue was found to be significantly reduced in concentration) in WD tissue. WD hampered reproductive functionality by the reduction in pollen viability and germination with the progression of stress and reduction was less in the drought tolerant genotype (AC0805#4912) compared to the sensitive one (11NF010c-4). This was also demonstrated WD caused developmental impairment in stamen and pistil where pistil appeared more sensitive than stamen. These findings suggested that in addition to pollen viability, pistil function regulates pod development and nutritional quality in faba bean resilient to WD.
7.1 Introduction

Faba bean is the world's sixth important grain legumes grown over a wide range of environments. The yield of faba bean has doubled over the past 50 years whereas the planted area declined by almost half (Foyer et al., 2016). The most common factor for this decline was irregular rainfall over the growing season. Faba bean is the most WD sensitive cultivated grain legume and yield loss was recorded as much as 50% (Mwanamwenge et al., 1999). Drought tolerance in faba bean has been investigated via physiological characterisation and biological markers (Annicchiarico and Iannucci, 2008; Khan et al., 2007; Khazaei et al., 2013b). Direct measurement of physiological parameters; hold great potential for successful drought tolerance screening for multiple faba bean genotypes (Ammar et al., 2015; Kabbadj et al., 2017).

A range of environmental factors of which WD is critical drives the impact of climate change on global crop production. Yield reduction due to WD is influenced by crop growth stage and the severity of the stress. Plants deploy an array of physiological and biochemical strategies to cope with WD. Physiologically, stomata play an important role whilst biochemically, plants may produce metabolites to maintain cell turgor and prevent cell dehydration. To investigate the impact of WD, tools need to be developed and deployed that can quantify the physiological and biochemical changes in different plant organs (leaf, grain and root) to promote and predict crop production under limited water environments.

One common response of plants against stress conditions is to accumulate minerals and synthesise osmoprotectants in the form of stress metabolites (Samarah et al., 2004). Stress metabolites commonly fall into a limited number of chemical groups attributable to their chemical properties. Among many functions, the accumulation of such compounds assists in the maintenance of cell turgor pressure during WD conditions (Hare et al., 1998). Metabolites
types and their concentration significantly differ in reducing the osmotic potential among plant species. Rapid accumulation of metabolites in plant tissues may indicate stress conditions (either through the accumulation in response to the stress or as a consequence of it) whilst also influencing nutritional quality. Identification of these may hold importance for the development of chemically based tools for detection and/or mitigation of stress conditions.

The availability of water and the ability of plants to uptake nutrients are intrinsically linked. In addition, reduced plant growth imparts less capacity for roots to explore the soil profile and access mineral nutrition. Metabolite profiling holds great potential for detection of stress conditions and informs the timing and magnitude of management required.

In flowering plants, flower production followed by seed formation is linked with yield in any crop. WD hampers the productivity of all growing stages although the incidence during reproductive and grain filling stage is most sensitive for legumes causing significant yield loss (Fang et al., 2010; Kong et al., 2015). This yield loss is often boosted with the sensitive genotype and/or intensity of the stress. In our study, we investigated the chemical composition of the leaf and grain in contrasting faba bean genotypes during WD, as they may inform the remedial actions that can be used or indicate superior genotypes for promotion in plant breeding programs. We also focused on physiological changes of reproductive organs (pistil, pollen) in response to WD at anthesis to explain how WD affects pod development at this stage for faba bean.

Considering, the importance of faba bean as food, feed and in conservation agriculture this study was undertaken to define how WD changes the concentration of metabolites in different plant tissues that underpin yield quality and quantity. Specifically, our objectives were a) to identify biochemical indicators from leaf and grain tissue of contrasting faba bean genotypes; b) to test whether WD can impact on nutritional quality of faba bean grain and c) to identify
the impact of WD on reproductive biology i.e. pollen viability, pollen germination and pistil function towards pod formation. The critical observation of our study would lead to identifying potential biochemical markers for WD tolerance in faba bean breeding programs.

7.2 Methodology

7.2.1 Plant materials and growth conditions

The experiment was carried out at the Centre for Carbon, Water and Food, University of Sydney. Faba bean seeds of three contrasting genotypes (for their leaf δ13C) were used in this experiment. These three genotypes (AC0805#4912, 11NF010c-4 and PBA Warda) were chosen for their diverse leaf δ13C value. Plants were grown in nine L plastic pots filled with Osmocote® potting mix (Scotts, Australia). The soil water content at 100% FC was pre-determined by flooding with water in free draining pots, then allowing pots to leach the excess water. This process was completed for at least five pots for both well-watered and water deficit treatment. All the pots were weighed with an electrical balance to quantify their weight at full field capacity. Seeds were germinated in trays and were transplanted into individual 9L pots after three weeks. At planting, the potting mix in each pot was inoculated with Nodule N® Strain WSM 1455, (a traditional sterile peat formulation for seed coating prior to planting). Immediately after transplanting, the surface of each pot was covered with a plastic wrap to minimize soil evaporation. Eight pots were used for each genotype comprising both WW and WD treatment and each pot was considered as a replicate. Thus, each treatment and genotype had four replicates. Control plants were watered on a daily basis to soil full water holding capacity until final harvest. WD plants received full irrigation until three pod stage and restricted irrigation for two weeks or until they showed wilting symptom at three pod stage. WD plant then received 20% FC until maturity to harvest seed from the treated plants.
The temperature of the growth chamber was maintained at 25 ± 2 °C for 14 h, and 15 ± 2 °C for 10 hours for light and dark period respectively. Light intensity and relative humidity were fixed at 400 μmol m⁻²s⁻¹ and 60% ± 5% and respectively.

