

# **Variability in Mesophyll Conductance to CO<sub>2</sub> in Grain Legumes**

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## Statement of Originality

I hereby declare that this thesis is my own work and to the best of my knowledge and belief, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and does not contain material published or written previously by another person, except where due acknowledgment has been made in the text.

A handwritten signature in black ink, appearing to read 'Arjina Shrestha', written in a cursive style.

**Signature: Arjina Shrestha**

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## Thesis Summary

Mesophyll conductance to CO<sub>2</sub> ( $g_m$ ) limits the diffusion of CO<sub>2</sub> from sub-stomatal cavities to the carboxylation sites and is a significant limitation to photosynthesis. Recently there has been growing interest in  $g_m$  for its potential to increase photosynthesis and photosynthetic water-use efficiency. However, there is a lack of complete understanding of  $g_m$  variability and its regulation under different environmental conditions, and relevant studies in grain legumes are scarce. Grain legumes are widely recognized as having an important role in agricultural and food systems. Thus, my research projects aimed to characterize genetic variability in  $g_m$  among different genotypes of important grain legumes, to characterize the response of  $g_m$  to short- and long-term changes in environment, to understand the mechanistic bases of  $g_m$  variation, and to assess the relationship between  $g_m$  and leaf water-use efficiency. I was also interested in exploring if  $g_m$  shares diffusion pathway with leaf hydraulic conductance ( $K_{leaf}$ ) by examining the response of both traits to different growth conditions when measured simultaneously.

Mesophyll conductance varied significantly between genotypes for most of the legume species studied under non-limiting environments. Genotypes differed in their  $g_m$  response to growth environments including water availability and nitrogen source (biologically fixed or inorganic-nitrogen supplied).  $g_m$  increased with rapid increases in light intensity and temperature but decreased under short-term exposure to blue light. However, genotypes differed in their interactive response of  $g_m$  between short- and long-term environmental changes. The mechanistic bases of this variability in  $g_m$  are not clear. Environmentally driven variation in leaf anatomical traits including leaf thickness, surface area of mesophyll or chloroplasts facing intercellular air spaces and the fraction of intercellular air space were not the major factors determining  $g_m$ , but genotypes differed in the degree to which leaf anatomy influenced  $g_m$ . Similarly, the influence of leaf temperature on chloroplast ultrastructure was examined in soybean and *Arabidopsis thaliana* to understand the temperature response of  $g_m$ . However, there was no clear indication of the formation of temperature-induced chloroplast protrusions in this study. To further assist with understanding  $g_m$  regulation, simultaneous stable carbon ( $\Delta^{13}C$ ) and oxygen isotope discrimination ( $\Delta^{18}O$ ) techniques were used to estimate  $g_m$ , and its component conductances (cell

wall/plasma membrane conductance and chloroplast membrane conductance). Chloroplast membrane conductance ( $g_{cm}$ ) was found to vary within and between legume species. Both components of  $g_m$  varied similarly with changes in growth environments.  $g_m$  and its component conductances were not correlated to leaf hydraulic conductance for faba bean genotypes grown under different environmental conditions.  $g_m$  was strongly associated with leaf photosynthetic rate but the relationships between  $g_m$  and leaf intrinsic water use efficiency and between  $g_m$  and stomatal conductance depended on legume species and environmental conditions. Further, there was variability in the short-term temperature response of  $g_m$  between individual leaves within a single genotype. This may reflect instrument noise during  $g_m$  measurements or the sensitivity of  $g_m$  calculation across different environmental conditions.

The results of this project can provide useful information for crop genetic improvement through  $g_m$  in legumes under climate change scenarios. Increasing mesophyll conductance in legumes will increase photosynthetic rate and possibly water-use efficiency, when there is no increase in stomatal conductance. Concurrent  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  estimates showed the potential to enhance understanding of  $g_m$  regulation by providing better insight into the relative contribution of  $g_m$  components, but the location of  $\text{CO}_2\text{-H}_2\text{O}$  equilibration needs to be identified to be able to correctly interpret  $\Delta^{18}\text{O}-g_m$  estimates, as  $\Delta^{18}\text{O}-g_m$  relates to the conductance of  $\text{CO}_2$  to the site of  $\text{CO}_2\text{-H}_2\text{O}$  equilibration. More studies on  $g_m\text{-}K_{\text{leaf}}$  relationships across different environmental conditions and species are needed before arriving at definite conclusion of the proposed coordination. Furthermore, it will be necessary in future studies to address the sensitivity of the model parameters used for  $g_m$  calculation under a range of measurement conditions.

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## 1. General Introduction and Outline

Legumes are members of Leguminosae (or Fabaceae) botanical family, and are broadly defined by their unusual flower structure and podded fruit and their ability to fix atmospheric nitrogen (Graham & Vance, 2003). Different legume species include important grain, pasture, and agroforestry species (Graham & Vance, 2003). Grain legumes are now widely known to play an important role in human nutrition and sustainable agriculture (Graham & Vance, 2003, Foyer *et al.*, 2016). Drought is one of the major abiotic constraints limiting legume crop productivity and yield stability worldwide (Daryanto *et al.*, 2015, Farooq *et al.*, 2017). The projected warmer and drier conditions under climate change (Postel, 2000, Mpelasoka *et al.*, 2008, Rosenzweig & Hillel, 2008, Beebe *et al.*, 2011) and the rapidly increasing global population (UN DESA, 2015) emphasize the challenges ahead for improving crop productivity and efficiency of water use.

One potential strategy for concomitant increase of photosynthesis ( $A$ ) and leaf-intrinsic water use efficiency (the ratio of photosynthetic rate to stomatal conductance to water vapour;  $A/g_{sw}$ ) would be to enhance the mesophyll conductance to  $CO_2$  (Barbour *et al.*, 2010, Flexas *et al.*, 2016). Mesophyll conductance to  $CO_2$ , abbreviated as  $g_m$ , is the ease of  $CO_2$  diffusion from the substomatal cavities to the carboxylation site in the chloroplast stroma (Evans *et al.*, 2009). Many studies have shown that  $g_m$  is finite and not constant, and is a significant limitation to photosynthesis (Flexas *et al.*, 2008). Barbour *et al.* (2010) first reported a positive correlation between  $g_m$  and  $A/g_{sw}$  in barley and suggested that selecting for increased  $g_m$  has unexplored potential to provide improvement in  $A/g_{sw}$ . A large variability in  $g_m$  has been found between plant functional groups, genera, species and genotypes (Flexas *et al.*, 2008, Barbour *et al.*, 2010, Jahan *et al.*, 2014).  $g_m$  is a dynamic leaf trait which may vary in the short term (within seconds to minutes) or the in long term (growth conditions) in response to different environmental factors (Warren *et al.*, 2007, Loreto *et al.*, 2009, Bunce, 2010, Douthe *et al.*, 2011, Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016) although the response may vary between species or genotypes (Singsaas *et al.*, 2004, von Caemmerer & Evans, 2015, Barbour & Kaiser, 2016). There are conflicting results between studies for environmental response of  $g_m$ , such as the short term response of  $g_m$  to light and  $CO_2$ , with some studies showing significant response but not others (Tazoe *et al.*, 2009, Douthe *et al.*, 2011). There is a considerable variability in the temperature response of  $g_m$  between species (Flexas *et al.*, 2008, von Caemmerer & Evans, 2015). Moreover,



previous studies have mostly focussed on cross-species variation, and studies in legumes are relatively scarce. More studies are needed to extend our knowledge of genotypic and environmental variation of  $g_m$  and to evaluate whether this variability translates into variation in photosynthesis and leaf WUE.

Variability in maximum values of  $g_m$  observed among species and genotypes and in response to growth conditions could partly be explained by the variation in leaf structure and anatomical properties like leaf thickness, mesophyll and/or chloroplast surface area facing intercellular air spaces, cell wall thickness (Evans *et al.*, 2009, Tosens *et al.*, 2012b, Tomás *et al.*, 2013). However, some studies did not find relationships between  $g_m$  and leaf anatomy (Evans & Vellen, 1996, Hanba *et al.*, 2001, Hanba *et al.*, 2002, Hanba *et al.*, 2004, Miyazawa *et al.*, 2008, Tomás *et al.*, 2014). Carbonic anhydrase and aquaporins have been proposed as the potential candidates for the dynamic changes in  $g_m$  (Flexas *et al.*, 2013a). However, mechanisms that regulate  $g_m$  variation remain unclear (Flexas & Diaz-Espejo, 2015, von Caemmerer & Evans, 2015). Simultaneous stable carbon and oxygen isotope techniques to estimate  $g_m$ , and its component conductances, have the potential to enhance understanding of  $g_m$  regulation (Gillon & Yakir, 2000, Barbour *et al.*, 2016b). Gillon and Yakir (2000) demonstrated that measurements of oxygen isotope composition ( $\delta^{18}\text{O}$ ) of  $\text{CO}_2$  and water inside the leaves could be used to estimate conductance to  $\text{CO}_2$  diffusion from the substomatal cavities to the site of  $\text{CO}_2\text{-H}_2\text{O}$  isotopic equilibration, and the combined carbon and oxygen isotope discrimination methods allow partitioning of total mesophyll conductance into the components before and after  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium. Recent research (Barbour *et al.*, 2016b) coupled laser absorption spectrometers with gas exchange systems to partition total  $g_m$  into its component conductances in  $\text{C}_3$  plants. This technique is in its preliminary stage and needs further studies to discern the relative importance of the  $g_m$  components on a range of species and environments.

A review by Flexas *et al.* (2013b) observed a general positive correlation between mesophyll conductance and leaf hydraulic conductance ( $K_{\text{leaf}}$ ) across species, and suggested a potential coordination between these two leaf internal conductances. There are few, but conflicting studies on  $g_m$  and  $K_{\text{leaf}}$  relationships. More studies on a range of species are needed to confirm the

generality of the proposed coordination between  $g_m$  and  $k_{leaf}$ , and whether the relationship is due to anatomical or biochemical properties is also not clear.

### **1.1. Aims**

The major aims of this thesis were to

1. measure the degree of variation in  $g_m$  among crop legume species and genotypes,
2. assess genetic variation in the  $g_m$  response to growth environment,
3. quantify the  $g_m$  response to short-term changes in temperature, light quality and light quantity, and determine genotypic and growth environment effects on the sensitivity of  $g_m$  to short-term environmental changes,
4. determine the degree to which leaf anatomy influences  $g_m$  in crop legumes,
5. measure the level of variability in chloroplast membrane conductance among crop legume species and genotypes and with growth environment,
6. determine the closeness of correlation between mesophyll and leaf hydraulic conductances,
7. assess the degree to which mesophyll conductance is related to photosynthetic rate and leaf intrinsic water use efficiency, and
8. identify methodological and instrumental limitations to  $g_m$  estimation.

### **1.2. Thesis outline**

The thesis is composed of four research chapters, preceded by a detailed introduction with literature review (Chapter 2), research methodology (Chapter 3) and finally a general discussion including a synthesis of the results and overall conclusion (Chapter 8). Chapter 3 covered methods used to estimate mesophyll conductance to  $CO_2$  using carbon and oxygen isotope techniques. Plant material, growth conditions, experimental design and other methods used specifically for each experiment are included in their respective research chapters. The first research chapter (Chapter 4) examines the variation in  $g_m$  and its components (determined by partitioning  $g_m$  into cell wall plus plasma membrane and chloroplast membrane conductance) within and among grain legume species under non-limiting environments. Chapter 5 investigates the correlation between  $g_m$  and  $K_{leaf}$  under differing growth environments in faba bean genotypes. This chapter examines if the environmentally driven changes in leaf anatomy have any influence on the variation in  $g_m$  or  $K_{leaf}$  and their relationship. Chapter 6 includes  $g_m$  response

to short-term (light intensity and light quality) and long-term (water availability or nitrogen sources) variation in environmental conditions in different genotypes of chickpea. Chapter 7 includes the temperature response of  $g_m$  in soybean and common bean genotypes at different leaf age and also discusses the variability in response within a genotype. This chapter also examines the occurrence of chloroplast protrusions in response to temperature. The final chapter of the thesis synthesises the main findings of the research chapters and identifies some of the research gaps and future directions for understanding  $g_m$  regulation.

## 2. Detailed Introduction and Literature Review

### 2.1. Grain legumes and their importance

Grain legumes, as defined by the Food and Agriculture Organization of the United Nations, are annual leguminous crops harvested only for dry grain (FAO, 1994), and they play a critical role in human health, agriculture and environment (Graham & Vance, 2003, Foyer *et al.*, 2016). The global population is continuously increasing and the current world population of 7.3 billion (mid 2015) is expected to reach 9.7 billion by 2050 (UN DESA, 2015). The growing population will require 70 percent more food production between 2005/07 and 2050, while adopting efficient and sustainable use of limited natural resources and adapting to global climate change (FAO, 2009). Grain legumes have an important role in food security, since they are affordable sources of plant-based protein for the human diet, and are significant sources of vitamins and minerals for millions of people around the world, especially in developing countries (Asif *et al.*, 2013, Mudryj *et al.*, 2014, Sanchez-Chino *et al.*, 2015, Temba *et al.*, 2016). Grain legumes contribute about 33% of the dietary protein nitrogen needs of humans and under subsistence conditions the percentage can be higher (Graham and Vance 2003).

Grain legumes have the ability to fix atmospheric nitrogen ( $N_2$ ) by forming a symbiotic relationship with rhizobia bacteria (Giller, 2001, Biswas & Gresshoff, 2014), thus playing a potentially significant role in enhancing the productivity and sustainability of farming systems (Evans *et al.*, 1991, Howieson *et al.*, 2000, Graham & Vance, 2003, Crews & Peoples, 2005, Peoples *et al.*, 2009, Foyer *et al.*, 2016). Nitrogen is the primary nutrient limiting plant production (Vitousek & Farrington, 1997, Lebauer & Treseder, 2008). The amount of nitrogen fixed in the roots of grain legumes has been estimated at 150-200 kg/ha, most of which is removed in the crop grain (Fisher, 1996). We have ample evidence of the benefits of inclusions of legumes in the cereal-production systems. Nitrogen stored in the legume crop residues is gradually released and is available to succeeding cereal crops (Vanotti *et al.*, 1997, Espinoza *et al.*, 2015, Peoples *et al.*, 2015, Foyer *et al.*, 2016), and there is evidence of increased biomass, N content and yield of cereal crops grown after legumes compared to cereals grown after cereals (Evans *et al.*, 1991). Preissel *et al.* (2015), in their review of different experiments in Europe, stated that inclusion of a grain legume in crop rotation can reduce nitrogen fertilizer requirements

by 23-31 kg ha<sup>-1</sup>, and cereal yields are between 0.5 and 1.6 Mg ha<sup>-1</sup> higher than after cereal pre-crops.

The nitrogen-fixing ability of legumes provides an option for reducing the use of synthetic nitrogen fertilizers, thereby for reducing adverse impacts of chemical fertilizer use on environments (Bergeron, 2010). Globally, more than 50 percent of the fertiliser N applied to cropland is lost by volatilisation or leaching, wasting the resource, causing air, water and soil pollution, and generating greenhouse gas emissions (Luis *et al.*, 2014). N losses from legume sources are lower than from fertilizers (Crews & Peoples, 2005). An analysis of the historical data from 1961 to 2009 from 124 countries by Luis *et al.* (2014) showed that cereals have greater nitrogen-use efficiency (NUE; mass grain dry yield divided by mass nitrogen fertilizer applied) when a larger proportion of nitrogen additions are from residues of a preceding legume crop than are from chemical fertilizers. Greenhouse gas emissions from wheat production declined by 56% on a per-hectare basis when a grain legume crop (lupin) was included in a cropping rotation in a semi-arid environment (Barton *et al.*, 2014). Legume-cereal rotation or intercropping improves soil organic matter and productivity (Vanotti *et al.*, 1997). Similarly, some grain legumes, through their root exudates, make phosphorus more readily available to the cereal under legume-cereal rotation or intercropping system (Bais *et al.*, 2006).

## **2.2. Grain legumes in Australian cropping system**

Grain legumes are relative newcomers in Australian cropping system (Pulse Australia, 2017a). Traditional Australian farming system consisted of cereal production rotated with a number of years of legume-based pastures which were grazed by livestock (Siddique & Sykes, 1997). During the 1970s and 1980s, the emphasis of farming practices shifted to inclusion of grain legumes in cropping rotations (Siddique & Sykes, 1997). The area under grain legume production has increased from less than 0.08 million ha in 1971 (Siddique *et al.*, 2013) to about 1.8 million hectares in 2015, producing 2.2 million metric tonnes of grain, worth A\$1.2 billion in exports (Pulse Australia, 2017a). Australia is now one of the world's largest exporters of grain legumes (Pulse Australia, 2017b).

Nationally, grain legumes average just under 10 per cent of the total crop area, however they can occupy as much as 25% of the total crop area in favourable production areas (Pulse Australia, 2017a). They are grown throughout the southern and northern regions of Australia. The six major grain legume groups grown in Australia are chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), lentil (*Lens culinaris* Medikus), lupin (*Lupinus angustifolius* L.) and mungbean (*Vigna radiate* (L.) R. Wilczek). The grain legume industry's growth is important for the future sustainability of the whole Australian grains industry due to the importance of grain legumes in enhancing the cereal cropping system (AEGIC, 2017). The potential for grain legumes in Australia, assuming all constraints are overcome, is to increase its current size to 4.2 million tonnes (Pulse Australia, 2017a).

### **2.3. Grain legumes constraints**

Globally, the area planted with grain legumes has been gradually increasing in the past 50 years, but grain legumes lag behind cereals in terms of cultivation, productivity and genetic improvements (Graham & Vance, 2003, Nedumaran *et al.*, 2015, Foyer *et al.*, 2016). For all the grain legumes, production increases were primarily due to increase in the land area planted rather than the yield increase (Gowda *et al.*, 2009, Foyer *et al.*, 2016). The global demand for chickpea is projected to be 18.3 million tons in 2050 compared with a supply of 9.4 million tons in 2010 (Krishnamurthy *et al.*, 2013).