7.2.2 Leaf gas exchange measurements

Four plants from each genotype and treatment were measured with a LI-COR 6400 XT infra-red gas analyser (LI-COR, Lincoln, NE, USA). Leaf-level photosynthesis was measured from 10:00 to 15:00 on the same leaves at least four times over the day. Leaves were chosen from the main stem. Leaf temperature, chamber CO₂ concentration along with air humidity was set at tracking mode. Data were completed on two consecutive days where day one was allocated for WW and day two for WD. After insertion of leaves into the gas exchange chamber, leaves were allowed to stabilise with chamber conditions until steady-state gas exchange rates were established. The daily integral of assimilation-weighted ci was calculated according to the method outlined in (Cernusak et al., 2005).

7.2.3 Tissue collection and carbon isotope analysis

Leaf samples were collected on the same day after gas exchange measurement between 4.00 to 5.00 pm. From each pot, leaves which were used to measure gas exchange, were collected with a sharp razor and immediately put into a 2 ml Eppendorf tube and transferred into an icebox to reduce metabolism. Within 2 hours, leaf samples were placed in an oven at 65 °C for 48 h to completely dry. An oscillating matrix mill was used to grind the dried leaf sample. Approximately 3 mg of ground leaf was then placed into silver capsules (IVA Analysentechnik, Meerbusch, Germany) and then analysed to determine the Δ and % N of samples according to the protocol outlined in Smith (2018). Grain samples were harvested at maturity and stored at
-20 °C for further analysis. Whole grain was initially ground with a traditional coffee grinder and further ground using an oscillating matrix mill. The protocol which was applied for leaf also applied for grain. The precision for the standard materials was between 0.04 ‰ and 0.08 ‰.

7.2.4 Quantification of Soluble sugar, Amino acids and Nutrients

Soluble sugar and amino acid analysis were performed according to the protocol outlined in Smith (2018). For the extraction process, 40 mg of milled leaf/grain samples were poured into a screw cap microtube. The methanol, chloroform and water (MCW) extraction was performed according to the protocol outlined in Merchant et al. (2006a). Samples were stored at -80 °C for further gas chromatography triple quadrupole mass spectrometer (GC-QQQ) analysis which was performed according to Merchant et al. (2006a). Soluble sugar and amino acid determination from extracted leaf sample was done through an Agilent 7890A gas chromatograph coupled to a triple quadrupole mass spectrometer with a HP5 column (Agilent Technologies, Santa Clara, CA, USA).

Determination of soluble nutrients in extracted samples was completed using an inductively coupled plasma optical emission spectrometer (Varian Vista, Agilent Technologies, Santa Clara, CA, USA) as per Merchant et al. (2010). Samples were prepared with a dilution of 400 μl of supernatant in 10 ml of ultra-pure Milli-Q water. Nutrients; calcium, iron, potassium, magnesium, phosphorus, sodium, sulphur and zinc were chosen for analysis. Any results lower than the detection limits of the instrument were not considered for analysis.
7.2.5 Pollen study

*In vitro* pollen viability and germination was measured at 0, 3, 6, 9, 12 and 15 d after the water deficit treatments. Five unopened and unfertilized flowers from each genotype and treatment were collected and placed in a petri dish with a cover and carried over laboratory for pollen viability analysis. Pollen grains were spread through soft pointed paintbrush on microscope slide having a drop of the stain and covered with a cover-slip to observe under the microscope (Ahmad and Martin, 2017). Stain consisted of 2% w/v carmine (Sigma C1022) in 45% acetic acid. On each slide 100 pollen grains were examined based on their stain retention and count percentage of viability. Unstained pollen grains were scored as non-viable. Five more flowers were collected from each genotype and treatment to carry out *in vitro* pollen germination. Pollen germination medium was prepared according to the recipe outlined in Bishop et al. (2016). The experimental process was adopted (Ahmad and Martin, 2017). Viability and germination were tested around 10.00-12.00 am each sampling day.

A reciprocal *in vivo* cross-pollination was also completed to determine the influence of WD on the pistil/pollen function. Twenty flowers from each genotype and treatment were emasculated at 16:00 to 18:00 h on the previous day of pollination. The following morning (approximately 12 h later), fresh pollen from fully opened flowers was used to pollinate those emasculated flowers according to genotype and treatment. The number of pollinated flowers and the total number of formed pods were recorded.

7.3 Statistical analysis

The effects of treatments on amino acids and soluble sugar concentration were examined by analysis of variance (ANOVA) using GenStat 18th Edition (VSN International, Hemel Hempstead, UK). Fisher's unprotected least significant difference (LSD) test was used to
determine the treatment effects. Regression analysis including the line of best fit, $R^2$ and P values for the relationship of WUE calculated using predicted and measured protocols was determined using GraphPad Prism7 (GraphPad Software, San Diego, CA, USA).

7.4 Results

7.4.1 Water deficit induced changes in isotopic abundance of leaf but not in the grain

Significant genotypic differences were observed for both plants’ tissues, i.e. leaf and grain. Among the plant organs, the leaf was enriched with $\delta^{13}C$ significantly by WD for all genotypes ($P < 0.05$) at the three pod stage (Figure 7.1A). Genotypic differences were also observed for leaf $\delta^{13}C$. Grain $\delta^{13}C$ appeared relatively consistent against treatments with no statistically significant differences (Figure 7.1B) but was found to be significant for genotypes.
Figure 7.1 Carbon isotope abundance ($\delta^{13}C$) ($\%o$) for leaf (A) and grain (B) measured in three faba bean *Vicia faba* L genotypes to well-watered (WW, black bars) and water deficit (WD, grey bars) treatment. Treatment was applied at the three pod stage. Standard errors were calculated on four replicates for each treatment and genotype. Statistically significant ($P < 0.05$) differences were observed for treatment on leaf samples (denoted with a single asterisk) and among genotypes (denoted with lettering).