Abiotic stress, which includes multiple stresses such as drought, salinity, waterlogging, high temperature and chilling, negatively impact legume crop productivity and cause more than 50% of crop loss worldwide (Reddy *et al.*, 2012, Latef & Ahmad, 2015). Agricultural drought refers to conditions when plant available water is insufficient to meet potential transpiration due to high atmospheric demand and/or limited soil moisture, leading to below-average yields (Stone, 2011). Drought has been one of the major abiotic factors reducing legume crop productivity and yield worldwide, through adverse effects on the rate of net photosynthesis, total biomass, flowering and reproductive development, grain set and grain development (Siddique, 2005, Micheletto *et al.*, 2007, Fang *et al.*, 2010, Siddique *et al.*, 2012, Daryanto *et al.*, 2015, Pang *et al.*, 2016, Farooq *et al.*, 2017). The magnitude of drought-induced yield reduction varies with the grain

legume species, crop phenological stage, soil texture, and the duration and intensity of the stress (Daryanto *et al.*, 2015, Fahad *et al.*, 2017, Farooq *et al.*, 2017); also see Table 2.1.

**Table 2.1** Reduction in economic yield by drought stress in some important grain legumes

<b>Crop</b>	<b>Growth stage</b>	<b>Yield reduction (%)</b>
Mashbean ( <i>Vigna mungo</i> L.)	Flowering	31–57
	Reproductive	26
Chickpea ( <i>Cicer arietinum</i> L.)	Late ripening	49–54
	Anthesis	27–40
	Reproductive	45–69
Common bean ( <i>Phaseolus vulgaris</i> L.)	Reproductive	58–87
	Flowering	49
	Pod filling	40
Cowpea ( <i>Vigna unguiculata</i> (L.) Walp.)	Reproductive	34–66
	Pod filling	29
Faba bean ( <i>Vicia faba</i> L.)	Grain filling	68
Lentil ( <i>Lens culinaris</i> L.)	Reproductive	24
	Pod development	70
Pigeon pea ( <i>Cajanus cajan</i> L.)	Flowering	42–57
Soyabean ( <i>Glycine max</i> (L.) Merr.)	Grain filling	42
	Onset of pod set	45–50

Source: (Farooq *et al.*, 2017)

Grain legume crops are prone to drought as, globally, more than 90% of grain legumes are grown under rainfed conditions (Siddique *et al.*, 2001, Graham & Vance, 2003, Siddique, 2005, Sinclair & Vadez, 2012). Half of the total arable land area over Australia is regularly affected by drought (Smithson & Sanchez, 2001). Study has shown that most parts of Australia are vulnerable to climatic extremes, and Australia's agricultural ecosystems are much more susceptible to drought than native shrublands and grasslands (Ma *et al.*, 2015). Drought problems for legumes are likely to aggravate with the predicted increase in the intensity and frequency of drought episodes and heat waves under ongoing global climate change (Postel, 2000, Mpelasoka *et al.*, 2008, Rosenzweig & Hillel, 2008, Beebe *et al.*, 2011). Rainfall has been predicted to decrease in the mid-latitudes where most food legumes are grown; and extreme precipitation events are predicted in tropical regions with more frequent periods of within-season drought (Christensen *et al.*, 2007). Global warming, with the prediction of a 2–4°C increase in temperature over the next

century, will further escalate drought due to increased transpiration at higher temperatures, which in rain-fed agriculture can directly limit plant productivity (Lawlor & Mitchell, 2000, Peng *et al.*, 2004).

Thus, rapidly increasing world population, global climate change, and irrigation water scarcity have emphasized the challenges ahead to increase the productivity and efficiency of water use for grain legume yield improvement.

#### **2.4. Physiological improvement of water use efficiency**

Improving water use efficiency has been a primary target for agronomists, plant breeders, and plant physiologists (Sadras & McDonald, 2012, Kirkegaard *et al.*, 2014). Water use efficiency (WUE) is an important index of the relationship between water consumption and yield of interest (either biomass production or grain yield) (Evans & Sadler, 2008). Complementing agronomic approaches (Hatfield *et al.*, 2001, Gregory, 2004), WUE can be improved at the physiological level (Parry *et al.*, 2005), by consideration of leaf-intrinsic WUE; the ratio of photosynthetic rate ( $A$ ) to stomatal conductance to water vapour ( $g_{sw}$ ).

Genotypic variation in  $A/g_{sw}$  has been reported in different grain legume crops, including bean (White, 1993, Polania *et al.*, 2016), faba bean (Khan *et al.*, 2007), cowpea (Ismail & Hall, 1992), chickpea (Krishnamurthy *et al.*, 2013, Sadras *et al.*, 2016) and soybean (Gilbert *et al.*, 2011b). Differences in  $A/g_{sw}$  and WUE between genotypes have been reported to have a genetic basis (Martin *et al.*, 1989, Masle *et al.*, 2005). There have been considerable gains in WUE, notably in cereals, from improved  $A/g_{sw}$  (Farquhar & Richards, 1984, Rebetzke *et al.*, 2002, Richards *et al.*, 2002). Nevertheless, high water-use efficiency at the leaf level may not always translate into increased whole-plant water-use efficiency (Condon *et al.*, 2004, Medrano *et al.*, 2015) and the gap between the leaf-level and crop-level estimates of WUE might depend on photosynthate allocation to the harvested plant organs, crop developmental stage and the environmental conditions (Barbour *et al.*, 2010). The relationship between crop yield and leaf WUE (measured as  $\Delta^{13}C$ ) has also been shown to be highly variable (Condon *et al.*, 2002, Vadez *et al.*, 2012, Vadez & Ratnakumar, 2016).



The current knowledge of plant physiological responses to drought stress have been reviewed in agronomically important plants including some grain legumes (Turner *et al.*, 2001, Reddy *et al.*, 2003, Feller & Vaseva, 2014, Osakabe *et al.*, 2014, Farooq *et al.*, 2017). Mechanisms of the regulation of stomatal conductance (inverse of stomatal resistance) have been extensively studied (Farquhar & Sharkey, 1982, Collatz *et al.*, 1991, Jones, 1998, Comstock, 2002). Under water stress conditions or high leaf-to-air vapour pressure deficit (VPD), stomata rapidly adjust their aperture so as to control the transpiration water loss, thereby providing an opportunity to increase WUE through decreased transpiration, but this occurs at the expense of decreased photosynthetic carbon fixation and, potentially, decreased yield (Flexas *et al.*, 2004). The CO<sub>2</sub> required for photosynthesis diffuses from the atmosphere into the substomatal cavities (with CO<sub>2</sub> concentration  $C_i$ ) via the stomata, the same points at which water molecules diffuses out, and thus the reduced stomatal conductance simultaneously reduces transpiration and photosynthetic rate (Gaastra, 1959). Increasing WUE while maintaining or increasing yield would require high  $A$  (high rates of carbon fixation) when  $g_{sw}$  and  $C_i$  are low (Flexas *et al.*, 2013a). At the leaf level, increasing mesophyll conductance to CO<sub>2</sub> has been suggested as one of the ways to simultaneously increase  $A$  and  $A/g_{sw}$  (Barbour *et al.*, 2010), which will be discussed further later in this chapter.

## 2.5. Mesophyll conductance to CO<sub>2</sub>

Stomatal resistance, which restricts the diffusion of CO<sub>2</sub> from the atmosphere ( $C_a$ ) to substomatal cavities ( $C_i$ ) via the stomata, is the first major barrier in the CO<sub>2</sub> pathway. CO<sub>2</sub> has to move further from the substomatal cavities through air spaces, cell walls, cytosol, and chloroplast envelopes to finally reach the site of carboxylation inside the chloroplast stroma ( $C_c$ ) where it is fixed by Rubisco (Evans & von Caemmerer, 1996, Evans & Loreto, 2000, Evans *et al.*, 2009). Together, the conductance of these pathways from the substomatal cavities to the stroma constitute mesophyll conductance to CO<sub>2</sub>, abbreviated as  $g_m$  (Kaldenhoff, 2012). Evidence gathered over the past two decades suggests that  $g_m$  is sufficiently small as to significantly decrease  $C_c$  relative to  $C_i$  (Flexas *et al.*, 2008), (for example,  $C_i/C_a = 0.7$  and  $C_c/C_a = 0.5$  (Evans *et al.*, 1986)).  $g_m$  is a significant and variable limitation to photosynthesis, imposing diffusional limitations of a quantitative magnitude similar to (and in some cases greater than) that of the

stomatal and biochemical (CO<sub>2</sub> carboxylation) limitations (Flexas *et al.*, 2008, Warren, 2008c, Flexas *et al.*, 2012a, Flexas *et al.*, 2013a).

$g_m$  has been studied intensively and the current understanding of  $g_m$  has been reviewed several times in the past decade addressing different aspects of  $g_m$  including variability among species, short-term and long-term responses to environmental changes and mechanism of  $g_m$  regulation (Flexas *et al.*, 2008, Warren, 2008c, Flexas *et al.*, 2012a, Flexas *et al.*, 2014). These studies have confirmed that  $g_m$  is a complex leaf trait including leaf anatomical and biochemical properties which may vary among species and in response to short term (within seconds to minutes) or long term changes in different abiotic factors. In addition, specific reviews and commentary on the methodology (Pons *et al.*, 2009), the potential errors in estimating  $g_m$  (Gilbert *et al.*, 2011a, Tazoe *et al.*, 2011, Tholen *et al.*, 2012, Tholen *et al.*, 2014), and the mechanistic basis of  $g_m$  (Tholen & Zhu, 2011, Flexas & Diaz-Espejo, 2015, von Caemmerer & Evans, 2015) have been published, highlighting the complexity and the controversial aspects on  $g_m$  estimation and its regulation. Recently, there has been a growing interest in  $g_m$  for increasing photosynthesis and photosynthetic WUE in C<sub>3</sub> plants (Barbour *et al.*, 2010, Flexas *et al.*, 2013a, Flexas *et al.*, 2016). More comprehensive studies on the variability and regulation of  $g_m$  are required before it can be recommended as selection criteria for breeders for improving WUE.

## **2.6. Current knowledge and challenges for estimating $g_m$ and its components**

There are currently three major approaches to estimate  $g_m$ , namely, the online carbon isotope discrimination method (Evans *et al.*, 1986, Tazoe *et al.*, 2009), the combined chlorophyll fluorescence and gas exchange method (Harley *et al.*, 1992), and the curve-fitting method (Ethier & Livingston, 2004). The advantages and limitations of each method are reviewed in detail in Pons *et al.* (2009), and the selection of the appropriate method depends on the experimental materials, objectives and instrumentation availability (Pons *et al.*, 2009). However, all techniques rely on models with different assumptions, and the reliability of the calculated  $g_m$  depends on the validity of the model assumptions (which are not fully tested) and sensitivity of parameter values (Pons *et al.*, 2009, Tholen *et al.*, 2012, Gu & Sun, 2014). CO<sub>2</sub> from mitochondrial respiration and photorespiration diffusing into the chloroplast provides an additional source of CO<sub>2</sub> for photosynthesis and thus can bias measurements and CO<sub>2</sub> and oxygen sensitivity of  $g_m$

(Tholen *et al.*, 2012). Several studies have measured  $g_m$  under low  $O_2$  to limit the photorespiration-induced uncertainties (von Caemmerer & Evans, 1991, Loreto *et al.*, 1992, Flexas *et al.*, 2007, Douthe *et al.*, 2011, Douthe *et al.*, 2012). Recently, Yin and Struik (2017) extended the framework of Tholen *et al.* (2012) by considering various scenarios for the intracellular arrangement of chloroplasts and mitochondria and suggested that the sensitivity of  $g_m$  to the ratio of photorespiratory ( $F$ ) and respiratory ( $R_d$ )  $CO_2$  release to net  $CO_2$  uptake ( $A$ ) lies between no sensitivity in the classical method and high sensitivity in the Tholen *et al.* model.

As mentioned in the previous section,  $g_m$  is a combination of gaseous diffusion through intercellular air spaces and diffusion of dissolved  $CO_2$  through the cell wall, plasma membrane, cytosol, and chloroplast envelope to the site of carboxylation. However, the aforementioned techniques to quantify  $g_m$  are not yet able to decompose  $g_m$  into its components due to current technical limitations. Gillon and Yakir (2000) proposed that measurements of  $\delta^{18}O$  of  $CO_2$  and  $\delta^{18}O$  of water inside the leaves could be used to estimate conductance to  $CO_2$  diffusion from the substomatal cavities to the site of  $CO_2$ - $H_2O$  isotopic equilibration, which was assumed to be at the chloroplast surface. Water inside leaves becomes enriched in  $H_2^{18}O$  during transpiration (Cernusak & Kahmen, 2013). Carbonic anhydrase (CA) catalyses exchange of oxygen atoms from  $^{18}O$ -enriched water to leaf-dissolved  $CO_2$ , so that  $CO_2$  leaving the leaf chamber is enriched in  $^{18}O$  compared to that entering the leaf chamber. Gillon and Yakir (2000) used a combination of  $^{18}O$  estimate of  $g_m$  and  $\Delta^{13}C-g_m$  (total conductance estimated using the carbon isotope discrimination method) to partition total mesophyll conductance into cell wall/plasma membrane and chloroplast membrane components.

More recently, Barbour *et al.* (2016b) demonstrated that  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  can be rapidly and easily measured in  $C_3$  plants by coupling traditional gas exchange with laser absorption spectrometers that measure the stable carbon and oxygen isotope composition of  $CO_2$  and the stable oxygen isotope composition of transpired water vapour. However, they highlighted that the interpretation of the conductance from the  $\Delta^{18}O$  technique should be made cautiously as it depends on the sites of  $CO_2$ - $H_2O$  equilibrium, which further depends on the location and activity of CA, and the partitioning technique may not be possible in all species. Simultaneous measurement of  $\Delta^{18}O-g_m$  and  $\Delta^{13}C-g_m$  has the potential to provide better understanding of the

relative magnitude of the  $g_m$  components before and after CO<sub>2</sub>–H<sub>2</sub>O equilibration, which would be valuable in identifying targets to genetically manipulate  $g_m$ . This technique is in preliminary stage and measurements in other major crops under different environmental conditions are imperative in the near future. Combined measurements of  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  in different grain legume genotypes will be discussed in Chapter 4 and 5.

## **2.7. $g_m$ variability within and among plant species**

The range of  $g_m$  variation in plants was reviewed by Flexas *et al.* (2008), who observed general trends in  $g_m$  when comparing different plant functional groups and significant variability in  $g_m$  within a single group, genus and species.  $g_m$  can be less than 0.1 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> in woody evergreen gymnosperms, and higher than 1 mol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> in fast-growing herbaceous crops. Significant genotypic variation in  $g_m$  has been reported in important crop species, such as cereals (Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014), *Castanea sativa* (Lauteri *et al.*, 1997), *Solanum lycopersicum* (Galmés *et al.*, 2011) and *Vitis vinifera* (Tomás *et al.*, 2014) but the information is very limited in grain legumes. Flowers *et al.* (2007) observed genotypic variation in  $g_m$  among snap bean (*Phaseolus vulgaris*) genotypes but only after exposure to high ozone concentration. This suggested that  $g_m$  might be involved in the differences in the rate of net photosynthesis between different species and genotypes. Genotypic variability in  $g_m$  among grain legumes will be presented in Chapter 4.

## **2.8. $g_m$ response to environmental changes**

In addition to being widely variable within and among species,  $g_m$  varies in response to the short- and long-term changes in environmental conditions. Water stress has often been found to reduce  $g_m$  (Flexas *et al.*, 2008, Warren, 2008b, Flexas *et al.*, 2009, Galle *et al.*, 2009, Cano *et al.*, 2014, Olsovska *et al.*, 2016), although recent studies have shown that  $g_m$  was not as sensitive to drought as  $g_{sw}$ , mostly during moderate water stress (Bunce, 2009, Flexas *et al.*, 2010, Theroux-Rancourt *et al.*, 2014). Genotypic variability in the  $g_m$  response to nitrogen and water availability was observed for wheat by Barbour and Kaiser (2016). Long-term changes in  $g_m$  have been observed under salinity (Flexas *et al.*, 2004), nutrient supplement (Warren, 2004, Bown *et al.*, 2009, Li *et al.*, 2012, Xiong *et al.*, 2015a), and light level (Hanba *et al.*, 2002, Piel *et al.*, 2002, Laisk *et al.*, 2005, Warren *et al.*, 2007). Fini *et al.* (2016) showed that  $g_m$  is maximum at the light

intensity at which plant species have developed. Similarly, in the long term Singaas *et al.* (2004) reported decreased  $g_m$  with higher CO<sub>2</sub> in some species and not in others.

Rapid responses of  $g_m$  to changes in CO<sub>2</sub> concentration (Flexas *et al.*, 2007, Flexas *et al.*, 2008, Douthe *et al.*, 2011, Flexas *et al.*, 2012a, Xiong *et al.*, 2015a) and light intensity (Flexas *et al.*, 2008, Douthe *et al.*, 2011, Douthe *et al.*, 2012, Flexas *et al.*, 2012a, Xiong *et al.*, 2015a) have frequently been reported. Bunce (2010) observed substantial differences in the sensitivity of  $g_m$  to  $C_i$  between two legume species (common bean and soybean) and suggested that light level during leaf development strongly affected their  $g_m$  response to  $C_i$ . In contrast, several other studies have reported  $g_m$  to be stable in response to changes in CO<sub>2</sub> concentration (von Caemmerer & Evans, 1991, Tazoe *et al.*, 2009) and light intensity (Tazoe *et al.*, 2009). There has been speculation that the rapid changes in  $g_m$  with light intensity are a methodological artefact (Evans, 2009, Tholen *et al.*, 2012, Gu & Sun, 2014). Nevertheless, Douthe *et al.* (2012) concluded that the rapid response of  $g_m$  to light is unlikely to be a computation artefact in the carbon isotope method since using different values for the parameters of the discrimination model in the isotope method changed the absolute values of  $g_m$  but did not affect the relative response to light intensity in *Eucalyptus* species. Two studies examined the effect of light colour on  $g_m$  (Loreto *et al.*, 2009, Pallozzi *et al.*, 2013) and found that exposure to blue light rapidly reduces  $g_m$ . Positional movements of chloroplasts are known to be influenced by blue light (Banaś *et al.*, 2012) which might affect  $g_m$ , although  $g_m$  reduction was faster than any possible chloroplast redistribution (Loreto *et al.*, 2009).  $g_{sw}$  increases strongly in response to blue light, but blue light induces lower photosynthetic rates than red light at the same  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Sharkey & Raschke, 1981, Pieruschka *et al.*, 2010).

$g_m$  responds to both measurement and growth temperature changes. In response to increased leaf temperature,  $g_m$  increases linearly, or increases to an optimum, depending on the species and acclimation conditions, and then becomes constant or decreases thereafter (Flexas *et al.*, 2008, Evans & von Caemmerer, 2013, Walker *et al.*, 2013). von Caemmerer and Evans (2015) studied the temperature response of  $g_m$  in nine species with contrasting characteristics grown under the same conditions and using a single methodology for the estimation of  $g_m$ . They found that the responses varied from a 2-3 fold increase in  $g_m$  between 15 and 40°C for some species to almost

no change in others. It is very important to understand  $g_m$  response to temperature, light and  $[\text{CO}_2]$  because these response functions are used to predict photosynthetic rates at higher scales, and at the moment, the photosynthesis models assume  $g_m$  is a constant.

Therefore, understanding  $g_m$  variability is crucial to incorporate  $g_m$  in photosynthesis models and thus accurately predict the carboxylation rate (Niinemets *et al.*, 2009a); and to understand the mechanistic reasons behind the plant responses to different environments and the variability in these responses, which would be relevant in terms of maximizing crop yield and water use efficiency. Short-term or long-term response of  $g_m$  to some of the important environmental variables will be briefly discussed in the subsequent chapters.

## **2.9. Mechanistic basis of $g_m$ variation**

Despite considerable evidence for  $g_m$  variability within and among species and in response to environmental conditions, the mechanisms that regulate these variations are poorly understood. There is a lack of comprehensive understanding of the structural and molecular control of  $g_m$  (Flexas *et al.*, 2013a). Studies have shown that  $g_m$  is more strongly influenced by the liquid phase compared to the gas phase conductance (Flexas *et al.*, 2008). The variability in  $g_m$  observed among species and genotypes and their responses to growth environments have been associated with leaf structure and anatomical properties particularly, leaf thickness, mesophyll ( $S_{\text{mes}}$ ) and/or chloroplast ( $S_c$ ) surface area facing intercellular air spaces, cell wall thickness ( $T_{\text{cw}}$ ), chloroplast thickness (Evans *et al.*, 1994, Evans *et al.*, 2009, Scafaro *et al.*, 2011, Terashima *et al.*, 2011, Tholen & Zhu, 2011, Peguero-Pina *et al.*, 2012, Tosens *et al.*, 2012a, Muir *et al.*, 2014, Xiong *et al.*, 2015a, Peguero-Pina *et al.*, 2016). Reductions in chloroplast size have been shown to induce a decline in  $g_m$  in rice under nitrate nutrition with water stress (Li *et al.*, 2012). Two backcrossed inbred lines of rice had higher mesophyll conductance than their parental varieties due to higher number of mesophyll cells per leaf area and more highly developed lobes of mesophyll cells in the inbred lines (Adachi *et al.*, 2013).

Tomás *et al.* (2013) evaluated the relative importance of different anatomical traits in constraining  $\text{CO}_2$  diffusion using a quantitative model. They found that  $g_m$  was most strongly correlated with  $S_c$  and  $T_{\text{cw}}$ , but the importance of different anatomical traits in the restriction of

CO<sub>2</sub> diffusion varied depending on foliage structure. Gillon and Yakir (2000) were able to partition  $g_m$  into cell wall and plasma membrane ( $g_w$ ) and chloroplast membrane conductance ( $g_{ch}$ ). They showed that  $g_w$  was lower than  $g_{ch}$  in thick leaves of oaks, but  $g_{ch}$  was lower than  $g_w$  in the mesophytic leaves of soybean and tobacco. In a study by Giuliani *et al.* (2013),  $g_m$  was not significantly correlated with any of the measured individual structural traits in rice and its wild relatives, leading the authors to suggest that changes in  $g_m$  depend on covariations of multiple leaf and mesophyll cell structural traits. There are other studies that showed no relationship between  $g_m$  and the observed anatomical variability. The correlations between  $g_m$  and  $S_c$  were poor in *Acer* cultivars (Hanba *et al.*, 2002) and the authors suspected that other factors including the structural and chemical composition of cell walls, conductance across cell and chloroplast membranes may contribute to the  $g_m$  response to the growth light level. Similarly, Tomás *et al.* (2014) did not find any effect of water stress on the distribution of chloroplasts in grapevine cultivars and speculated that genotype-dependent and water stress-induced differences in cell and chloroplast membrane permeability could be a likely cause for the observed variations of  $g_m$ .

Moreover, changes in leaf structural properties cannot explain the rapid variation observed in response to varying environmental conditions. Changes in the leaf enzymatic processes including membrane permeability have been proposed to be responsible for some of the rapid response of  $g_m$  to environments (Flexas *et al.*, 2008, Evans *et al.*, 2009, Tholen & Zhu, 2011). Based on a temperature response coefficient ( $Q_{10}$ ) of approximately 2.2 for  $g_m$  in tobacco leaves, Bernacchi *et al.* (2002) speculated that  $g_m$  must be driven by an enzyme or protein-facilitated process, since such  $Q_{10}$  value is observed if enzymes such as carbonic anhydrase (CA) or aquaporin are involved in the CO<sub>2</sub> diffusion to the site of carboxylation.

Aquaporins are membrane proteins that can facilitate membrane water transport (Maurel & Chrispeels, 2001). Aquaporins are the most abundant protein in plant cell membranes and some aquaporins may function as CO<sub>2</sub> channels (Uehlein *et al.*, 2003, Uehlein *et al.*, 2008, Uehlein *et al.*, 2012, Mori *et al.*, 2014, Groszmann *et al.*, 2017, Uehlein *et al.*, 2017). Deactivation of aquaporins with HgCl<sub>2</sub>, an inhibitor of aquaporins, reduced  $g_m$  in faba bean and common bean leaves (Terashima & Ono, 2002) and in tobacco plants growing under long-term drought (Miyazawa *et al.*, 2008), suggesting that aquaporins might enhance CO<sub>2</sub> permeability (although

effects of  $\text{HgCl}_2$  were not specific). Other studies (Hanba *et al.*, 2004, Flexas *et al.*, 2006) reported that the antisense suppression and overexpression of aquaporin genes were respectively associated with reductions and increases in  $g_m$ . Furthermore, a study by Heckwolf *et al.* (2011) on a mutation of the *Arabidopsis thaliana* aquaporin *AtPIP1;2* gene, characterized as a non-water transporting but  $\text{CO}_2$  permeable aquaporin in the *Saccharomyces cerevisiae* heterologous expression systems showed that *AtPIP1;2* gene reduced  $\text{CO}_2$  diffusion and photosynthesis in leaves. However, we do not have conclusive evidence for the function of aquaporins in membrane permeability. Recently, Zhao *et al.* (2017) measured water and  $\text{CO}_2$  permeability ( $P_{os}$ ,  $P_{CO_2}$ ) using stopped flow spectrofluorimetry on plasma membrane vesicles isolated from *Pisum sativum* and *Arabidopsis thaliana* leaves and found a weak positive correlation between  $P_{os}$  and  $P_{CO_2}$ . They suggested that aquaporins may facilitate  $\text{CO}_2$  transport across plasma membranes, but probably via a different pathway than water because inhibitors of  $P_{os}$  did not alter  $P_{CO_2}$ .

Carbonic anhydrase catalyses the reversible interconversion of  $\text{CO}_2$  with  $\text{HCO}_3^-$ , that differ in their diffusivities, pH and temperature dependencies (Flexas *et al.*, 2012a). CA could improve  $\text{CO}_2$  diffusion through the stroma by allowing carbon to diffuse in the form of  $\text{HCO}_3^-$  (Tholen & Zhu, 2011, Flexas *et al.*, 2012a). CA contributes to  $g_m$  by maintaining a nearly constant  $\text{CO}_2$  concentration throughout the stroma, allowing a more effective use of the available Rubisco (Tholen & Zhu, 2011). Despite the suggested involvement in  $g_m$  some time ago, only a few studies have investigated the effect of CA activity on  $g_m$ . Gillon and Yakir (2000) suggested that contribution of CA to  $g_m$  is species dependent, and their role may become more important when  $g_m$  is low as in sclerophyllous species. More recently, Momayyezi and Guy (2017) provided the first empirical evidence of an important role for carbonic anhydrase in photosynthesis. Using a chemical inhibitor of CA, they demonstrated that CA activity is tightly correlated with latitudinal variation in mesophyll conductance of *Populus trichocarpa* Torr. & Gray.

Recent work by von Caemmerer and Evans (2015) highlighted the lack of complete understanding of the mechanistic bases of  $g_m$  responses. They observed temperature response of  $g_m$  differed greatly between species, and proposed this may be due to variation in the activation energy for membrane permeability to  $\text{CO}_2$  (suggesting the involvement of fast biochemical



components like aquaporins in the regulation of  $g_m$ ) and the effective pathlength for liquid phase diffusion (referring mostly to the cell wall thickness). Flexas and Diaz-Espejo (2015) (commentary article on von Caemmerer and Evans (2015)) summarized three potential mechanisms for the rapid response of  $g_m$ : changes in cell wall properties (the nature of chemical interactions inside cell wall pores), regulation of membrane properties, and reshaping and redistribution of chloroplasts. Previously, Tholen *et al.* (2008) found that short-term chloroplast avoidance response, induced by blue light, reduced  $S_c$  thereby reducing  $g_m$ . A few studies (Holzinger *et al.*, 2007a, Buchner *et al.*, 2015) observed an increase in chloroplast protrusions in leaf mesophyll cells in response to an increase in temperature, leading to a dynamic enlargement of the chloroplast surface area. This might explain the  $g_m$  response to temperature but has not been demonstrated with concurrent gas exchange measurements. However, Moser *et al.* (2015) demonstrated that in *Ranunculus glacialis* L., chloroplast protrusions were not a result of heat or light stress but were most abundant under moderate temperature and non-stress irradiation conditions.

Changes in leaf structural properties, chloroplast positioning, aquaporins and CA activity may all act together to control  $g_m$ , while their relative importance might depend on species and environments. Understanding the determinants of  $g_m$  is imperative to understand its regulation and for it to be useful as a selective trait.

## **2.10. Coordination of mesophyll conductance and leaf hydraulic conductance**

A review by Flexas *et al.* (2013b) suggested that mesophyll conductance and leaf hydraulic conductance ( $K_{\text{leaf}}$ ) might share portions of their transport pathways within leaves, and thus may respond to changes in environmental conditions in a similar or coordinated way. Leaf hydraulic conductance ( $K_{\text{leaf}}$ ,  $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ) is a measure of how efficiently water is transported through the leaf.  $K_{\text{leaf}}$  is determined as the ratio of the water flow rate per unit leaf area to the water potential gradient across the leaf (Sack and Holbrook, 2006). It is related to transpiration by following equation:  $K_{\text{leaf}} = E / (\Psi_{\text{stem}} - \Psi_{\text{leaf}})$ , where  $E$  is the transpiration rate,  $\Psi_{\text{stem}}$  is the stem water potential and  $\Psi_{\text{leaf}}$  is the leaf water potential.  $K_{\text{leaf}}$  has two components: the vein xylem hydraulic conductance ( $k_x$ ) and the outside-xylem hydraulic conductance ( $K_{\text{ox}}$ ). The outside-xylem compartment includes all living tissues (xylem parenchyma, bundle sheath, and

mesophyll) that transport water from xylem vessels to the point outside the xylem that corresponds to operational measurements of  $\Psi_{\text{leaf}}$  (Buckley *et al.*, 2017).

$K_{\text{leaf}}$  and light-saturated net  $\text{CO}_2$  assimilation rate ( $A_{\text{max}}$ ) are correlated across the diverse range of plant species (Brodribb *et al.*, 2005, Franks, 2006). Low leaf hydraulic conductance restricts water flow to the epidermis, which reduces stomatal conductance and photosynthesis but increases WUE (Sinclair *et al.* 2008). Low  $K_{\text{leaf}}$  also restricts water flow to the mesophyll and thus reduces mesophyll water potential, which may in turn directly reduce photosynthetic rate.  $K_{\text{leaf}}$  varies among plant species and is dependent on the anatomy, development and age of the leaf.  $K_{\text{leaf}}$  shows rapid response to environmental variables such as leaf hydration, light, temperature and vapour pressure deficit (Sack *et al.*, 2002, Brodribb & Holbrook, 2003, Sack *et al.*, 2004, Cochard *et al.*, 2007, Levin *et al.*, 2007, Scoffoni *et al.*, 2008, Scoffoni *et al.*, 2011). The mechanisms of  $K_{\text{leaf}}$  response to specific environmental conditions include transcriptional regulation of aquaporin and changes in their abundance, trafficking, and intrinsic activity (Prado & Maurel, 2013). The manipulation of  $K_{\text{leaf}}$  could help optimize the entire plant performance and its adaptation to extreme conditions over short and long time scales.

Flexas *et al.* (2013b) surveyed the coordination of  $g_m$  and  $K_{\text{leaf}}$  and observed a generally positive relationship across plant species. They showed that  $g_m$  and  $K_{\text{leaf}}$  have widely agreed trends in the responses to a number of environmental variables such as temperature, light level and water status. However, the response of  $K_{\text{leaf}}$  to  $\text{CO}_2$  have not been found to parallel those of  $g_m$ . Locke *et al.* (2013) found no effect of growth at high  $\text{CO}_2$  on  $K_{\text{leaf}}$  in soybeans, while higher growth  $\text{CO}_2$  has mostly been shown to reduce  $g_m$  (Singsaas *et al.*, 2004) consistent with partially independent pathways for movement of water and  $\text{CO}_2$  inside leaves. Based on their parallel responses to a number of environmental variables, along with the partially distinct dynamics of these traits ( $\text{CO}_2$  response), Flexas *et al.* (2013b) proposed that  $g_m$  is linked to the outside-xylem component of  $K_{\text{leaf}}$  ( $K_{\text{ox}}$ ), and the lack of correlation between  $K_{\text{leaf}}$  and vein density implies the independence of the coordination between  $g_m$  and xylem component of  $K_{\text{leaf}}$ .

The mechanisms for coordinated dynamics of  $g_m$  and  $K_{\text{leaf}}$  are not clear (Flexas *et al.*, 2012a, Griffiths & Helliker, 2013). The outside-xylem component was found to depend on mesophyll

structure and physiology (Aasamaa *et al.*, 2005), a trait which also influences  $g_m$ . Nevertheless, water flow outside the xylem is poorly understood. Once in the bundle sheath, water may move apoplastically (through the cell walls), symplastically (through plasmodesmata), or transcellularly (through aquaporins in cell membranes) (Steudle *et al.*, 1993) and the relative contribution of these pathways might affect the response of  $K_{ox}$  to environmental variables and leaf anatomy (Buckley, 2015, Buckley *et al.*, 2015). Isotopic studies also indicate some coordination of regulation of  $g_m$  and  $K_{leaf}$ , particularly in aquaporin-dependent pathways. Ferrio *et al.* (2012) demonstrated a strong linear relationship between  $g_m$  and  $K_{ox}$  and the effective path length ( $L$ ) for water transport from xylem vessels to the sites of evaporation in grapevines. In another study (Theroux-Rancourt *et al.*, 2014),  $K_{leaf}$  decreased in concert with  $g_{sw}$  with drought stress, whereas  $g_m$  remained relatively constant up to a certain  $g_{sw}$  threshold, supporting the hypothesis of a partial hydraulic isolation of the mesophyll from the main transpiration pathway. Recently, Xiong *et al.* (2017) observed a linear correlation between  $g_m$  and  $K_{ox}$  across all rice genotypes and both were closely related to mesophyll structural traits (fraction of leaf mesophyll volume occupied by intercellular air space,  $S_{mes}$ ,  $S_c$ ,  $T_{cw}$ ). In contrast, Loucos *et al.* (2017) found a weak correlation between  $g_m$  and  $K_{leaf}$  only across growth treatment in cotton (one cultivar). They did not find a correlation between  $K_{leaf}$  and chloroplast membrane conductance, estimated with simultaneous measurement of  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$ . However,  $\Delta^{18}O-g_m$  was significantly, but weakly correlated to  $K_{leaf}$ , suggesting limited coordinated regulation of  $CO_2$  and water transport across the cell wall and plasma membrane. Analysis of the relationship between  $g_m$  and  $K_{leaf}$  is still at an embryonic stage with conflicting results. More studies are needed to confirm the  $g_m$  and  $K_{leaf}$  relationships in other species and growth environments. Coordination between  $g_m$  and  $K_{leaf}$  will be further discussed in Chapter 5.

### **2.11. Mesophyll conductance and water use efficiency**

There has been growing interest in  $g_m$  for increasing photosynthesis ( $A$ ) and leaf water use efficiency ( $A/g_{sw}$ ). Barbour *et al.* (2010) reported positive correlation between  $g_m$  and  $A/g_{sw}$  in barley and suggested that breeding for higher  $g_m$  has the potential to increase leaf WUE. Flexas *et al.* (2013a), based on their review of values of  $g_m$  in the literature, suggested that simultaneous improvement of  $A$  and WUE is possible by improving  $g_m$  over  $g_{sw}$ .  $A$  is strongly influenced by both  $g_{sw}$  and  $g_m$ ; however, which of the two conductances plays the most important role may

depend on species and environmental conditions. Several studies have shown a correlation between  $g_m$  and  $g_{sw}$  (Flexas *et al.*, 2002, Flexas *et al.*, 2008, Centritto *et al.*, 2009, Barbour *et al.*, 2010, Galmés *et al.*, 2011, Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016). On the other hand, there are studies indicating the lack of correlation between  $g_m$  and  $g_{sw}$  (Bunce, 2009, Jahan *et al.*, 2014). Co-regulation between  $g_m$  and  $g_{sw}$  might differ between species and with the intensity of stress, as suggested by Warren (2008b). The combination of low  $g_{sw}$  and high  $g_m$  would produce high leaf water-use efficiency (Barbour *et al.*, 2010, Buckley & Warren, 2014, Cano *et al.*, 2014, Flexas *et al.*, 2016).