7.4.2 Gas exchange measurements tightly follow leaf level isotopic signatures

Gas exchange measurement across the light period showed a significant reduction in WD plants compared to WW plants for all tested genotypes (Figure 7.2A, 7.2B). Genotype AC0805#4912 had the highest $A$ and $g_s$, which were closely followed by PBA Warda, whilst genotype
11NF010c-4 had the lowest $A$ and $g_s$ among genotypes. The ratio of the internal concentration of CO$_2$ to atmospheric CO$_2$ ($C_i/C_a$) also varied with the genotypes as well as treatments. Genotype 11NF010c-4 had the highest ($C_i/C_a$) among tested genotypes.

**Figure 7:2** Net photosynthesis ($A$), stomatal conductance ($g_s$) and $C_i/C_a$ (internal CO$_2$ to atmospheric CO$_2$ ratio) for three contrasting faba bean genotypes under well-watered (closed symbols) and water deficit (open symbols) treatments at the three pod stage. Data were recorded sequentially four times over the day. Standard errors were calculated on four replicates for each treatment and genotype.
7.4.3 Water deficit impact on nutrient composition in leaf and grain

Among the tested genotypes, we didn’t find any significant difference for most of the detected nutrients in the leaf samples (Figure 7.3). For treatment, all identified nutrients from WW and WD plants were non-significant except phosphorus, which was statistically significant for the genotypes 11NF010c-4 and AC0805#4912 (Figure 7.3A, 7.3B). Among the nutrients, potassium was found at the highest concentration followed by calcium and magnesium. Iron was absent in leaf samples but detected in grain in a tiny amount.

For grain nutrient abundance, no statistically significant differences were observed among genotypes (Figure 7.4). Treatments significantly influenced sulphur concentrations in grain for all tested genotypes (Figure 7.4). Potassium was found to be most abundant among identified nutrients followed by phosphorus and sulphur for grain samples. Percentage nitrogen exhibited a higher value in grain than in the leaf but had no statistically significant difference between treatments.
Figure 7:3 Soluble nutrient concentration in leaves (mg g\(^{-1}\)) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. Statistically significant differences were observed for treatments in the leaf sample only for phosphorus (denoted with a single asterisk) (P < 0.05).
Figure 7:4 Soluble nutrient concentrations in grains (mg g⁻¹) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. Statistically significant (P < 0.05) differences were observed for treatment in the leaf sample only for sulphur (denoted with a single asterisk).
7.4.4 Water deficit impact on sugar abundance in leaf and grain

Individual sugar and polyol concentrations were similar for all tested genotypes in leaves and grain, among which there were few treatments or genotypic differences (Figure 7.5 & 7.6). Sugar concentrations in the leaf soluble fraction were almost double that of the soluble grain tissue (Figure 7.5 & 7.6). Generally, leaf tissue from WD plant contained a higher concentration of sugars though they were not statistically significant except myo-Inositol (P<0.05). In grain tissue, sugar concentration was significantly higher in WW plants. Overall, sucrose concentration was found to be the most abundant both in leaf and in grain tissue. In leaf tissue, myo-Inositol responded against treatment for genotypes 11NF010c-4 and AC0805#4912 but not for PBA Warda (Figure 7.5). In grain tissue, sucrose had a significant treatment effect (P < 0.05), i.e. WW plants had the higher concentration for all tested genotypes (Figure 7.6).
Figure 7.5 Soluble leaf (mg g$^{-1}$) metabolite concentrations of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. *myo-Inositol* concentration had significant (P < 0.05) treatment difference which is denoted with an asterisk.
Figure 7:6 Soluble grain leaf (mg g⁻¹) metabolite concentrations of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. Sucrose concentration had significant (P < 0.05) treatment difference, which is denoted with an asterisk.
7.4.5 Water deficit impact on amino acid abundance in leaf and grain

The concentration of amino acids varied between leaf and grain tissue (Figure 7.7 & 7.8). Leaf tissue contained almost ten times higher amounts of amino acids compared to grain tissue. Thirteen amino acids were detected in leaf tissue; they were alanine, asparagine, aspartic acid, glutamine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine and valine. Proline was found to be the most abundant in leaf tissue for all tested genotypes (Fig. 7.7 A, B, C). Ten amino acids were detected in grain tissue, they amino acids; alanine, asparagine, aspartic acid, glutamine, glutamic acid, isoleucine, leucine, phenylalanine, proline and valine were in grain tissue, glutamine and phenylalanine were found to be the most abundant (Fig. 7.8 A, B, C). In leaf tissue, alanine and serine were found to be higher in WD plants for all tested genotypes (P < 0.05). No statistically significant differences among treatments were observed in grain tissue for any amino acid. The concentrations of amino acids followed a regular pattern neither among genotypes nor between treatments. In addition, methionine, serine and threonine were completely absent in grain tissue.
Figure 7.7 Soluble amino acids concentration in leaf (mg g⁻¹) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. Amino acids namely, alanine and serine concentrations had significant (P < 0.05) treatment differences, which are denoted with an asterisk.
Figure 7:8 Soluble amino acids concentration in grain (mg g⁻¹) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. Error bars were calculated from four replicates. No significant differences were observed.
7.4.6 Water deficit impact on pollen viability and pollen germination of faba bean genotypes

*In vitro* pollen viability was tested using acetocarmine stain and counted as percentage. Pollen viability of tested faba bean genotypes reduced with increasing severity of stress (Figure 7.9). During the two weeks of stress imposition, pollen viability was more or less similar in WW and WD plants for the first six days (Figure 7.9). Pollen viability dropped sharply after 9 days of the stress treatment and reduced to almost 60% in 12 days WD plants compare to WW for all genotypes after two weeks of the stressed period. Pollen viability for the tolerant genotype AC0805#4912 recorded 95± 2.16 and 49± 4.08 in WW and WD plant respectively. The drought sensitive genotype 11NF010c-4 had lowest (42 ± 1.70) pollen viability after the stress period.