Regarding crop genetic improvement through  $g_m$ , Barbour *et al.* (2016a) reported the first hints of genetic control of mesophyll conductance in bread wheat. The multigene nature of leaf anatomy makes it difficult to manipulate  $g_m$  through leaf morphological alterations (Flexas *et al.*, 2016). There is evidence of increased  $g_m$  through altered aquaporin expression or activity (Hanba *et al.*, 2004, Uehlein *et al.*, 2008, Secchi & Zwieniecki, 2013, Sade *et al.*, 2014), but the concomitant increase in  $g_{sw}$  cancelled any improvement in  $A/g_{sw}$ . Flexas *et al.* (2016), in their review, speculated that same aquaporins might be involved in water transport as well as in  $CO_2$  diffusion and control both  $g_{sw}$  and  $g_m$ . However, Zhao *et al.* (2017) suggested that aquaporins may facilitate  $CO_2$  transport across plasma membranes, but probably via a different pathway than for water. In legumes, the effects of varying  $g_m$  on  $A$  and  $A/g_{sw}$  have been experimentally tested only in soybean cultivars (Tomeo & Rosenthal, 2017), where a strong positive correlation between mesophyll and stomatal conductance among soybean cultivars interfere with the potential to improve WUE through selection on  $g_m$ . Despite some complexity, the positive relationships between  $A$  and  $g_m$  and  $A/g_{sw}$  and  $g_m$  under the growth conditions, would demonstrate the potential of improving  $A$  and WUE within a crop improvement program.

## **2.12. Summary and further research**

Grain legumes play an important role in Australian agriculture. Drought is a major constraint in all grain legume growing regions of Australia, and climate change is expected to exacerbate this problem. Improving WUE has thus become a mandatory subject of research for sustainable legume production. Selection for higher WUE, generally considered in terms of  $g_{sw}$ , often resulted in reduced photosynthesis and yield. Barbour *et al.* (2010) found that mesophyll

conductance to CO<sub>2</sub> ( $g_m$ ), which regulates CO<sub>2</sub> diffusion from sub-stomatal cavities to the carboxylation site, was positively related to both  $A$  and  $g_{sw}$  and suggested that  $g_m$  has the potential to further improve  $A/g_{sw}$  while simultaneously maintaining productivity through crop breeding.

There has been a lack of techniques to partition  $g_m$  into its component conductances between the sub-stomatal cavities and the carboxylation site. This has made it difficult to determine the relative importance of the  $g_m$  components. Gillon and Yakin (2000) demonstrated that the oxygen isotope discrimination ( $\Delta^{18}O$ ) can be used to partition  $g_m$  between conductance before and after the chloroplast surface in C<sub>3</sub> plants. More recently, Barbour et al. (2016) demonstrated that  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  can be rapidly and easily measured by coupling traditional gas exchange with laser absorption spectrometers that measure the stable carbon and oxygen isotope composition of CO<sub>2</sub> and the stable oxygen isotope composition of transpired water vapour. However, the  $\Delta^{18}O$  technique to estimate  $g_m$  is in its embryonic stage, and measurements in other major crops are imperative in the near future.

Significant genotypic variation in  $g_m$  has been reported in several important crop species, but within grain legumes, genotypic variability in  $g_m$  has only been studied in *Phaseolus vulgaris* (Flowers *et al.*, 2007) and the variability was reported only after exposure to high ozone concentration.  $g_m$  can also respond to short and long term changes in environmental conditions such as water stress, salinity, temperature, CO<sub>2</sub> concentration, and light. However, evidence of rapid responses of  $g_m$  to light and CO<sub>2</sub> concentration are unclear, with positive relationship in some species and experiments but not others. The mechanisms of changes in  $g_m$  are poorly understood, and relevant studies in grain legumes are scarce. The variability of maximum values of  $g_m$  observed among species and genotypes associated with adaptive and acclimation responses often relate to leaf structure and anatomical properties, while the rapid responses of  $g_m$  has been attributed to carbonic anhydrase, aquaporins or chloroplast changes.

A recent review by Flexas *et al.* (2013b) suggested that  $g_m$  and outside xylem hydraulic conductance might share portions of their transport pathways within leaves, and there might be a functional linkage between  $g_m$  and  $K_{leaf}$ . However, recent studies showed contrasting results, with

a strong correlation between  $g_m$  and  $K_{leaf}$  in some studies while none or poor relationships in other studies (Loucos *et al.*, 2017). Comparisons between different species and genotypes and with different treatments might be a feasible approach to identify their coordination.

Therefore, before  $g_m$  can be recommended as selection criteria for breeders for improving WUE, there is a need for comprehensive research on  $g_m$  of grain legumes in terms of its association with water use efficiency. Future research should aim to answer some of the puzzling and often interrelated issues discussed in the previous sections. The degree of genotypic variation and co-variation of  $g_m$ ,  $g_m$  components and leaf WUE in different legume crops using the concurrent measurements of  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  and their response to different short-term and long-term environmental parameters would be valuable. Further investigation of the coordination of  $g_m$  and  $K_{leaf}$  in legumes cultivars under different growth conditions could provide new insights into the responses of these traits. With such an approach, development of grain legume genotypes better adapted to water-limited environments should progress more rapidly in the future.

### 3. Research Methodology

Mesophyll conductance to CO<sub>2</sub> ( $g_m$ ) was estimated using the online carbon discrimination ( $\Delta^{13}\text{C}$ ) method (Evans *et al.*, 1986, Tazoe *et al.*, 2009), for all the experiments in this thesis. The  $\Delta^{13}\text{C}$  method produced estimates of mesophyll conductance from intercellular air spaces to the sites of carboxylation, denoted hereafter as the  $\Delta^{13}\text{C}-g_m$ . In Chapter 4 and 5, CO<sub>2</sub> diffusional conductance from the intercellular air spaces to the sites of CO<sub>2</sub>-H<sub>2</sub>O equilibrium was estimated using oxygen isotope discrimination ( $\Delta^{18}\text{O}$ ) method (Gillon & Yakir, 2000, Barbour *et al.*, 2016b), denoted hereafter as  $\Delta^{18}\text{O}-g_m$ . This chapter covers the carbon and oxygen isotope methods, including the equations and fractionation factors used to estimate mesophyll conductance to CO<sub>2</sub>.

#### 3.1. Simultaneous gas exchange and mesophyll conductance measurements

Gas exchange measurements and regulation of leaf environmental conditions were conducted using a Li6400xt portable photosynthesis system (Li-Cor Biosciences, Lincoln, NE, USA). Leaf chambers and leaf environmental conditions within the chamber used for each experiment are included in their respective chapters. Gas exchange was recorded at 1min intervals. For experiments which measured both  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  (Chapter 4 and 5), the Li6400xt was coupled to a Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) and a Picarro water vapour isotope analyser (L1102-I, Picarro Inc., Sunnyvale, CA, USA). The TDL measured the stable carbon and oxygen isotope compositions of CO<sub>2</sub> (<sup>13</sup>CO<sub>2</sub>, C<sup>18</sup>O<sup>16</sup>O), as described by Barbour *et al.* (2007). The Picarro measured the stable oxygen isotope composition of transpired water vapour (H<sub>2</sub><sup>18</sup>O), as described by Simonin *et al.* (2013). Leaf chamber inlet and outlet air streams were sub-sampled to the TDL and a sub-sample of the leaf chamber outlet air stream was sent to the Picarro. The flow rate through the leaf chambers was maintained above 300 ml min<sup>-1</sup> to provide sufficient flow for the TDL (250 ml min<sup>-1</sup>) and the Picarro (25 ml min<sup>-1</sup>). All air streams were passed through a nafion drying tube prior to entering the TDL to obtain values at zero vapour concentration. For the experiments measuring  $\Delta^{13}\text{C}-g_m$  (Chapter 6 and 7), the Li6400xt was connected only to the TDL.

The TDL was calibrated using two calibration cylinders (or four calibration cylinders, in Chapter 5) spanning the range in concentrations of the isotopologues of the leaf chamber inlet and outlet air streams. Total CO<sub>2</sub> concentrations and isotope compositions of the calibration cylinders were measured using a stable isotope mass spectrometer at the National Institute of Water and Atmospheric Research, Wellington, New Zealand, and their absolute values are included in their respective chapters. Carbon isotope ratios are presented relative to the Vienna Pee Dee belemnite standard, and oxygen isotope ratios of CO<sub>2</sub> and water vapour are presented relative to the Vienna Standard Mean Oceanic Water (VSMOW) standard. The TDL received standards from the cylinders every 6 min and the raw values of the sample air streams within this time period were calibrated against these standards. Interchanging between calibration cylinders and the sample air streams was enabled by a manifold regulated by a datalogger (CR3000, Campbell Scientific Inc.).

The Picarro was calibrated following the procedure detailed in Simonin *et al.* (2013) using two standard water samples of known isotope values of -14.6 and 1.5‰ that spanned the range in δ<sup>18</sup>O of leaf chamber inlet and outlet air streams (standards were measured using a stable isotope mass spectrometer at The Australian National University, Canberra, Australia). To account for the concentration dependence of δ<sup>18</sup>O, after measurements were complete, each standard water sample was analysed at a range of concentrations to span the observed range of concentrations, as described in Simonin *et al.* (2013). The Li6400xt data, the TDL data and the Picarro measurements were synchronized by matching their timestamps. For the leaves not covering the entire leaf chamber, after each measurement, leaf area within the chamber was calculated from the digitized images of the leaf using ImageJ (NIH, Bethesda, MD, USA). Gas exchange variables were recalculated with the corrected leaf area.

Photosynthetic <sup>13</sup>CO<sub>2</sub> and C<sup>18</sup>O<sup>16</sup>O discrimination were calculated from the following equation (Evans *et al.*, 1986):

$$\Delta_{\text{obs}} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (1)$$

$$\text{where: } \xi = \frac{c_e}{c_e - c_o} \quad (2)$$



$c_e$  and  $\delta_e$  are concentrations and isotope compositions of CO<sub>2</sub> of dry air entering the leaf chamber and  $c_o$  and  $\delta_o$  are concentrations and isotope compositions of CO<sub>2</sub> of dry air exiting the chamber, respectively. Carbon and oxygen isotope compositions of CO<sub>2</sub> were obtained from the TDL.

### 3.2. Estimation of mesophyll conductance from $\Delta^{13}\text{C}$

Mesophyll conductance from the intercellular air spaces to the sites of carboxylation in the chloroplast stroma ( $\Delta^{13}\text{C-g}_m$ , mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>), was calculated from the difference between calculated carbon isotope discrimination assuming infinite  $g_m$  ( $\Delta^{13}\text{C}_i$ ), and that measured by the coupled system ( $\Delta^{13}\text{C}_{\text{obs}}$ ), using equations and fractionation factors presented in Barbour *et al.* (2016b) and including the ternary corrections as described by Farquhar and Cernusak (2012):

$$\Delta^{13}\text{C}_i = \frac{1}{1-t} \left[ a_b \frac{C_a - C_s}{C_a} + a_s \frac{C_s - C_i}{C_a} \right] + \frac{1+t}{1-t} \left[ b \frac{C_i}{C_a} - \frac{\alpha_b}{\alpha_\epsilon} \epsilon \frac{R_d}{A + R_d} \frac{C_i - \Gamma^*}{C_a} - \frac{\alpha_b}{\alpha_f} f \frac{\Gamma^*}{C_a} \right] \quad (3)$$

where  $C_a$ ,  $C_s$  and  $C_i$  are the ambient, leaf surface and intercellular CO<sub>2</sub> partial pressures,  $a_b$  and  $a_s$  are the fractionations during diffusion through the leaf boundary layer (2.9‰) (Evans *et al.*, 1986) and the stomata (4.4‰) (Farquhar & Richards, 1984) respectively,  $b$  is the fractionation associated with carboxylation (30‰) (Guy *et al.*, 1993),  $f$  is the fractionation associated with photorespiration (16.2‰) (Evans & von Caemmerer, 2013),  $\alpha_b$  is the fractionation factor for carboxylation ( $1 + b$ ),  $\alpha_\epsilon$  is the fractionation factor for day respiration ( $1 + \epsilon$ ),  $\alpha_f$  is the fractionation factor for photorespiration ( $1 + f$ ).  $R_d$  is the rate of day respiration and  $\Gamma^*$  is the compensation point in the absence of  $R_d$ . Both  $R_d$  and  $\Gamma^*$  were predicted from leaf temperature using the approach described by Bernacchi *et al.* (2002).

In Eqn 3,  $\epsilon$  is the fractionation associated with day respiration, corrected for source CO<sub>2</sub>  $\delta^{13}\text{C}$  (Tazoe *et al.*, 2009), and is given by:

$$\epsilon = e + \delta^{13}\text{C}_{\text{tank}} - \delta^{13}\text{C}_{\text{atmosphere}} \quad (4)$$

where  $e$  is assumed to be -3‰ (Tcherkez *et al.*, 2010),  $\delta^{13}\text{C}_{\text{tank}}$  was measured by the TDL, and  $\delta^{13}\text{C}_{\text{atmosphere}}$  was measured by the Picarro.

In Eqn 3,  $t$  is the ternary correction factor (Farquhar & Cernusak, 2012), and is given by:

$$t = \frac{\alpha_{ac}E}{2g_{ac}} \quad (5)$$

where  $E$  is the transpiration rate ( $\text{mmol m}^{-2} \text{s}^{-1}$ ),  $\alpha_{ac}$  is the fractionation factor of  $\text{CO}_2$  diffusion in air ( $1+\bar{a}$ ),  $\bar{a}$  is the weighted fractionation through the leaf boundary layer and stomata (Evans *et al.*, 1986).  $g_{ac}$  denotes the total conductance to  $\text{CO}_2$  diffusion including the boundary layer and stomatal conductance.

Then, mesophyll resistance ( $r_m$ ) is given by Farquhar and Cernusak (2012):

$$r_m = \frac{1-t}{1+t} (\Delta^{13}\text{C}_i - \Delta^{13}\text{C}_{\text{obs}}) \frac{C_a}{A \left( b - a_m - \frac{\alpha_p \epsilon - R_d}{\alpha_e (A + R_d)} \right)} \quad (6)$$

$\Delta^{13}\text{C}_{\text{obs}}$  is calculated from Eqns 1 and 2,  $A$  is the  $\text{CO}_2$  assimilation rate ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ),  $a_m$  is the fractionation factor for liquid phase  $\text{CO}_2$  diffusion and dissolution (‰).

Mesophyll conductance ( $\Delta^{13}\text{C}-g_m$ ), was calculated as:

$$\Delta^{13}\text{C}-g_m = 1/r_m \quad (7)$$

Then, chloroplast  $\text{CO}_2$  partial pressure ( $C_c$ ) was calculated as:

$$C_c = C_i - (A/\Delta^{13}\text{C}-g_m) \quad (8)$$

### 3.3. Estimation of mesophyll conductance from $\Delta^{18}\text{O}$

$\text{CO}_2$  diffusional conductance from the intercellular air spaces to the sites of  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium ( $\Delta^{18}\text{O}-g_m$ ) was estimated from oxygen isotope discrimination method, as described in Barbour *et al.* (2016b). Photosynthetic oxygen isotope discrimination ( $\Delta^{18}\text{O}_{\text{obs}}$ ) was calculated from TDL  $\delta^{18}\text{O}$  measurements, using Eqns 1 and 2. Water inside leaves becomes enriched in  $\text{H}_2^{18}\text{O}$  during transpiration. Carbonic anhydrase catalyses the interconversion of  $\text{CO}_2$  and  $\text{HCO}_3^-$  allowing isotopic exchange of  $^{18}\text{O}$  between leaf-dissolved  $\text{CO}_2$  and  $^{18}\text{O}$ -enriched  $\text{H}_2\text{O}$ , so that  $\text{CO}_2$  leaving the leaf chamber is enriched in  $^{18}\text{O}$  above  $\text{CO}_2$  entering the leaf chamber (Gillon & Yakir, 2000).

The oxygen isotopic composition of the CO<sub>2</sub> being assimilated ( $\delta^{18}\text{O}_A$ ) (Barbour *et al.*, 2016b) is given by :

$$\delta^{18}\text{O}_A = \frac{\delta^{18}\text{O}_o - \Delta^{18}\text{O}_{\text{obs}}}{1 + \Delta^{18}\text{O}_{\text{obs}}} \quad (9)$$

where  $\delta^{18}\text{O}_o$  is the oxygen isotope composition of the air leaving the chamber.

Here I assumed full equilibration between cytosolic CO<sub>2</sub> and local cytosolic water, due to the high rate of carbonic anhydrase activity commonly observed in C<sub>3</sub> plants. Studies to date have found CO<sub>2</sub> and H<sub>2</sub>O to be close to full equilibrium (Cernusak *et al.*, 2004, Kodama *et al.*, 2011). The oxygen isotope composition of CO<sub>2</sub> in the cytosol ( $\delta^{18}\text{O}_c$ ) is given by (Cernusak *et al.*, 2004):

$$\delta^{18}\text{O}_c = \delta^{18}\text{O}_e + \varepsilon_w \quad (10)$$

where  $\delta^{18}\text{O}_e$  is the oxygen isotope composition of the cytosolic leaf water and  $\varepsilon_w$  is the isotopic equilibrium between CO<sub>2</sub> and water (Brenninkmeijer *et al.*, 1983), and is given by:

$$\varepsilon_w(\text{‰}) = \frac{17604}{T_1} - 17.93 \quad (11)$$

In Eqn 11,  $T_1$  is the leaf temperature in Kelvin.

The cytosolic water was assumed to be isotopically the same as water at the sites of evaporation within leaves (Gillon & Yakir, 2000, Barbour & Farquhar, 2004, Tomás *et al.*, 2013). The stable oxygen isotope composition of water at the evaporation sites within leaves was estimated using the modified Craig–Gordon model of evaporative enrichment (Craig & Gordon, 1965, Flanagan *et al.*, 1991) for non-steady state conditions (Harwood *et al.*, 1998):

$$\delta^{18}\text{O}_e = \delta^{18}\text{O}_{\text{trans}} + \varepsilon^* + \varepsilon_k + \frac{e_a}{e_i} (\delta^{18}\text{O}_v - \varepsilon_k - \delta^{18}\text{O}_{\text{trans}}) \quad (12)$$

$\delta^{18}\text{O}_{\text{trans}}$  is the isotopic composition of transpired water vapour (measured by the Picarro) and  $\delta^{18}\text{O}_v$  is the vapour in the leaf chamber. In this study, the air entering the leaf chamber was dried

of H<sub>2</sub>O. Therefore, the humidity of the air leaving the leaf chamber was entirely derived from transpiration, and so  $\delta^{18}\text{O}_v = \delta^{18}\text{O}_{\text{trans}}$ .

$e_a/e_i$  is the ratio of ambient to intercellular vapour pressure.

$\varepsilon^*$  is the equilibrium fractionation factor during evaporation (Bottinga & Craig, 1968), and is given by :

$$\varepsilon^* = 2.644 - 3.206 \left( \frac{10^3}{T_1} \right) + 1.534 \left( \frac{10^6}{T_1^2} \right) \quad (13)$$

$\varepsilon_k$  is the kinetic fractionation during diffusion of vapour (Merlivat, 1978, Farquhar *et al.*, 1998, Luz *et al.*, 2009), and is given by:

$$\varepsilon_k = \frac{28g_s^{-1} + 19g_b^{-1}}{g_s^{-1} + g_b^{-1}} \quad (14)$$

where  $g_s$  and  $g_b$  are stomatal conductance and boundary layer conductance respectively.

The oxygen isotope composition of CO<sub>2</sub> in the intercellular air spaces, without ternary corrections, is given by Farquhar and Cernusak (2012):

$$\delta^{18}\text{O}_{i_o} = \delta^{18}\text{O}_A \left( 1 - \frac{C_a}{C_i} \right) (1 + a_{18}) + \frac{C_a}{C_i} (\delta^{18}\text{O}_{\text{out}} - a_{18}) + a_{18} \quad (15)$$

$$\text{where } a_{18} = \frac{(C_s - C_i)a_{18s} + (C_a - C_s)a_{18b}}{C_a - C_i} \quad (16)$$

$a_{18}$  and  $a_{18b}$  are the discriminations during diffusion through the stomata and the boundary layer for C<sup>18</sup>O<sup>16</sup>O, assuming fractionation factors of 8.8‰ and 5.8‰ respectively.

The oxygen isotope composition of CO<sub>2</sub> in the intercellular air spaces, including ternary corrections (Farquhar & Cernusak, 2012), is given by:

$$\delta^{18}\text{O}_i = \frac{\delta^{18}\text{O}_{i_o} + t \left[ \delta^{18}\text{O}_A \left( \frac{C_a}{C_i} + 1 \right) - \delta^{18}\text{O}_a \frac{C_a}{C_i} \right]}{1+t} \quad (17)$$

The CO<sub>2</sub> partial pressures at the sites of CO<sub>2</sub>-H<sub>2</sub>O equilibrium (C<sub>CH</sub>) may be calculated from the oxygen isotope composition of CO<sub>2</sub> in the intercellular air spaces (δ<sup>18</sup>O<sub>i</sub>) and the oxygen isotope composition of CO<sub>2</sub> at the sites of CO<sub>2</sub>-H<sub>2</sub>O equilibrium (δ<sup>18</sup>O<sub>c</sub>) (Barbour *et al.*, 2016b), and is given by :

$$C_{CH} = C_i \left( \frac{\delta^{18}O_i - a_{18w} - \delta^{18}O_A(1 + a_{18w})}{\delta^{18}O_c - a_{18w} - \delta^{18}O_A(1 + a_{18w})} \right), \quad (18)$$

where  $a_{18w}$  is the combined discriminations against C<sup>18</sup>O<sup>16</sup>O during liquid phase diffusion and dissolution (0.8‰).

Then, CO<sub>2</sub> diffusional conductance from the intercellular air spaces to the sites of CO<sub>2</sub>-H<sub>2</sub>O equilibrium (Δ<sup>18</sup>O- $g_m$ , mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>) is given by (Barbour *et al.*, 2016b):

$$\Delta^{18}O-g_m = A / (C_i - C_{CH}) \quad (19)$$

### 3.4. Estimation of chloroplast membrane conductance ( $g_{cm}$ )

The combined measurements of Δ<sup>18</sup>O- $g_m$  (from the Δ<sup>18</sup>O method) and Δ<sup>13</sup>C- $g_m$  (from the Δ<sup>13</sup>C method) allow partitioning of total conductance into the components before and after the sites of CO<sub>2</sub>-H<sub>2</sub>O equilibrium. If the CO<sub>2</sub>-H<sub>2</sub>O equilibrium occurred within the cytosol or at the chloroplast surface (Gillon & Yakir, 2000), Δ<sup>18</sup>O- $g_m$  would relate to cell wall and plasma membrane conductance. The combined measurements of Δ<sup>18</sup>O- $g_m$  and Δ<sup>13</sup>C- $g_m$  were then used to partition total CO<sub>2</sub> conductance into cell wall and membrane conductance and chloroplast membrane conductance ( $g_{cm}$ ) (Barbour *et al.*, 2016b), assuming no significant resistance to CO<sub>2</sub> diffusion in the gaseous leaf interior (Evans *et al.*, 1994).

Chloroplast membrane conductance ( $g_{cm}$ ) was calculated by:

$$g_{cm} = A / (C_c - C_{CH}) \quad (20)$$

where  $C_c$  was given by Δ<sup>13</sup>C (Eqn 8) and  $C_{CH}$  was given by Δ<sup>18</sup>O (Eqn 20).

However, the interpretation of Δ<sup>18</sup>O- $g_m$  must be made with caution, as highlighted by Barbour *et al.* (2016b), since estimates of Δ<sup>18</sup>O- $g_m$  depends on the location and activity of CA. CAs are

ubiquitous enzymes localized to the mesophyll cell chloroplast, cytosol, mitochondria and the plasma membrane in C<sub>3</sub> plants (Fabre *et al.*, 2007, Dimario *et al.*, 2017), and thus CO<sub>2</sub>-H<sub>2</sub>O equilibrium could occur at the plasma membrane and  $\Delta^{18}\text{O}-g_m$  would relate to conductance through the cell wall only. On the other hand, if the majority of CA activity localized to chloroplasts, then CO<sub>2</sub> would be fully equilibrated with water inside the chloroplast, in which case the partitioning technique would not be feasible. Assessing the location and activity of CA was not within the scope of this thesis, but certainly will prove useful in the future studies. I compared  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  measurements to see if  $\Delta^{18}\text{O}-g_m$  were higher than or close to  $\Delta^{13}\text{C}-g_m$  values. Higher  $\Delta^{18}\text{O}-g_m$  values will provide an indication that CO<sub>2</sub>-H<sub>2</sub>O exchange sites are not inside the chloroplast in the species studied.

## 4. Genetic variability in mesophyll conductance among crop legumes

### 4.1. Introduction

Improving crop performance under a changing climate requires concomitant increases in both photosynthesis and water use efficiency (Flexas *et al.*, 2016). Mesophyll conductance to CO<sub>2</sub> ( $g_m$ ) has been shown to limit the diffusion of CO<sub>2</sub> from sub-stomatal cavities to the carboxylation site and reduce rates of photosynthesis (Flexas *et al.*, 2008, Niinemets *et al.*, 2009b, Flexas *et al.*, 2013a, Flexas *et al.*, 2016) and manipulation of  $g_m$  has the potential to increase leaf intrinsic water-use efficiency;  $A/g_{sw}$  (Barbour *et al.*, 2010, Flexas *et al.*, 2013a).  $g_m$  is a dynamic leaf trait which may vary in the short term (within seconds to minutes) or the in long term in response to different abiotic factors (Warren *et al.*, 2007, Loreto *et al.*, 2009, Bunce, 2010, Douthe *et al.*, 2011, Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016) although the direction and sensitivity of the response may be species or genotype dependent (Singsaas *et al.*, 2004, von Caemmerer & Evans, 2015, Barbour & Kaiser, 2016).

Despite growing interest in  $g_m$  for increasing photosynthesis and  $A/g_{sw}$ , there are very limited studies on  $g_m$  in legume species. Grain legumes have important role in food security: because, they are a critical and inexpensive source of plant-based protein, vitamins and minerals and they fix their own nitrogen and can improve soil health (Foyer *et al.*, 2016). However, grain legume yields have not improved at the same rate as cereals, and grain legumes are high priorities for research and improvements in genetic potential (Graham & Vance, 2003, Nedumaran *et al.*, 2015, Foyer *et al.*, 2016). Water use efficiency varies among legume species (Suriyagoda *et al.*, 2010, Herzog & Chai-Arree, 2012). Genotypic variation in  $A/g_{sw}$  has been reported in different grain legume crops, including bean (White, 1993, Polania *et al.*, 2016), faba bean (Khan *et al.*, 2007) cowpea (Ismail & Hall, 1992), chickpea (Krishnamurthy *et al.*, 2013, Sadras *et al.*, 2016) and soybean (Gilbert *et al.*, 2011b). Differences in  $A/g_{sw}$  and WUE between genotypes have been reported to have a genetic basis (Martin *et al.*, 1989, Masle *et al.*, 2005). There is considerable scope for improvement of grain legume yields through breeding/crop genetic improvement. Genetic control of  $g_m$  was recently presented for common wheat for the first time by Barbour *et al.* (2016a), raising the possibility of selecting for high  $g_m$  to increase  $A/g_{sw}$ . Incorporating  $g_m$  in any breeding program aiming to increase photosynthesis or  $A/g_{sw}$  requires information on

genotypic variability of  $g_m$ . Significant genotypic variation in  $g_m$  has been reported in important crop species, including cereals (Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014), *Castanea sativa* (Lauteri *et al.*, 1997), *Solanum lycopersicum* (Galmés *et al.*, 2011) and *Vitis vinifera* (Tomás *et al.*, 2014) but information is very limited in grain legumes. Flowers *et al.* (2007) observed genotypic variation in  $g_m$  among snap bean genotypes but only after exposure to high ozone concentration.

$g_m$  is a combination of gaseous diffusion through intercellular air spaces and diffusion through the cell wall, through plasma membrane, the cytosol and through chloroplast envelope to the site of carboxylation (Evans *et al.*, 2009) and is thus influenced by a large number of physical and biochemical factors.  $g_m$  variability has been associated with leaf anatomical structure, particularly mesophyll structure, cell wall thickness, mesophyll ( $S_{mes}$ ) and/or chloroplast ( $S_c$ ) surface area facing intercellular air spaces (Evans *et al.*, 2009, Tosens *et al.*, 2012b, Tomás *et al.*, 2013), as well as with enzymatic processes affecting membrane permeability (Hanba *et al.*, 2004, Uehlein *et al.*, 2008, Perez-Martin *et al.*, 2014). Mesophyll conductance has been estimated using combined gas exchange and chlorophyll fluorescence method or the online carbon isotope discrimination method ( $\Delta^{13}C-g_m$ ) or by the curve-fitting method (Evans *et al.*, 1986, Harley *et al.*, 1992, Ethier & Livingston, 2004). These established techniques have been used to quantify  $g_m$ , providing estimates of total conductance to the carboxylation site in  $C_3$  plants (Pons *et al.*, 2009). Gillon and Yakir (2000) proposed that measurements of  $\delta^{18}O$  of  $CO_2$  and  $\delta^{18}O$  of water inside the leaves could be used to estimate conductance to  $CO_2$  diffusion from the intercellular air space to the site of  $CO_2$ - $H_2O$  isotopic equilibration, which was assumed to be at the chloroplast surface. Further, the  $^{18}O$  estimate of  $g_m$  (hereafter referred to as  $\Delta^{18}O-g_m$ ) combined with  $\Delta^{13}C-g_m$  (total conductance estimated using the carbon isotope discrimination method) could allow partitioning of total conductance into cell wall and membrane components (Gillon & Yakir, 2000). More recently, Barbour *et al.* (2016b) demonstrated that  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  can be rapidly and easily measured in  $C_3$  plants (tobacco, cotton and wheat) by coupling traditional gas exchange with laser absorption spectrometers that measure the stable carbon and oxygen isotope composition of  $CO_2$  and the stable oxygen isotope composition of transpired water vapour. Additional information on the components of  $g_m$  in  $C_3$  plants would be helpful in identifying targets to increase  $g_m$ . The goals of the present study were to quantify the variability



in  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  in common bean, faba bean, garden pea and field pea; to determine if  $g_m$  scales with photosynthetic rates and leaf WUE in these legume species under non-limiting conditions and finally to examine the utility of simultaneous carbon and oxygen isotope techniques to partition total  $g_m$  into its components.

## **4.2. Materials and Methods**

### **4.2.1. Plant material and growth conditions**

Five genotypes of common bean (*Phaseolus vulgaris* L.), two genotypes of faba bean (*Vicia faba* L.), three genotypes of garden pea (*Pisum sativum* L.) and ten genotypes of field pea (*Pisum sativum* L.) were grown in a controlled-environment growth cabinet at the University of Sydney, Centre for Carbon Water and Food (Camden, NSW, Australia). The growth cabinet was set to a 14 h photoperiod, 25/17°C day/night temperature, 75% relative humidity, 700  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density at plant height. Seeds were sown in 7 L pots filled with commercial potting mix supplemented with slow release fertilizer (Osmocote Exact, Scotts, NSW, Australia). After emergence, the plants were thinned to two per pot and were well watered throughout the experiment. The genotypes studied were (six replicate plants each) Common Bean: Brown Beauty, Cherokee Wax, Gourmet Delight, Vitalis, Westralia; faba bean: Cairo, PBA Warda; garden pea: Dwarf Sugar Snap, Blue Bantam, Greenfeast; and field pea: 07126, 08116, Gunyah, Maki, Prlas, Twillight, Walana, Yarrum, Oвра and Para. Oвра and Para were morphologically different to the other field pea genotypes, possessing narrow leaflets and stipules exhibiting the 'tare-leaf' trait, while the others were semi-leafless bearing normal stipules, but with leaflets replaced by profuse bunches of tendrils.

### **4.2.2. Concurrent gas exchange and mesophyll conductance measurements**

Leaf gas exchange and isotope discrimination measurements were conducted 4 weeks after planting. The youngest fully expanded leaves were used for gas exchange measurements using a LI6400XT portable photosynthesis system (Li-Cor Biosciences, Lincoln, NE, USA) fitted with a custom-built leaf chamber of area 38  $\text{cm}^2$  (Barbour *et al.*, 2007) and red-green-blue light source (Li6400 18A). The leaf chamber conditions were controlled to provide  $\text{CO}_2$  concentration of 320  $\mu\text{mol mol}^{-1}$  in the sample cell, leaf temperature at 25°C, and the photosynthetic photon flux

density (PPFD) of  $1300 \mu\text{mol m}^{-2} \text{s}^{-1}$  for all measurements. The LI-6400XT was coupled to two laser absorption spectrometers: Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) and Picarro water vapor isotope analyser (L1102-I, Picarro Inc., Sunnyvale, CA, USA), as described in Chapter 3. Two calibration cylinders were used to calibrate the TDL. Absolute values of  $^{12}\text{CO}_2$ ,  $^{13}\text{CO}_2$  and  $\text{C}^{18}\text{O}^{16}\text{O}$  were, respectively, 292.05, 3.17 and 1.20 ppm for low concentrations and 483.93, 5.26 and 1.99 ppm for high concentrations. Carbon isotope ratios are presented relative to the Vienna Pee Dee belemnite standard and oxygen isotope ratios relative to the VSMOW standard. The Picarro was calibrated using two standard water samples of known isotopic composition that spanned the range in  $\delta^{18}\text{O}$  of leaf chamber inlet and outlet air streams, following the procedure detailed in Simonin *et al.* (2013). For the leaves not covering the entire leaf chamber, after each measurement, leaf area within the chamber was calculated from the digitized images of the leaf using ImageJ (NIH, Bethesda, MD, USA). Gas exchange variables were recalculated with the corrected leaf area.

Mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ) was estimated using the online carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) method and oxygen isotope discrimination ( $\Delta^{18}\text{O}$ ) method as described in Chapter 3. I assumed that  $\text{CO}_2$ – $\text{H}_2\text{O}$  exchange occurs at the chloroplast surface, as suggested by Gillon and Yakir (2000), and the chloroplast membrane conductance ( $g_{cm}$ ) was calculated from the combined measurements of  $\Delta^{18}\text{O}$ - $g_m$  and  $\Delta^{13}\text{C}$ - $g_m$  as described in Chapter 3.

#### **4.2.3. Estimation of $V_{\text{cmax}}$ , $J_{\text{max}}$ and $R_d$**

Photosynthetic  $\text{CO}_2$  response curves at saturating light were measured by controlling  $\text{CO}_2$  concentration of the reference air stream from 50 to 1200 ppm. The  $\text{CO}_2$  concentration in the leaf chamber started at 400 ppm, was lowered to 50 and then increased to 1200 ppm in 8 steps (400, 50, 100, 150, 200, 300, 400, 800, 1000 and 1200 ppm). These response curves were measured in three replicate plants for each genotype (out of six replicates used for  $g_m$  measurements). For the majority of plants, the response curves and  $g_m$  measurements were made on the same leaf, and within three days of each other. Response curves were produced under PAR of  $1300 \mu\text{mol m}^{-2} \text{s}^{-1}$  and flow rate of  $500 \mu\text{mol s}^{-1}$ . Then  $C_c$  was calculated from measured  $A$  and  $C_i$ , and calculated values of  $\Delta^{13}\text{C}$ - $g_m$ . Maximum carboxylation rate by Rubisco ( $V_{\text{cmax}}$ ) and electron transport rate

( $J_{\max}$ ) were fitted using the spreadsheet form (Sharkey *et al.*, 2007), but using estimated  $\Delta^{13}\text{C}-g_m$  for each leaf, and assuming  $\Delta^{13}\text{C}-g_m$  did not vary with  $\text{CO}_2$  concentration (Tazoe *et al.*, 2009).

Photosynthetic light response curves were obtained by decreasing photosynthetic photon flux density (PPFD) from 100 to 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in 10 steps (100, 90, 80, 70, 60, 50, 40, 30, 20, 0  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), under  $\text{CO}_2$  concentrations of 400  $\mu\text{mol mol}^{-1}$ . The slope of these relationships typically declines abruptly at a PPFD near the light compensation point (i.e., a 'Kok' effect); the ordinate intercept of the regression of  $A$  vs PPFD above this slope change was assumed to be the rate of non-photorespiratory light respiration ( $R_d$ ) (Kok, 1948).

#### 4.2.4. Statistical analysis

Significant differences between values were assessed using analysis of variance, as implemented by GENSTAT 16th edn SP1 (VSN International Ltd, London, UK), and means were compared using Fisher's unprotected least significant difference test. Differences were considered statistically significant when  $p < 0.05$ .