Pollen germination in WW and WD plants were similar for the pollen collected from the first six days of the treatment period (Figure 7.10). Germination in WD plants had a sharp decline from day nine and reduced by almost 33% at the end of the treatment period (Figure 7.10). The drought tolerant genotype AC0805#4912 had germinated pollen (83±1.29 %) and (37±2.38 %) in WW and WD plants after two weeks of the stressed period. Drought sensitive genotype had similar germination in WW plants (82±3.86 %) but was reduced in WD plants (30±2.21 %) compared to the drought tolerant genotype.
Figure 7:9 Percentage pollen viability of three contrasting faba bean genotypes subjected to well-watered (WW) and water deficit (WD) treatments at the three-pod stage. Error bars were calculated from four replicates. Pollen viability was assessed by acetocarmine histochemical method where percentage counts of three fields on each of three slides.
Figure 7:10 Percentage pollen germination of three contrasting faba bean genotypes subjected to well-watered (WW) and water deficit (WD) treatments at the three pod stage. Error bars were calculated from four replicates. Pollen germination was assessed based on the percentage counts of three fields in each of three petri dishes.
7.4.7 Water deficit impacts the pod formation of faba bean genotypes

*In vivo* reciprocal cross-pollination was completed between male and female parents. Pollen from WW and WD plants were used to pollinate the stigma of WW and WD plants. A similar pattern of pollen viability and germination were also observed in pod formation. During the first six days of the treatment period, pod formation was more or less similar 80-85% in WD plants irrespective of genotypes. A slight reduction was observed among WD plants from day six. In drought tolerant genotype (AC0805#4912), 35% pod formation was found with the pollen from WW plants to stigma from WD plants but reduced to 10% when both (pollen and stigma) involved WD plants (Figure 7.11 A). There was no pod formation observed in WD sensitive genotype (11NF010c-4) while WD flowers were pollinated with WD pollen (Figure 7.11 B). However, when stigmas of WW plants were pollinated with the pollen from WD plants almost 30% pods were developed (Figure 7.11 B).
Figure 7:11 Percentage pod formation of three contrasting faba bean genotypes subjected to well-watered (WW, closed) and water deficit (WD, open) treatments at three pod stage. Twenty (20) flowers were pollinated per treatment combination. Treatment combinations were as follows: WW+WW= stigmas of WW plants pollinated with pollen from WW plants. WW+WD= stigmas of WW plants pollinated with pollen from WD plants. WD+WW= stigmas of WD
7.5 Discussion

Under the conditions experienced in this study, water deficit impacted plant nutrient and carbohydrate allocation as well as the viability of reproductive organ development (pistil and stamen). Within this response, genotypic variation in stress responses was observed among tested faba bean genotypes on physiological, biochemical and reproductive properties which may assist to identify superior genotypes and thus improve faba bean production in sub-optimal environments.

7.5.1 Water deficit impacts on physiological characters

Faba bean genotypes that demonstrated contrasting drought responses showed significant differences in their physiological characters for WD as well as WW conditions (Figure 7.2). These differences were found to be more prominent among genotypes in WW conditions. Physiological characteristics such as A, gs, and ci/ca were reduced under WD conditions compared to WW (Figure 7.2). Reduced photosynthesis is usually the product of either stomatal limitations or non-stomatal limitations, or both (Farquhar and Sharkey, 1982b). If the decrease in photosynthesis is primarily governed by stomatal limitations, gs and ci/ca decrease in parallel whereas, for non-stomatal limitations, increased or stable ci/ca is observed with decreased gs. In our study, a distinctive stomatal limitation was observed for net photosynthesis in accordance with water deficit treatment. This result is in line with the findings from Yan et al. (2013) where reduced photosynthesis was observed in a low sink (removed pods and flowers) faba bean genotypes. Reduction in gs, as well as A under WD conditions, are critical approaches to restrain water content in leaves (Farooq et al., 2009) with genotypes that maintain higher photosynthesis thought to be more productive. In our study, genotype AC0805#4912 showed higher A, gs and ci/ca compare to genotype 11NF010c-4 both in WW
and WD condition (Figure 7.2) suggesting superior adaptability of AC0805#4912 in both conditions. These findings are in agreement with Alghamdi et al. (2015) where drought tolerant genotypes maintained water content in their leaf tissue through reduced stomatal activity under drought stress.

WD impacted significantly on leaf level $\delta^{13}$C of contrasting faba bean genotypes between WW and WD treatments. However no, such difference was observed on carbon isotope composition of grain (Figure 7.1). Significant genotypic differences for leaf material and grain material among tolerant and sensitive genotypes were demonstrated in our experiment (Figure 7.2A & 7.2B). The relatively small shift in isotopic abundance suggested carbon isotope fractionation did not significantly alter over time and can be used as a robust character to select plant materials in the vegetative stage. Our findings closely align with previous studies in faba bean (Khan et al., 2007) and common bean (Smith, 2018).

7.5.2 Impact of water deficit on nutrient, carbohydrate and polyol content in leaf and grain

Availability of soil water and acquisition through roots are major determinants of nutrient accumulation and mobilisation within and among plant tissue. One of the objectives of this study was to examine the biochemical changes that occur among three contrasting faba bean genotypes under WD condition and to uncover robust biological indicators linked to improved plant growth under WD conditions. In general, nutrient concentration among different genotypes as well as within soluble leaf and grain did not change significantly in our study. One possible explanation may be the prevalence of the isohydric response limiting water uptake hence restricting the bulk flow of solutes from the soil.