### 4.3. Results

#### 4.3.1. Variations in leaf gas exchange and mesophyll conductance

There was significant variation in net  $\text{CO}_2$  assimilation rate ( $A$ ) between the legume species ( $p < 0.001$ ), with the highest  $A$  measured in faba beans (genotype average = 24.3  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and the lowest in field peas (genotype average = 13.3  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Variation in  $A$  was significant between common bean genotypes ( $p < 0.05$ ) and between field pea genotypes ( $p < 0.05$ ) but the differences were not significant within faba beans or garden peas (Figure 4.1a). Among field peas, 'Ovra' and 'Para' had significantly higher  $A$  than other genotypes (but not statistically different from 'Walana' and 'Prlas'). Overall, the highest  $A$  was observed in 'Cairo' (faba bean). Variation in stomatal conductance ( $g_{sw}$ ) was significant between the legume species ( $p < 0.001$ ) as well as between genotypes within each species (Figure 4.1b, common bean:  $p < 0.001$ , faba bean:  $p < 0.05$ , garden pea:  $p < 0.05$ , field pea:  $p < 0.016$ ). Similarly, leaf intrinsic water use efficiency ( $A/g_{sw}$ ) values were significantly different between species ( $p < 0.001$ ) and between genotypes within each species (Figure 4.1c, common bean:  $p < 0.001$ , faba bean:  $p < 0.05$ , garden pea:

$p < 0.05$ , field pea:  $p < 0.001$ ). ‘Cherokee wax’ (common bean) had the highest  $A/g_{sw}$  across the species while ‘Para’ had the highest  $A/g_{sw}$  within field pea.

The carbon isotope technique produced estimates of mesophyll conductance from intercellular air spaces to the sites of carboxylation ( $\Delta^{13}\text{C}-g_m$ ). Genotypic variation for  $\Delta^{13}\text{C}-g_m$  was observed in faba bean ( $p < 0.05$ ), garden pea ( $p < 0.05$ ) and in field pea ( $p < 0.001$ ) but not in common bean (Figure 4.2a). The  $\Delta^{13}\text{C}-g_m$  values for field peas ranged from 0.18 to 0.54 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>. Faba bean and garden pea genotypes had significantly higher  $\Delta^{13}\text{C}-g_m$  than most of the common bean and field pea genotypes ( $p < 0.001$ ). Overall,  $\Delta^{13}\text{C}-g_m$  varied by about 4-fold, with the highest value measured in ‘Cairo’ (0.79 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>) and the lowest in ‘Prlas’ (0.18 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>).

#### 4.3.2. Partitioning resistance to CO<sub>2</sub> diffusion

Assuming the CO<sub>2</sub>-H<sub>2</sub>O equilibrium occurred at the chloroplast surface in the investigated genotypes, the total mesophyll conductance to CO<sub>2</sub> was partitioned into cell wall and plasma membrane conductance ( $\Delta^{18}\text{O}-g_m$ , Figure 4.2b) and chloroplast membrane conductance ( $g_{cm}$ , Figure 4.2c). I did not find genotypic variation for  $\Delta^{18}\text{O}-g_m$  across the legume species (Figure 4.2b). Typically,  $\Delta^{18}\text{O}-g_m$  values were lower in field peas compared to other legume species ( $p < 0.001$ ).  $\Delta^{18}\text{O}-g_m$  was significantly higher than  $\Delta^{13}\text{C}-g_m$  for most of the genotypes across the legume species except for ‘Ovra’ and ‘Para’ (Figure 4.2a, Figure 4.2b). I found significant positive correlation between  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  (Figure 4.4c,  $R^2 = 0.51$ ,  $p < 0.001$ ) and between  $\Delta^{13}\text{C}-g_m$  and  $g_{cm}$  (Figure 4.4d,  $R^2 = 0.52$ ,  $p < 0.001$ ) across all the genotypes.

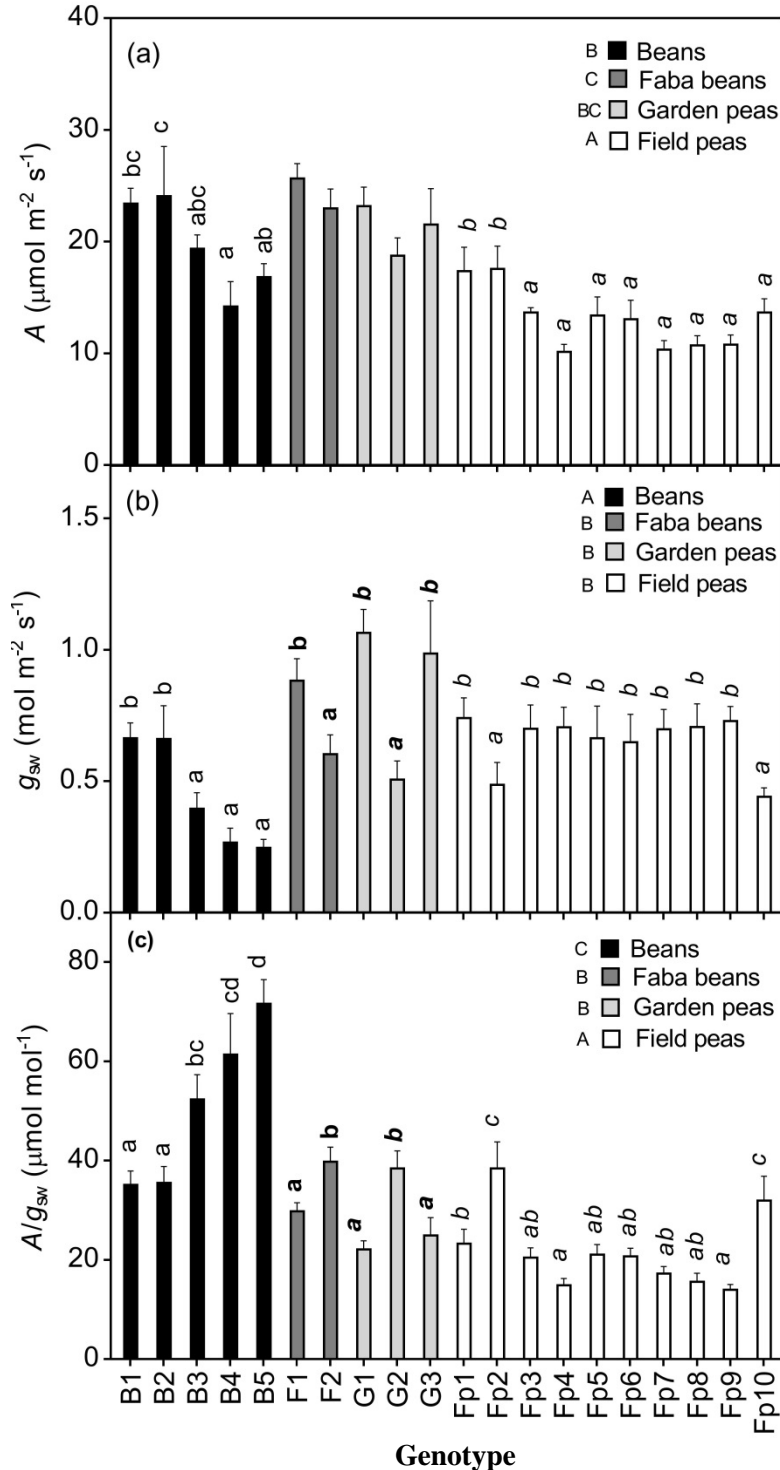
Resistance to CO<sub>2</sub> diffusion was partitioned into the stomatal ( $L'_{sc}$ ), cell wall/plasma membrane ( $L'_{cw}$ ) and the chloroplast membrane components ( $L'_{mem}$ ) as shown in Figure 4.3. To compare mesophyll resistance directly to the stomatal resistance to CO<sub>2</sub>, values for the mesophyll conductance were multiplied by atmospheric pressure, to correct for bar<sup>-1</sup> in the  $g_m$  units. The fraction of stomatal resistance to CO<sub>2</sub> was typically higher than the fraction of mesophyll resistance to CO<sub>2</sub> ( $L'_{cw} + L'_{mem}$ ) for most genotypes of common bean, faba bean and garden pea (except in ‘Gourmet Delight’ and ‘Vitalis’ of common bean and ‘Greenfeast’ of garden pea, where  $L'_{sc} \approx L'_{cw} + L'_{mem}$ ). Among the field pea genotypes,  $L'_{sc}$  was significantly lower than  $L'_{cw} + L'_{mem}$  for some genotypes, while stomatal and mesophyll resistances were of similar magnitude

for other genotypes (Figure 4.3d). Of the mesophyll resistance to CO<sub>2</sub>,  $L'_{cw} > L'_{mem}$  in faba bean genotypes, while  $L'_{cw} \approx L'_{mem}$  for most of the genotypes in common beans, garden peas, and field peas. The proportion of cell wall/plasma membrane resistance was significantly higher than the proportion of chloroplast membrane resistance in ‘Vitalis’ and ‘Westralia’ (common beans), ‘Greenfeast’ (garden pea) and in ‘Ovra’ (field pea).

### 4.3.3. Relationships between mesophyll conductance, photosynthetic rate and water use efficiency

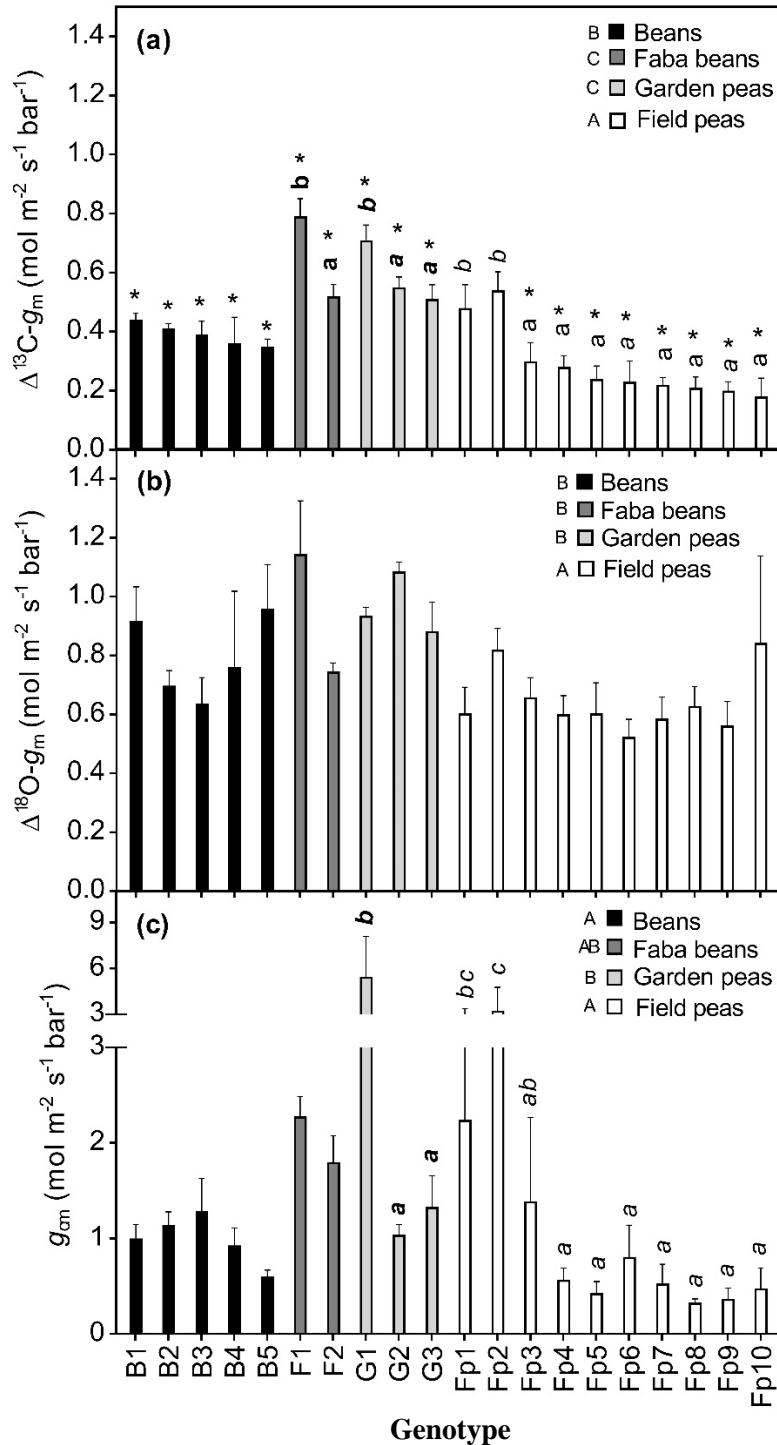
There was a significant positive relationship between  $\Delta^{13}\text{C}-g_m$  and  $A$  across all the genotypes and species (Figure 4.4a,  $R^2 = 0.72$ ,  $p < 0.001$ ). There was no significant relationship between  $\Delta^{13}\text{C}-g_m$  and  $g_{sw}$  or between  $\Delta^{13}\text{C}-g_m$  and  $A/g_{sw}$  (Figure 4.4b) across the genotypes. When genotype averages were calculated, stomatal conductance explained 57% of the observed variability in  $A/g_{sw}$  ( $p < 0.001$ ). Within field peas,  $\Delta^{13}\text{C}-g_m$  was weakly but positively correlated to  $A/g_{sw}$  ( $R^2 = 0.2$ ,  $p = 0.001$ ), but not related to  $g_{sw}$  across the genotypes.

Genotypic variation in  $V_{cmax}$  was significant except for faba beans (Figure 4.5a, common beans:  $p = 0.016$ , garden peas:  $p = 0.003$  and field peas:  $p < 0.001$ ).  $J_{max}$  varied significantly between genotypes in common beans ( $p < 0.035$ ) and field peas ( $p < 0.001$ ) (Figure 4.5b). Similarly, variation in non-photorespiratory respiration rate was significant between genotypes in common beans ( $p < 0.001$ ) and field peas ( $p < 0.001$ ).  $V_{cmax}$ ,  $J_{max}$  and  $R_d$  also differed between species ( $p < 0.001$ ). Photosynthetic rate was positively related to both  $V_{cmax}$  ( $R^2 = 0.60$ ,  $p < 0.001$ ) and  $J_{max}$  ( $R^2 = 0.67$ ,  $p < 0.001$ ) across all genotypes.

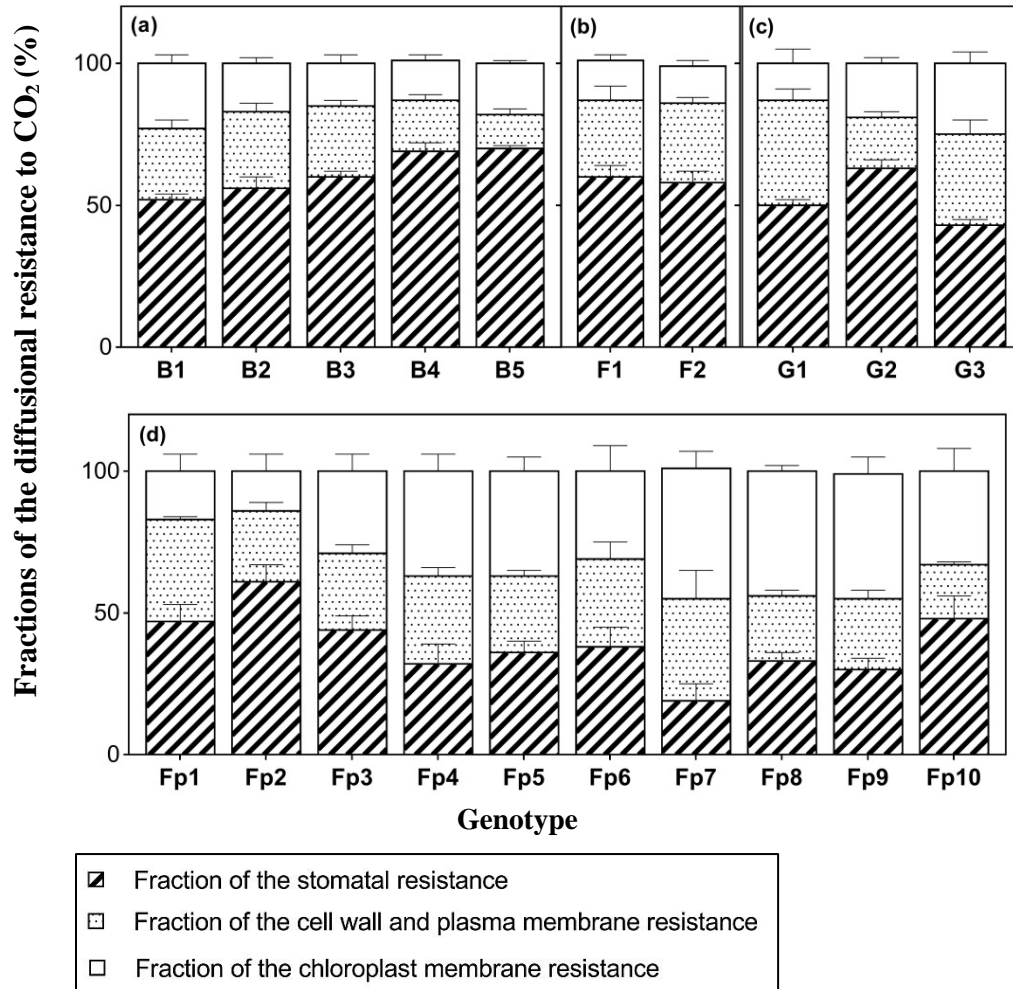


**Figure 4.1** Light-saturated photosynthetic rate ( $A$ ; a), stomatal conductance ( $g_{sw}$ ; b) and leaf-intrinsic water use efficiency ( $A/g_{sw}$ ; c) for common bean, faba bean, garden pea and field pea genotypes. Values are means  $\pm$  s.e.,  $n=6$ . Uppercase letters in the legend indicate significant differences ( $p<0.05$ ) between species. Lowercase letters indicate significant differences ( $p<0.05$ ) between genotypes within each species.

The genotypes were: B1-Gourmet Delight, B2-Vitalis, B3-Westralia, B4-Brown Beauty, B5-Cherokee Wax, F1-Cairo, F2-PBA Warda, G1- Greenfeast, G2-Dwarf Sugar Snap, G3-Blue Bantam, Fp1-Ovra, Fp2-Para, Fp3-Walana, Fp4-Twillight, Fp5-Yarrum, Fp6-08116, Fp7-Maki, Fp8-07126, Fp9-Gunyah and Fp10-Prlas.

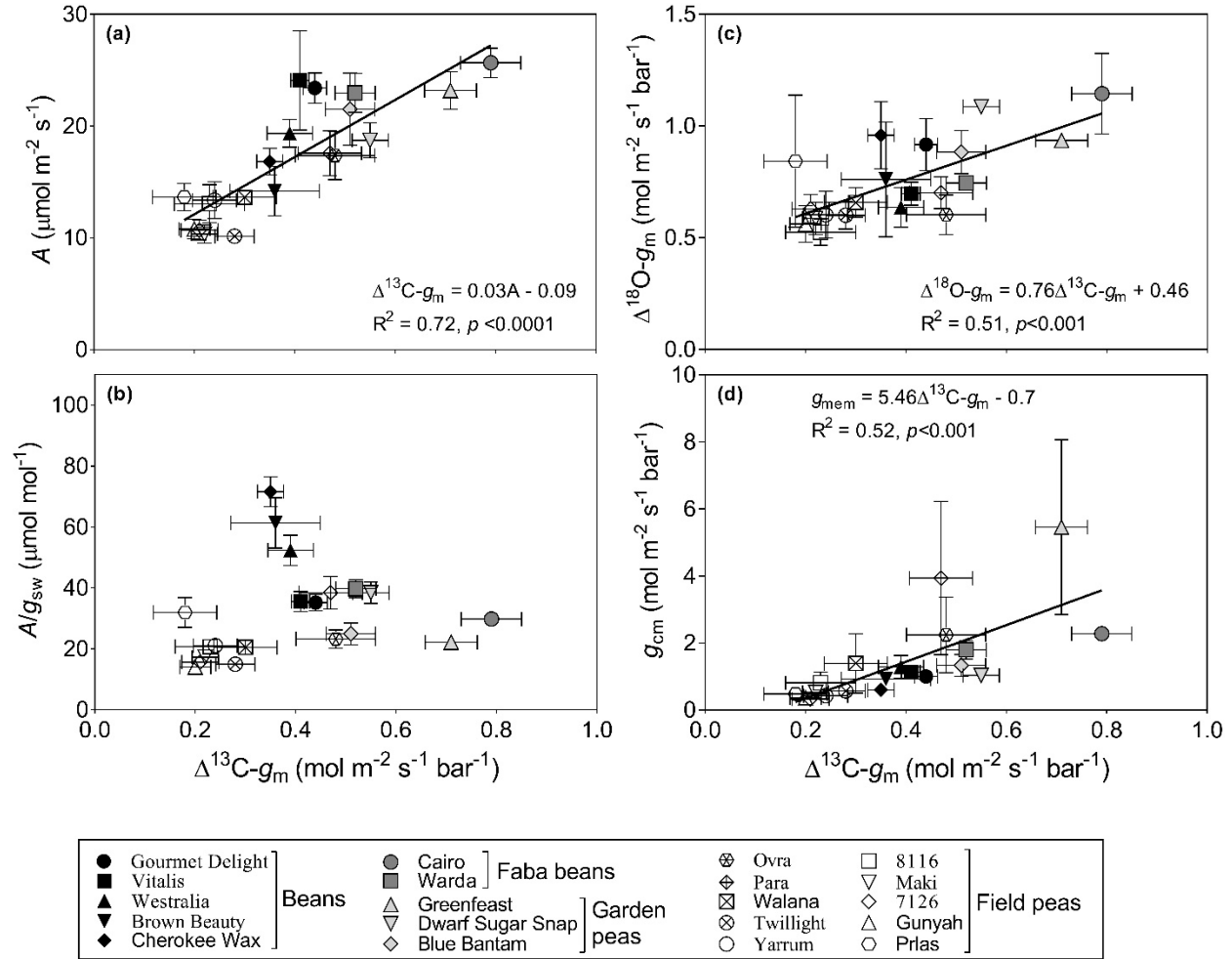


**Figure 4.2** Mesophyll conductance estimated from carbon isotope technique ( $\Delta^{13}\text{C}-g_m$ ; a), from oxygen isotope technique ( $\Delta^{18}\text{O}-g_m$ ; b) and chloroplast membrane conductance ( $g_m$ ; c) for common bean, faba bean, garden pea and field pea genotypes. Values are means  $\pm$  s.e.,  $n=4-6$ . Uppercase letters in the legend indicate significant differences ( $p<0.05$ ) between species. Lowercase letters indicate significant differences ( $p<0.05$ ) between genotypes within each species. Asterisks mark the genotypes for which significant differences in  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  were found. The genotypes were: B1-Gourmet Delight, B2-Vitalis, B3-Westralia, B4-Brown Beauty, B5-Cherokee Wax, F1-Cairo, F2-PBA Warda, G1- Greenfeast, G2-Dwarf Sugar Snap, G3-Blue Bantam, Fp1-Ovra, Fp2-Para, Fp3-Walana, Fp4-Twilight, Fp5-Yarrum, Fp6-08116, Fp7-Maki, Fp8-07126, Fp9-Gunyah and Fp10-Prlas.

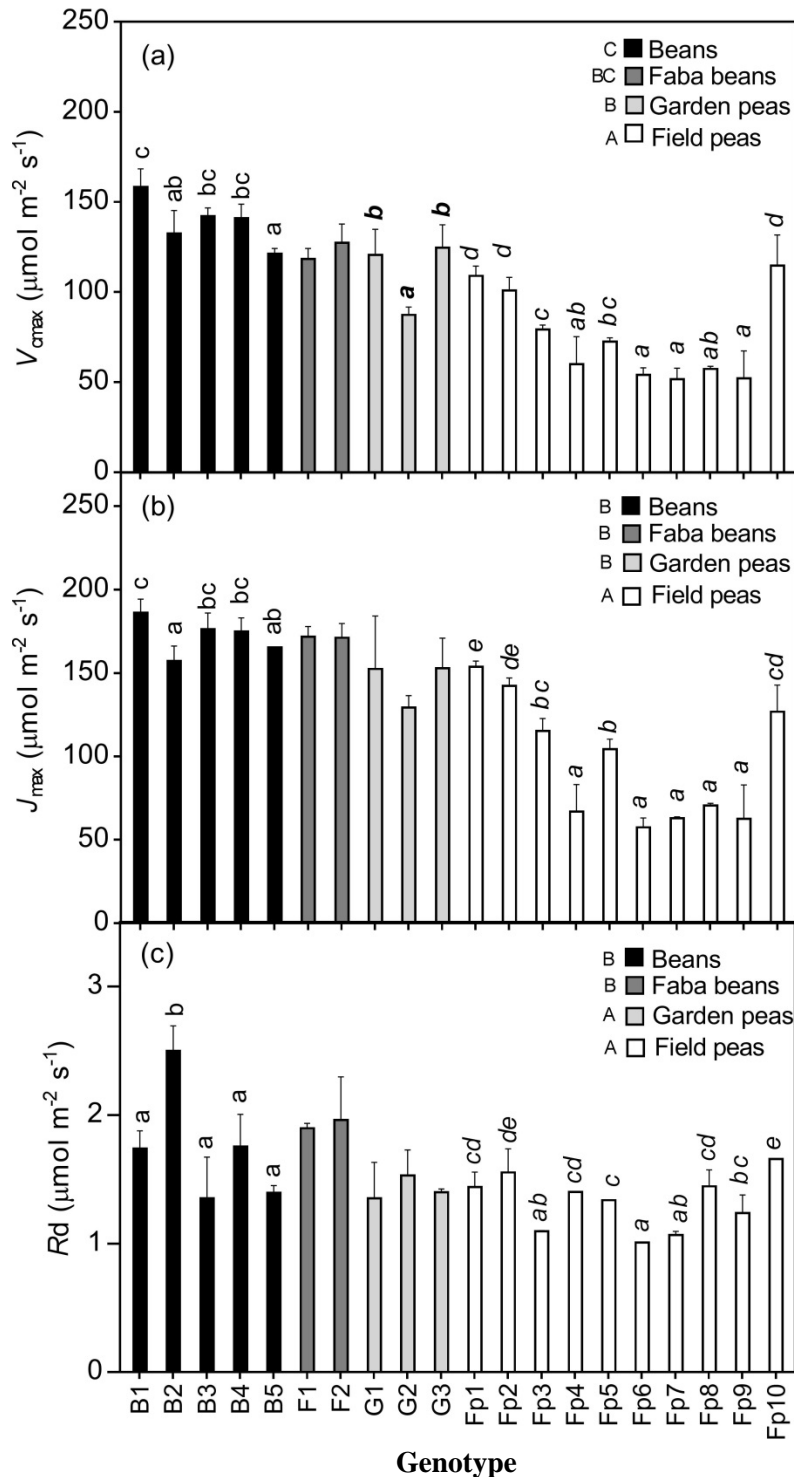


**Figure 4.3** Fractions of the stomatal, cell wall/plasma membrane and chloroplast membrane resistances to CO<sub>2</sub> for common bean (a), faba bean (b), garden pea (c) and field pea genotypes (d). Values are means  $\pm$  s.e.,  $n=4-6$ . The genotypes were: B1-Gourmet Delight, B2-Vitalis, B3-Westralia, B4-Brown Beauty, B5-Cherokee Wax, F1-Cairo, F2-PBA Warda, G1- Greenfeast, G2-Dwarf Sugar Snap, G3-Blue Bantam, Fp1-Ovra, Fp2-Para, Fp3-Walana, Fp4-Twillight, Fp5-Yarrum, Fp6-08116, Fp7-Maki, Fp8-07126, Fp9-Gunyah and Fp10-Prlas.





**Figure 4.4** The relationships between mesophyll conductances estimated from the carbon isotope technique ( $\Delta^{13}\text{C}-g_m$ ) and photosynthetic rate ( $A$ ; a) and leaf-intrinsic water use efficiency ( $A/g_{sw}$ ; b) and between  $\Delta^{13}\text{C}-g_m$  and mesophyll conductance from the oxygen isotope technique ( $\Delta^{18}\text{O}-g_m$ ; c) and the chloroplast membrane conductance ( $g_{cm}$ ; d) for common bean, faba bean, garden pea and field pea genotypes. Values are means  $\pm$  s.e.,  $n=4-6$ . Linear regressions are shown when significant ( $p < 0.05$ ).



**Figure 4.5** Maximum carboxylation rate ( $V_{cmax}$ ; a), electron transport rate ( $J_{max}$ ; b) and non-photorespiratory light respiration ( $R_d$ ; c) at 25°C for common bean, faba bean, garden pea and field pea genotypes. Values are means  $\pm$  s.e.,  $n=3$ . Uppercase letters in the legend indicate significant differences ( $p<0.05$ ) between species. Lowercase letters indicate significant differences ( $p<0.05$ ) between genotypes within each species.

The genotypes were: B1-Gourmet Delight, B2-Vitalis, B3-Westralia, B4-Brown Beauty, B5-Cherokee Wax, F1-Cairo, F2-PBA Warda, G1- Greenfeast, G2-Dwarf Sugar Snap, G3-Blue Bantam, Fp1-Ovra, Fp2-Para, Fp3-Walana, Fp4-Twillight, Fp5-Yarrum, Fp6-08116, Fp7-Maki, Fp8-07126, Fp9-Gunyah and Fp10-Prlas.

## 4.4. Discussion

### 4.4.1. $g_m$ varies within and among legume species

In the present study, mesophyll conductance was estimated for different genotypes of four important crop legumes (common bean, faba bean, garden pea and field pea), grown and measured under non-limiting conditions.  $g_m$  varied among the legume species, with significantly higher  $\Delta^{13}\text{C}-g_m$  values in faba bean and garden pea than in common bean and field pea genotypes. Genotypic variation in  $\Delta^{13}\text{C}-g_m$  was observed within each legume species except in common bean. Genotypic variation in  $g_m$  has been reported for cereals (Centritto *et al.*, 2009, Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014) and other crop species (Lauteri *et al.*, 1997, Galmés *et al.*, 2011, Tomás *et al.*, 2014). Flowers *et al.* (2007) found significant differences in  $g_m$  between common bean genotypes only after exposure to high ozone concentration. More recently, Tomeo and Rosenthal (2017) reported genetic variation for  $g_m$  in soybean edamame (*Glycine max*).  $g_m$  values currently available in the literature for common bean show variability from 0.19 to 0.39 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (von Caemmerer & Evans, 1991, Hanba *et al.*, 2003, Lucia *et al.*, 2003, Singsaas *et al.*, 2004, Flowers *et al.*, 2007).  $g_m$  values measured in faba bean genotypes in this study were slightly higher (0.5-0.8 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup>) than the values reported in the other studies; 0.34 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (Loreto *et al.*, 1992) and 0.46 mol m<sup>-2</sup> s<sup>-1</sup> bar<sup>-1</sup> (Terashima & Ono, 2002). These differences in  $g_m$  among different studies may be due to the genotypes used in the studies or difference in the growth or measurement conditions or the  $g_m$  estimation technique employed. Among field peas,  $A$  and  $g_m$  values were higher in tare-leaf genotypes compared to the semi-leafless genotypes. Armstrong and Pate (1994) also found the highest mean photosynthetic rates of the leaflets or combined green area (leaflets + stipules + tendrils) for the tare-leaved field pea genotypes than for the semi-leafless types.

Despite evidences for  $g_m$  variability between and within species, regulation of  $g_m$  is poorly understood. The observed variation in  $g_m$  could result from variation in a number of leaf traits including leaf anatomical structure, particularly- the mesophyll structure, the surface area of chloroplasts exposed to the intercellular spaces ( $S_c$ ), cell wall and chloroplast thickness, and chloroplast distributions spaces (Evans *et al.*, 2009, Tosens *et al.*, 2012b, Tomás *et al.*, 2013), or the variation in enzymatic effects on membrane permeability (Hanba *et al.*, 2004, Uehlein *et al.*,

2008, Perez-Martin *et al.*, 2014). Aquaporins (AQPs) are membrane proteins that facilitate membrane water transport (Maurel & Chrispeels, 2001). There is evidence that certain AQPs can facilitate CO<sub>2</sub> permeation across the plasma and chloroplast membranes (Uehlein *et al.*, 2003, Kaldenhoff, 2012, Mori *et al.*, 2014, Maurel *et al.*, 2015, Groszmann *et al.*, 2017, Uehlein *et al.*, 2017, Zhao *et al.*, 2017). Terashima and Ono (2002) provided indirect evidence regarding a possible role for aquaporins in faba beans and common beans by reducing mesophyll conductance to CO<sub>2</sub> using a non-specific inhibitor of some aquaporins (HgCl<sub>2</sub>). Tomeo and Rosenthal (2017) reported that the genetic variation for  $g_m$  was highly coordinated with leaf photosynthetic physiology and, to a lesser extent, with coarse leaf structure in soybean.

#### **4.4.2. Genetic variation in $g_m$ may be related to chloroplast membrane conductance**

Gillon and Yakir (2000) proposed that the combined measurement of both  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  could be used to partition the total mesophyll conductance into cell wall/plasma membrane ( $g_{cw}$ ) and chloroplast conductance ( $g_{cm}$ ). In this study,  $\Delta^{18}\text{O}-g_m$  was higher than  $\Delta^{13}\text{C}-g_m$  for most of the genotypes (except Ovrá genotype), indicating that CO<sub>2</sub>-H<sub>2</sub>O isotopic equilibration did not occur in the chloroplast. If the assumption of CO<sub>2</sub>-H<sub>2</sub>O equilibrium at the chloroplast surface (Gillon & Yakir, 2000) is true in these legume genotypes, then  $\Delta^{18}\text{O}-g_m$  values relate to the cell wall and plasma membrane conductance to CO<sub>2</sub>. I did not find genetic variation for  $\Delta^{18}\text{O}-g_m$  across the genotypes and species. The presence of genetic variation for total mesophyll conductance in faba bean, garden pea and field peas but no genetic variation in cell wall and plasma membrane conductance suggests that the variation in  $g_m$  might be related to the variation in chloroplast membrane conductance, which might be due to variation in  $S_c$  or chloroplast membrane permeability via aquaporin.

However, as Barbour *et al.* (2016b) highlighted, the interpretation of the  $\Delta^{18}\text{O}-g_m$  should be made cautiously.  $\Delta^{18}\text{O}-g_m$  relates to the conductance to CO<sub>2</sub> from intercellular air spaces to the location of CO<sub>2</sub>-H<sub>2</sub>O equilibrium, which is influenced by the location and activity of carbonic anhydrase (CA). CA catalyses exchange of oxygen atoms from <sup>18</sup>O-enriched water to leaf-dissolved CO<sub>2</sub> and has been reported to be localized to the chloroplast, the cytosol, the mitochondria and the plasma membrane in C<sub>3</sub> plants (Fabre *et al.*, 2007). If CO<sub>2</sub> had fully equilibrated with H<sub>2</sub>O at the plasma membrane, then  $\Delta^{18}\text{O}-g_m$  estimates in the present study

would relate to conductance through the cell wall only, and the observed variation in total mesophyll conductance in this study, might relate to plasma membrane permeability as well as chloroplast membrane permeability.

Assuming CO<sub>2</sub> equilibrates with water at the chloroplast surface, the results of this study suggested that the total diffusional resistance to CO<sub>2</sub> is divided nearly equally between cell wall/plasma membrane and chloroplast membrane for most of the genotypes in common beans, garden peas, and field peas, while most resistance (65-70%) lies in cell wall/plasma membrane in faba bean genotypes. The fraction of cell wall/plasma membrane resistance was significantly higher in 'Vitalis' and 'Westralia' (common beans), 'Greenfeast' (garden pea) and in 'Ovra' (field pea). The relative contribution of cell wall/plasma membrane and chloroplast membrane conductance has been found to vary among species. Gillon and Yakir (2000) have shown that  $g_{cw}$  was lower than  $g_{cm}$  in oaks, but  $g_{cm}$  was lower than  $g_{cw}$  in soybean and tobacco. Barbour *et al.* (2016b) calculated  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  in three C<sub>3</sub> species and found that resistance is divided nearly equally between the cell wall/plasma membrane and the chloroplast membrane for tobacco, and 40% of the total resistance lies in the chloroplast membrane for cotton. Barbour *et al.* (2016b) did not find significant difference between  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  in wheat, and suggested that the assumption of equilibration at the chloroplast surface may be incorrect in wheat, as the chloroplast membrane must impose some resistance to diffusion in all species.

Stomatal resistance played the most important role in CO<sub>2</sub> diffusion in common bean, faba bean and garden pea genotypes, while mesophyll resistances were generally more important than stomatal resistances in field pea genotypes. Flexas *et al.* (2013a), in their review, also suggested that the most limiting of the conductances was dependent on the species and treatment. They reported that in grapevine genotypes, most genotypes under irrigation were most limited by  $g_m$ , while under water stress, they were limited by stomatal conductance for CO<sub>2</sub> ( $g_{sc}$ ); in tomato, all genotypes were mostly limited by  $g_{sc}$ , while the rice genotypes were co-limited by the two conductances.