Overall, nutrient concentration did not change significantly in both leaf and grain tissue in response to treatment except for phosphorus (P) in leaf (Figure 7.3A & 7.3B) and sulphur (S)
in grain (Figure 7.4A & 7.4B). No genotypic differences were observed. A minor change in nutrient concentration demonstrated that faba bean could adjust under WD condition by the maintenance of leaf water content. In our experiment, we found increased P in WW plants for the genotype AC0805#4912 and 11NF010c-4. Increased P in leaves of WW plants may be due to the function of stomatal apparatus and/or genetic control of specific genotypes. It is reported that P accumulation in common bean during WD condition is driven by stomatal and enzymatic function as well (Santos et al., 2004) which is an agreement with our findings. Daoui et al. (2012) also described P acquisition and consumption is controlled genetically among faba bean genotypes. Grain mineral concentration also had no significant genotypic effect, but treatment had a significant impact on S accumulation. WW plants of all studied genotypes had a higher concentration of S compare to their counterpart. In legumes, the concentration of S within plants is quite crucial as S regulates the metabolism of sulphur rich amino acids like methionine and cysteine (see for example Scherer (2001). In our study, reduction in S due to WD condition may lead to down-regulated metabolism of S containing amino acids which are also an agreement with Hawkesford and De Kok (2006).

Generally, increases or decreases of a given carbohydrate depends on the species as well as the water content of the tissue in WD condition (Amede et al., 2003). Increased myo-inositol in WD plants is due to the function of polyols which support osmoregulation. Presence of myo-inositol as a significant osmoticum was found in stressed common bean leaf (Lockhart et al., 2016) and faba bean (Amede et al. (2003). In our study, sucrose was the most abundant metabolite in both leaf and grain tissue but only significant changes in response to WD in grain tissue. Sucrose degradation in faba bean was usually due to the action of sucrose synthase under optimal condition (Weber et al., 1996). Our study aligns with the findings of Abid et al. (2017) where sucrose accumulates in stressed faba bean leaf presumably for osmotic adjustment. The
offset in sucrose concentration between leaf and grain suggested that carbohydrate partitioning is impacted by WD which is supported by Cuellar-ortiz et al. (2008).

Amino acid composition of studied genotypes indicated the presence of 13 amino acids in leaf tissue and ten amino acids in grain tissue (No significant differences within treatments were observed for grain tissue (Figure 7.8). For leaves, two non-essential (for human nutrition) amino acids (alanine and serine) were found to differ significantly among genotypes (11NF010c-4 and PBA Warda) but they did not follow any specific pattern. For example, genotype 11NF010c-4 had a higher concentration in WW sample whereas PBA Warda had opposite in WW samples (Figure 7.7A & 7.7C). Among detected amino acids, proline was the most abundant and its accumulation in response to WD in faba bean has been observed previously (Abid et al., 2017; Siddiqui et al., 2015). The present study did not detect any treatment effect neither for proline nor other amino acids in our study. A possible explanation may have been insufficient treatment severity to elicit significant changes in nutrient availability. In this experiment, stressed plants received minimum moisture (20% of the control) after two weeks of treatment period to harvest seed and plants were grown in pots, which may have an additional impact.

7.5.3 Impact of water deficit on the reproductive biology of contrasting faba bean genotypes

Plant developmental stage has critical importance to consider yield under stress conditions. Faba bean is mostly prone to drought in pod set and early pod filling stage (Khan et al., 2010). Our study demonstrated that WD reduced in vitro pollen viability as well as germination with the progression of WD. Though pollen viability mostly regulated by environmental factors (Johri and Vasil, 1961) in our study it was also regulated by a genetic factor, demonstrated by higher pollen viability and germination in genotype AC0805#4912. A similar result was
observed in chickpea for heat stress (Devasirvatham et al., 2012). In vegetative tissue plants are able to adjust WD by reducing water loss or increasing water uptake but no such mechanism was involved in reproductive tissue. Another reason for reduced pollen viability may be the lower water potential of flowers than of leaves which was also proven by Kokubun et al. (2001) in soybean. The critical period for faba bean pollen viability and germination starts on from day six and after that period microsporogenesis supposed to be impacted by water deficit which leads to the reduced pollen viability and germination which was also observed by Shen and Webster (1986) in common bean. A substantial decrease in pollen viability and germination would lead to less seed formation and subsequently yield reduction. For example, in common bean (Shen and Webster, 1986) and chickpea (Fang et al., 2010) WD affects pollen viability, germination and pod development.

We also demonstrated WD caused impairment in stamen and pistil where pistil sensitivity was appearing more dominant. A reciprocal cross confirmed the incapability of stigma grown under WD to fertilise and form a pod irrespective of whether the pollen was from WD or WW treatments (Figure 7.11). Genotypic differences were observed and drought tolerant genotype AC0805#4912 was able to develop seed (10%) when pistil of WD plants was pollinated with WD plants, but no pod formation was observed in sensitive genotypes in the same condition, i.e. WD+WD (Figure 7.1A &7.1B). This impairment suggested that stigma functionality is more critical for pod development in faba bean which is also echoed with Fang et al. (2010) for chickpea. It was also demonstrated by Nayyar et al. (2005a) in chickpea that stigma is more sensitive to stress compared to pollen and this sensitivity is probably due to the presence of higher level of abscisic acid in female parts of a flower.
7.6 Conclusion

Understanding the nutritional status of crops during stress conditions has significant consequences for plant adaptation strategies under changing climatic conditions. As a rainfed crop that is sensitive to drought, faba bean production is at risk due to changes in climate. Overall, WD effect on nutrient acquisition elicited through isohydric behaviour of faba bean. With a little change in their carbon isotope abundance, faba bean did not replicate significant treatment differences in WUE or nutritional quality. The abundance of selected metabolites in leaves responded to WD treatment. However, this was not observed in the grain. Most critically for yield loss, WD significantly reduced the formation of viable pods due to reduced pollen viability. Genotypic difference was significant to retain pollen viability during drought stress indicating selection for this trait is possible. Combined, these responses suggest that whilst WD conditions are likely to reduce the volume of faba bean production, the nutritional quality of that production appears resilient to its effects.
Chapter 8
General Discussion

Drought tolerance breeding programs commonly select for yield and yield components across genotypes subjected to drought conditions. However, the effectiveness of this trait-based selection is often curtailed due to low heritability of the multigenic trait expressed under a variable environment. Nevertheless, selection based on secondary/physiological traits has been shown to increase breeding efficiency in faba bean (Khan et al., 2007; Khazaei, 2014).

Plasticity of target traits is an important component of adaptation/survival but is not commonly considered due to an inability to place numerical values on its capacity. Further, metabolic processes can be altered due to stress and may link with physiological trait modification. To address these challenges, this thesis focused on quantifying the key morphological, physiological and biochemical parameters under both controlled and field conditions to identify drought tolerant faba bean genotypes for future breeding programs. I achieved this through a stepwise process.

First, I constructed a review with the current information of biochemical and physiological markers for legume yield improvement. I determined the pros and cons of these traits for their implementation in large-scale characterisations across genotypic lines whilst maintaining precision under field conditions. Identification and quantification of specific stress related metabolites has significant potential to increase selection efficiency. The review concluded with a statement that no single trait and no single approach are adequate to improve yield against drought; instead a combination of characteristics and methods are required.

I then quantified carbon isotope discrimination and illustrated that is an important trait to be considered as a selective trait in enhancing drought tolerance through a breeding program. I observed leaf-level carbon isotope discrimination ($\Delta$) to predict grain yield under field conditions and suggest $\Delta$ as a selective trait across a large population (Chapter 3). Diversity
of traits is a prerequisite for any successful breeding program. I observed a large genotypic variation for $\Delta$ (16.84% – 21.96%) which can be utilised at scale.

Consideration of the target environmental conditions is useful in choosing the most appropriate crop variety (Agegnehu et al., 2006). Five genotypes (AC0805#4912, 11NF008b-15, 11NF010c-4, 11NF020a-1 and PBA Warda) were selected based on their diverse $\Delta$ value from my field trial (Chapter 3) to investigate adaptive traits and suitable phenology that contributed to grain yield under field conditions. I characterised potentially predictive morphological and physiological traits for yield with plant height along with 100 seed weight the strongest predictors of yield (0.60**). In addition, lower pod filling duration can indicate rapid pod development, which is a valuable trait for drought tolerance and useful in terminal drought escape.

Using the same five genotypes, I then investigated possible physiochemical changes under rainfed and irrigated conditions. Here, I also compared between $\Delta$ from bulk leaf and gas exchange to predict water use efficiency (WUE). Accumulation of chemical entities due to stresses may give an indication of plant responses against specific stress (Weckwerth, 2011). My key findings from this study was that leaf level soluble chemistry grown under field conditions provides an overall indication of plant performance, in particular $\Delta$ and WUE determined by gas exchange. Thus, bulk leaf-level carbon isotope abundance to calculate WUE may be used as an effective and accurate selection criterion for improving WUE in faba bean breeding programs under field conditions.

In chapter six, I developed a seedling root-based phenotyping protocol in order to enhance the speed at which selection traits may be applied. A major challenge to this is a lack of root related genetic information, and established, reliable screening protocols (Belachew et al., 2018). I
screened 96 genotypes grown under hydroponics and confirmed and standardised this by concurrently growing the same genotypes in sand. Genotypes with advantageous traits identified under field conditions (Chapter 3) showed similar behaviour and superiority under hydroponic conditions but their degree of tolerance change slightly. Hydroponics was found to be a reliable phenotyping platform to identify drought tolerant faba bean genotypes and would increase the pace of selection efficiency.

Phenotyping of plants under controlled environments allow the imposition of uniform stress conditions (Tuberosa, 2012). To standardise my previous results, I executed an experiment under controlled conditions to monitor physiological and biochemical changes that occur during water deficit conditions. I also investigated changes in the nutritional properties and reproductive biology of this crop under water deficit conditions. I found little changes in grain carbon isotope fractionation from leaf carbon isotope which demonstrated carbon isotope heterotrophic fractionation was not significant under the imposed growth conditions suggesting it as a robust property to select plant material. For leaf chemistry, higher concentrations of myo-inositol in water deficit leaf tissues and lower sucrose concentrations in water deficit grain tissue may be useful as quantitative bio-indicators of stress. Grain nutrient content tended to be resilient to drought stress. Also, water deficit reduced pod development due to reduced pollen viability but genotypic difference existed to retain pollen viability during drought stress indicating selection for this trait is possible.

Overall, a major challenge to my objectives was the characterisation of traits at meaningful scales under field conditions (identified at several points throughout this thesis). Addressing this challenge requires two major foci for future research. Firstly, developing appropriate tools in which the collection of meaningful data at appropriate spatial and temporal scales is crucial
in gaining an understanding of variation in target traits for the purposes of breeding programs. Adequate collection of such data must encompass hundreds of genotypes at timescales that are relevant for characteristics such as photosynthesis and carbon allocation which are subject to temporal variation. Secondly, my thesis presents tangible evidence of variation in physiological and morphological traits that govern yield production. Refining our understanding of these traits, their significance during key stages of the developmental cycle, and incorporating them into breeding program screening trials will ultimately enhance the capacity and resilience of genotypes to maintain growth and production under a variable climate. Besides this, genotypes (AC0805#4912, 11NF008b-15, IX564c/1-7, 11NF026c-18 and 11NF011a-3) that were selected as drought tolerant from this thesis based on their morphology, physiology and root characteristics would be useful resource in future faba bean improvement program either as parent and/or dissecting further molecular characteristics.

Of particular note, my thesis highlighted that:

1. Genotypic variation in leaf carbon isotope content was observed among faba bean genotypes from different environmental origins
2. Carbon isotope discrimination can be used as potential predictor of grain and drought tolerance in faba bean.
3. Plant height and 100 seed weight are trait of interest for yield improvement in faba bean breeding along with shorter pod filling duration for drought tolerance breeding.
4. The suitability of carbon isotope to an alternate option of gas exchange to predict water use efficiency has been demonstrated.
5. The capability of carbon isotope discrimination and soluble metabolites to reflect physiological status of faba bean in field condition was shown.
6. Identification of drought tolerant genotype at seedling stage through hydroponic system validated and hydroponic platform suggested an efficient screening tool.
7. The resilience to nutrient content in grain tissue against water deficit has been demonstrated.

8. The sensitivity of the pistil is more dominant compare to stamen during water deficit was demonstrated.

Combined, this represents the first broad scale characterisation of morphological, biochemical and physiological traits for consideration in future breeding programs and an assessment of key traits under field, controlled environment and hydroponic growth conditions. Ultimately this will contribute to the enhancement of yield as well as production in this important crop.
References


effective (Fix+) and ineffective (Fix−) rhizobia. *Soil Biology and Biochemistry* **36**, 1975-1981.


### 3. App. Table 1: List of germplasm along with pedigree in field trial 2014

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<td>152</td>
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<td>11NF010d-4</td>
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<td>Fiord</td>
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5. **App. Table 1.** Mean net photosynthetic rate \((A, \mu\text{mol m}^{-2}\text{ ms}^{-1})\), Stomatal conductance \((g_s, \text{mmol m}^{-2}\text{ ms}^{-1})\) and ratio between intercellular carbon concentrations \((C_i)\) to atmospheric carbon concentration \((C_a)\) measured among five contrasting faba bean genotypes under rainfed and irrigated conditions at Narrabri, NSW. \((n = 4)\)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Irrigated A</th>
<th>g_s</th>
<th>Ci/ca</th>
<th>Rainfed A</th>
<th>g_s</th>
<th>Ci/ca</th>
</tr>
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<tbody>
<tr>
<td>11NF010c-4</td>
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<td>0.69</td>
<td>13.06</td>
<td>163.34</td>
<td>0.72</td>
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<tr>
<td>11NF008b15</td>
<td>12.72</td>
<td>180.07</td>
<td>0.69</td>
<td>8.94</td>
<td>174.39</td>
<td>0.61</td>
</tr>
<tr>
<td>AC0805#4912</td>
<td>15.36</td>
<td>161.98</td>
<td>0.74</td>
<td>11.22</td>
<td>152.07</td>
<td>0.66</td>
</tr>
<tr>
<td>11NF020a-1</td>
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<td>0.71</td>
<td>9.11</td>
<td>103.06</td>
<td>0.74</td>
</tr>
<tr>
<td>PBA Warda</td>
<td>12.87</td>
<td>188.90</td>
<td>0.73</td>
<td>11.74</td>
<td>140.84</td>
<td>0.60</td>
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</table>

5. **App. Table 2.** Average metabolite concentrations (mg g\(^{-1}\) leaf material) in five contrasting faba bean genotypes under rainfed and irrigated trials at Narrabri, NSW. \((n = 4)\)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Irrigated Fructose</th>
<th>Glucose</th>
<th>myo-Inositol</th>
<th>Sucrose</th>
<th>Rainfed Fructose</th>
<th>Glucose</th>
<th>myo-Inositol</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>11NF010c-4</td>
<td>6.65</td>
<td>6.12</td>
<td>1.24</td>
<td>357.83</td>
<td>7.37</td>
<td>8.52</td>
<td>1.64</td>
<td>409.75</td>
</tr>
<tr>
<td>11NF008b15</td>
<td>7.22</td>
<td>5.90</td>
<td>1.19</td>
<td>391.71</td>
<td>6.45</td>
<td>7.54</td>
<td>1.32</td>
<td>397.01</td>
</tr>
<tr>
<td>AC0805#4912</td>
<td>6.50</td>
<td>4.11</td>
<td>1.39</td>
<td>378.91</td>
<td>6.69</td>
<td>6.49</td>
<td>1.75</td>
<td>451.61</td>
</tr>
<tr>
<td>11NF020a-1</td>
<td>5.27</td>
<td>5.30</td>
<td>1.12</td>
<td>364.57</td>
<td>4.82</td>
<td>6.12</td>
<td>1.54</td>
<td>413.04</td>
</tr>
<tr>
<td>PBA Warda</td>
<td>6.19</td>
<td>3.08</td>
<td>0.99</td>
<td>392.84</td>
<td>5.54</td>
<td>4.92</td>
<td>1.46</td>
<td>462.69</td>
</tr>
</tbody>
</table>

7. **App. Table 1.** Mean net photosynthetic rate \((A, \mu\text{mol m}^{-2}\text{ ms}^{-1})\), Stomatal conductance \((g_s, \text{mmol m}^{-2}\text{ ms}^{-1})\) and ratio between intercellular carbon concentrations \((C_i)\) to atmospheric carbon concentration \((C_a)\) measured among three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. \((n = 4)\)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Well-Watered (WW) A</th>
<th>g_s</th>
<th>Ci/ca</th>
<th>Water Deficit(WD) A</th>
<th>g_s</th>
<th>Ci/ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>11NF010c-4</td>
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<td>0.71</td>
<td>2.05</td>
<td>0.02</td>
<td>0.52</td>
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<tr>
<td>AC0805#4912</td>
<td>8.25</td>
<td>0.11</td>
<td>0.62</td>
<td>5.36</td>
<td>0.04</td>
<td>0.61</td>
</tr>
<tr>
<td>PBA Warda</td>
<td>8.37</td>
<td>0.07</td>
<td>0.62</td>
<td>5.24</td>
<td>0.03</td>
<td>0.44</td>
</tr>
</tbody>
</table>
7. App. Table 2. Soluble nutrient concentration in leaves and grain (mg g\(^{-1}\)) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. (n = 4)

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Well-Watered (WW)</th>
<th>Water Deficit (WD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11NF010c-4</td>
<td>AC0805#4912</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>8.24</td>
<td>5.46</td>
</tr>
<tr>
<td>Mg</td>
<td>5.89</td>
<td>4.40</td>
</tr>
<tr>
<td>Na</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>P</td>
<td>0.44</td>
<td>0.92</td>
</tr>
<tr>
<td>S</td>
<td>1.88</td>
<td>1.31</td>
</tr>
<tr>
<td>Si</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>Sr</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Zn</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>%N</td>
<td>3.99</td>
<td>4.85</td>
</tr>
<tr>
<td>Grain</td>
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<td></td>
</tr>
<tr>
<td>Ca</td>
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<td>0.03</td>
</tr>
<tr>
<td>Fe</td>
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<td>0.01</td>
</tr>
<tr>
<td>K</td>
<td>5.51</td>
<td>6.03</td>
</tr>
<tr>
<td>Mg</td>
<td>0.30</td>
<td>0.35</td>
</tr>
<tr>
<td>Na</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>P</td>
<td>1.05</td>
<td>1.33</td>
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<tr>
<td>S</td>
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<td>0.66</td>
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<tr>
<td>Zn</td>
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<td>0.04</td>
</tr>
<tr>
<td>%N</td>
<td>4.49</td>
<td>5.28</td>
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</table>
7. App. Table 3. Mean soluble metabolites concentrations in leaf and grain (mg g\(^{-1}\)) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. (n = 2)

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Well-Watered (WW)</th>
<th>Water Deficit (WD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11NF010c-4</td>
<td>AC0805#4912</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>3.56</td>
<td>5.89</td>
</tr>
<tr>
<td>Fructose</td>
<td>6.89</td>
<td>6.27</td>
</tr>
<tr>
<td>myo-</td>
<td>2.53</td>
<td>7.11</td>
</tr>
<tr>
<td>Inositol</td>
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<td>398.20</td>
</tr>
<tr>
<td>Sucrose</td>
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<td></td>
</tr>
<tr>
<td>Grain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.71</td>
<td>0.56</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.69</td>
<td>3.36</td>
</tr>
<tr>
<td>myo-</td>
<td>0.53</td>
<td>0.52</td>
</tr>
<tr>
<td>Inositol</td>
<td>144.62</td>
<td>104.05</td>
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<tr>
<td>Sucrose</td>
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<td></td>
</tr>
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</table>
### 7. App. Table 4

Mean soluble amino acids concentration in leaf and grain (mg g\(^{-1}\)) of three contrasting faba bean genotypes under well-watered (WW) and water deficit (WD) conditions at the three pod stage. (n = 4)

<table>
<thead>
<tr>
<th>Amino Acids</th>
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<th>Water Deficit(WD)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>11NF010c-4</td>
<td>AC0805#4912</td>
</tr>
<tr>
<td>Leaf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2.89</td>
<td>3.21</td>
</tr>
<tr>
<td>Asn</td>
<td>0.58</td>
<td>5.68</td>
</tr>
<tr>
<td>Asp</td>
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<td>0.76</td>
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<tr>
<td>Gln</td>
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<td>1.04</td>
</tr>
<tr>
<td>Glu</td>
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<td>0.78</td>
</tr>
<tr>
<td>Ile</td>
<td>1.15</td>
<td>1.21</td>
</tr>
<tr>
<td>Leu</td>
<td>1.66</td>
<td>1.55</td>
</tr>
<tr>
<td>Met</td>
<td>3.36</td>
<td>4.24</td>
</tr>
<tr>
<td>Phe</td>
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<td>0.34</td>
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<tr>
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<td>8.25</td>
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<tr>
<td>Ser</td>
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<tr>
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<td>2.30</td>
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<tr>
<td>Val</td>
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<td>1.49</td>
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<tr>
<td>Grain</td>
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<td></td>
</tr>
<tr>
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<tr>
<td>Asn</td>
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<tr>
<td>Asp</td>
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<td>0.83</td>
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<tr>
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<td>Glu</td>
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<td>Ile</td>
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<td>0.59</td>
</tr>
<tr>
<td>Leu</td>
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<td>0.62</td>
</tr>
<tr>
<td>Val</td>
<td>0.58</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Ala = Alanine, Asn = Asparagine, Asp = Aspartic acid, Gln= Glutamine, Glu = Glutamic acid, Ile = Isoleucine, Leu = Leucine, Met = Methionine, Phe = Phenylalanine, Pro = proline, Ser = Serine, Thr = Threonine and Val = Valine