#### 4.4.3. The relationship between mesophyll conductance, photosynthetic rate and water use efficiency

Significant positive correlations between photosynthetic rate and mesophyll conductance have been reported in many important crops. I also found a positive relationship between photosynthetic rate and mesophyll conductance across all the genotypes, as strong as reported in other crops (Barbour *et al.*, 2010, Flexas *et al.*, 2013a) and in soybean cultivars (Tomeo & Rosenthal, 2017). In the current study,  $g_m$  was significantly, though not strongly, related to  $A/g_{sw}$  across genotypes in field pea but not in other legume species or across species. Moreover,  $g_m$  was not related to  $g_{sw}$  within field pea or across species. Among the field pea genotypes, 'Para' had the highest  $A/g_{sw}$  and was among the highest for  $A$  and  $g_m$  and was among the lowest for  $g_{sw}$ . Barbour *et al.* (2010) reported a positive correlation between  $g_m$  and  $A/g_{sw}$  in barley and suggested that breeding for higher  $g_m$  has the potential to increase leaf WUE. Uncoupling  $g_m$  and  $g_{sw}$  would be advantageous for simultaneous increase of photosynthesis and WUE since high  $g_m$  would allow high  $A$  but low  $g_{sw}$  would prevent water loss (Barbour *et al.*, 2010, Buckley & Warren, 2014, Cano *et al.*, 2014, Flexas *et al.*, 2016). Tomeo and Rosenthal (2017) found a strong positive correlation between mesophyll and stomatal conductance among soybean cultivars which may interfere with the potential to improve WUE through selection on  $g_m$  in soybean. Correlations between  $g_m$  and  $g_{sw}$  have been found to differ between species and growth environments. Positive relationships between  $g_m$  and  $g_{sw}$  have been found in rice (Centritto *et al.*, 2009), barley (Barbour *et al.*, 2010), tomato (Galmés *et al.*, 2011) and wheat genotypes (Barbour & Kaiser, 2016), but the two conductances were unrelated in the wheat study by Jahan *et al.* (2014). In the present study, low  $g_{sw}$  was more important in driving high  $A/g_{sw}$  than was high  $g_m$  across species, suggesting that increasing  $g_m$  might improve  $A/g_{sw}$  only if  $g_{sw}$  remains unchanged.

Regarding crop genetic improvement through  $g_m$ , Barbour *et al.* (2016a) reported the first hints of genetic control of mesophyll conductance in common wheat. Peguero-Pina *et al.* (2012) observed that *Abies alba* had both larger maximum  $A$  and  $A/g_{sw}$  than its closely related species, *A. pinsapo*, mostly due to larger  $g_m$  which, in turn, was dependent on differences in leaf anatomical traits. Carbonic anhydrase catalyses the reversible interconversion of  $CO_2$  with  $HCO_3^-$ , that differ in their diffusivities, pH and temperature dependencies (Flexas *et al.*, 2012a). CA contributes to  $g_m$  by maintaining a nearly constant  $CO_2$  concentration throughout the stroma,

allowing a more effective use of the available Rubisco (Tholen & Zhu, 2011, Flexas *et al.*, 2012a). Evidence for a correlation between CA and  $g_m$  have been found (Gillon & Yakir, 2000, Perez-Martin *et al.*, 2014), but genetic manipulation of CAs has resulted in small effects on  $g_m$  and  $A$  (reviewed in Flexas *et al.* (2016)). Further, there is evidence of genetic manipulation of  $g_m$  through altered aquaporin expression or activity (Hanba *et al.*, 2004, Uehlein *et al.*, 2008, Secchi & Zwieniecki, 2013, Sade *et al.*, 2014). In most cases, manipulation of aquaporins resulted in increased  $g_m$  and  $A$ , but with concomitant increases in  $g_{sw}$ , which cancelled any improvements of  $A/g_{sw}$ . However, Zhao *et al.* (2017) found very weak correlation between water and CO<sub>2</sub> permeability when measured using stopped flow on plasma membrane vesicles isolated from *Pisum sativum* and *Arabidopsis thaliana* leaves and suggested that aquaporins may facilitate CO<sub>2</sub> transport across plasma membranes, but probably via a different pathway than for water.

#### 4.5. Conclusion

I investigated the genotypic variability of  $\Delta^{13}\text{C}-g_m$  and  $\Delta^{18}\text{O}-g_m$  in important crop legume species.  $\Delta^{13}\text{C}-g_m$  relates to the total mesophyll conductance to CO<sub>2</sub> from the intercellular air spaces to the chloroplast stroma, while  $\Delta^{18}\text{O}-g_m$  relates to the conductance to CO<sub>2</sub> from the intercellular air spaces to the CO<sub>2</sub>-H<sub>2</sub>O equilibration, assumed to be at the chloroplast surface. Genotypic variability in  $\Delta^{13}\text{C}-g_m$  was found in faba bean, garden pea and field pea but not in common bean.  $\Delta^{18}\text{O}-g_m$  was mostly higher than  $\Delta^{13}\text{C}-g_m$  suggesting that CO<sub>2</sub> equilibrates with water outside the chloroplast for those genotypes. I did not find genetic variation in  $\Delta^{18}\text{O}-g_m$  across species. This suggests that genetic variation in  $g_m$  may be related to variation in chloroplast membrane conductance. The  $\Delta^{18}\text{O}$  technique has the potential to discern the relative importance of the  $g_m$  components before and after CO<sub>2</sub>-H<sub>2</sub>O equilibration, which will advance our understanding of the mechanism of  $g_m$  regulation. Future studies of genotypic variability in  $g_m$  should include measurement of temperature response of  $\Delta^{18}\text{O}-g_m$  or quantification and localization of CA activity to assess the location of CO<sub>2</sub>-H<sub>2</sub>O equilibration. Measurement of leaf anatomical properties including chloroplast thickness,  $S_{mes}$ ,  $S_c$  or membrane permeability to CO<sub>2</sub> through aquaporins would further help in assessing the importance of these traits on  $g_m$ .  $g_m$  was strongly associated with leaf photosynthetic rates, supporting the recognition of possibility of enhancing  $A$  through selection for increased  $g_m$ .  $g_m$  was significantly, though not strongly, related to  $A/g_{sw}$ , but not related to  $g_{sw}$  across genotypes within field pea, indicating that simultaneous

improvement of  $A$  and  $A/g_{sw}$  may be possible in field peas. However,  $g_{sw}$  had a stronger effect on leaf water-use efficiency than did  $g_m$  across species. Despite the complexity and a lack of complete understanding of the mechanistic basis of  $g_m$ , the presence of genotypic variation in  $g_m$  and the positive relationships between  $A$  and  $g_m$  (within and among species) and  $A/g_{sw}$  and  $g_m$  (in field peas) demonstrate the potential of improving photosynthetic rates and leaf intrinsic water use efficiency within a crop improvement program.



## 5. Mesophyll conductance and leaf hydraulic conductance are not correlated in faba bean

### 5.1. Introduction

Increasing crop yield, while improving water use efficiency (WUE), i.e., greater crop yield per unit water used, is one of the most important challenges for global food production under current and future climate conditions. Climate change predictions include altered precipitation patterns, rising temperatures and elevated CO<sub>2</sub>, which have markedly affected carbon and water balance and crop productivity (Postel, 2000, Rosenzweig & Hillel, 2008, Lobell *et al.*, 2011, Penuelas *et al.*, 2013, Bagley *et al.*, 2015). In addition, an interaction between climate change and other environmental factors may intensify the adverse impacts (Penuelas *et al.*, 2013, Xu *et al.*, 2016). How plants leaves exchange and regulate water and CO<sub>2</sub>, and carbon and water balance is crucial to improve crop productivity and WUE. Coordination of CO<sub>2</sub> and water exchange between the leaf and the atmosphere through the stomata is reasonably well understood (Collatz *et al.*, 1991, Davies *et al.*, 2002, Liu *et al.*, 2005). However, we still lack a clear and complete understanding of the regulation of CO<sub>2</sub> and water movement within the leaf, which may be useful information for the optimization of photosynthesis and WUE.

After diffusing into the leaf through stomata, CO<sub>2</sub> moves further from the substomatal cavities through intercellular air spaces then diffuses in the liquid phase through the mesophyll cell walls, plasma membrane, cytosol, and chloroplast envelope to finally reach the site of carboxylation inside the chloroplast stroma. The ease of CO<sub>2</sub> diffusion from the substomatal cavities to the stroma constitute mesophyll conductance to CO<sub>2</sub>, abbreviated as  $g_m$  (Evans *et al.*, 2009). Mesophyll conductance is now recognized as a significant and variable limitation to photosynthesis (Flexas *et al.*, 2008, Flexas *et al.*, 2012a). Increasing  $g_m$  will result in higher chloroplastic CO<sub>2</sub> concentration and hence higher photosynthetic rates ( $A$ ), without any effect on leaf transpiration rate, so may result in simultaneous increase in leaf intrinsic WUE ( $A/g_{sw}$ ) (Barbour *et al.*, 2010, Flexas *et al.*, 2010, Flexas *et al.*, 2013a).  $g_m$  varies widely between species or genotypes and in response to short term or long term changes in different abiotic factors (Flexas *et al.*, 2008, Barbour *et al.*, 2010, Barbour & Kaiser, 2016). The observed  $g_m$  variability may be due to the variation in leaf anatomical properties, particularly chloroplast surface area

facing intercellular air spaces per unit of leaf area ( $S_c$ ) and cell wall thickness ( $T_{cw}$ ) (Evans *et al.*, 1994, Tosens *et al.*, 2012a, Tosens *et al.*, 2012b, Tomás *et al.*, 2013) or due to changes in membrane permeability through plant aquaporins (Bernacchi *et al.*, 2002, Hanba *et al.*, 2004, Uehlein *et al.*, 2008). Aquaporins (AQPs) are water channel proteins from a larger family of integral membrane proteins that facilitate the transport of water across membranes of plant cells (Maurel & Chrispeels, 2001), but certain AQPs have also been shown to facilitate CO<sub>2</sub> transport across the plasma and chloroplast membranes (Terashima & Ono, 2002, Uehlein *et al.*, 2003, Kaldenhoff, 2012, Mori *et al.*, 2014, Maurel *et al.*, 2015, Uehlein *et al.*, 2017).

Water moves through the xylem and then through the leaf mesophyll following, possibly, four alternative extra-xylary pathways for water movement to the sites of evaporation: liquid diffusion through the apoplast (cell walls), liquid diffusion through the symplast (plasmodesmata), transcellular transport (across membranes via aquaporin) (Stedde *et al.*, 1993), and gas diffusion through the intercellular air spaces (Rockwell *et al.*, 2014, Buckley, 2015). Leaf hydraulic conductance ( $K_{leaf}$ ) is a measure of how efficiently water is transported within the leaf and includes the liquid water flow through xylem and extra-xylary components and vapour transport through internal air spaces (Sack & Holbrook, 2006, Buckley, 2015). Low  $K_{leaf}$  restricts water flow to the epidermis, which reduces stomatal conductance and photosynthesis (Brodribb & Holbrook, 2003, Locke & Ort, 2014).  $K_{leaf}$  has been shown to be highly variable, by more than 65-fold across species, and this variation has been associated with the leaf morpho-anatomy including the variation in leaf thickness, leaf vein architecture, as well as the extra-xylary pathways through mesophyll to the sites of evaporation (Sack & Holbrook, 2006, Xiong *et al.*, 2015b).  $K_{leaf}$  has also been found to be relatively dynamic in response to various internal and external leaf environment e.g. temperature, irradiance and water availability (Sack & Holbrook, 2006, Sellin & Kopper, 2007, Gortan *et al.*, 2009, Sack & Scoffoni, 2012) either due to anatomical differences (Sack & Frole, 2006, Prado *et al.*, 2013, Buckley *et al.*, 2015) or due to changes in aquaporins expression and activity affecting the regulation of the transcellular pathway (Cochard *et al.*, 2007, Baaziz *et al.*, 2012, Lopez *et al.*, 2013, Pou *et al.*, 2013a).

Within a leaf, CO<sub>2</sub> and water vapour might share portions of their diffusion pathways (Ferrio *et al.*, 2012). The outside xylem hydraulic conductance depends on mesophyll anatomy (e.g. leaf

thickness, airspace fraction, cell wall thickness) (Aasamaa *et al.*, 2005, Buckley *et al.*, 2015), which also influences  $g_m$ . Flexas *et al.* (2013b) observed a generally positive relationship between  $g_m$  and  $K_{leaf}$  across plant species. They showed that  $g_m$  and  $K_{leaf}$  have widely agreed trends in response to a number of environmental variables such as temperature, light level and water status. They suggested one reason for the observed coordination may be due to the shared pathway for CO<sub>2</sub> and water, most probably along the extra-xylary pathway of water. However, the Flexas *et al.* (2013b) suggestion was based on published studies measuring either  $g_m$  or  $K_{leaf}$  but not both  $g_m$  and  $K_{leaf}$ . After the review, a few studies have assessed the correlation between  $g_m$  and  $K_{leaf}$  by simultaneously measuring these two parameters. Theroux-Rancourt *et al.* (2014) found that  $K_{leaf}$  decreased in concert with  $g_{sw}$  with drought stress, whereas  $g_m$  remained relatively constant up to a threshold  $g_{sw}$  among poplar clones. On the other hand, (Xiong *et al.*, 2017) observed a linear correlation between  $g_m$  and  $K_{leaf}$  across rice cultivars and both the parameters were closely related to specific mesophyll structural traits such as cell wall thickness, fraction of leaf mesophyll volume occupied by intercellular air space, mesophyll and chloroplast surface area exposed to intercellular air space. More recently, Loucos *et al.* (2017) found a weak correlation between  $g_m$  and  $K_{leaf}$  in cotton when variation was driven by leaf anatomy due to differing growth conditions. Analysis of the relationship between  $g_m$  and  $K_{leaf}$  is still at an embryonic stage with conflicting results. Moreover, previous studies have shown that plant species or even genotypes differ in their response to changes in growth environments (Hanba *et al.*, 2002, Barbour & Kaiser, 2016, Fini *et al.*, 2016, Peguero-Pina *et al.*, 2016). More studies on a range of species are needed to confirm the generality of the proposed coordination between  $g_m$  and  $K_{leaf}$ .

In this study, I assessed the responses of  $g_m$  and  $K_{leaf}$  to changes in growth environment, created by varying growth irradiance and CO<sub>2</sub> partial pressure, to create differences in leaf anatomy, physiology and biochemistry. The main objectives of this study were to determine if  $g_m$  and  $K_{leaf}$  are correlated among faba bean (*Vicia faba* L.) genotypes grown under differing environments, and further, if  $K_{leaf}$  is correlated to the cell wall plus plasma membrane conductance in faba bean, as observed by Loucos *et al.* (2017) in cotton. I was also interested to see if  $g_m$  scales with  $A$  and  $A/g_{sw}$  under differing growth conditions.

## 5.2. Materials and methods

### 5.2.1. Plant material and growth conditions

The experiment was conducted in a controlled environment facility at the University of Sydney, Centre for Carbon Water and Food in Camden NSW, Australia. Seeds of five faba bean (*Vicia faba* L.) genotypes (PBA Rana, Cairo, PBA Warda, Doza and 220d) were obtained from the University of Sydney, I. A. Watson Grains Research Centre, Narrabri. The seeds were germinated in 7L pots filled with commercial potting mix supplemented with slow release fertilizer (Osmocote Exact, Scotts, NSW Australia). Plants were grown in two controlled environment rooms; with CO<sub>2</sub> partial pressure ( $p_a$ ) set at 60.4 and 101 Pa respectively, and the average measured values of  $p_a$  across the entire period were 66.4 and 126.8 Pa, respectively, within each room. Half of the plants in each room were covered in a shade-cloth to lower the irradiance levels for those plants, which produced photosynthetic photon flux density (PPFD) of approximately 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (low irradiance) and 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (high irradiance). Both rooms were set to a 16 h photoperiod, 27/19°C day/night temperature and 75% relative humidity (RH). The growth environments were set to be matched except for CO<sub>2</sub> and light, however it is likely that temperature and RH varied a little between the environments. Hence, there was a need to be careful with data interpretation. After emergence, the plants were thinned to two per pot and were well watered throughout the experiment. Five genotypes (five individual plants each) were completely randomized within each growth environment.

### 5.2.2. Simultaneous gas exchange and mesophyll conductance measurements

Five weeks after planting, leaf gas exchange, mesophyll conductance and leaf hydraulic conductance were measured under the four growth conditions. Five youngest fully expanded leaves per genotype per growth environment were used for gas exchange measurements using a LI-6400XT portable photosynthesis system (Li-Cor Biosciences, Lincoln, NE, USA) fitted with a custom-built leaf chamber with an area of 38cm<sup>2</sup> (Barbour *et al.*, 2007). The LI-6400XT was coupled to two laser absorption spectrometers; a Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) and a Picarro water vapour laser (L1102-I, Picarro Inc., Sunnyvale, CA, USA), as described in Chapter 3.

The leaf chamber conditions were controlled to provide CO<sub>2</sub> at a  $p_a$  of 60.4 Pa or 101 Pa (using the Li-Cor CO<sub>2</sub> mixer) and irradiance at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  or 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, matching the growth conditions. All the measurements were made at a leaf temperature of 25°C. The flow rate through the leaf chambers was maintained above 300  $\text{ml min}^{-1}$  to provide sufficient flow for the TDL (250  $\text{ml min}^{-1}$ ) and the Picarro (25  $\text{ml min}^{-1}$ ). The airstream into the cuvette was dried of H<sub>2</sub>O, and thus the humidity of the air leaving the chamber was entirely derived from transpiration. Leaves remained in the chamber until a steady transpiration rate had been achieved (approximately 30 minutes). LI-6400XT data, TDL data and Picarro data were synchronized by matching timestamps of the measurements. After measuring the water potential, described later in this chapter, leaves were photographed and leaf area within the chamber was calculated from the digitized images of the leaf using ImageJ (1.45 s, NIH, Bethesda, MD, USA). Gas exchange variables were recalculated with the corrected leaf area.

The TDL was calibrated using four calibration cylinders spanning the range in concentrations of the isotopologues of the leaf chamber inlet and outlet air streams. Absolute values of <sup>12</sup>CO<sub>2</sub>, <sup>13</sup>CO<sub>2</sub> and C<sup>18</sup>O<sup>16</sup>O were, respectively, 292.05, 3.17 and 1.20 ppm (for low concentrations), 483.93, 5.26 and 1.99 ppm (for high concentrations), 127.79, 1.39 and 0.52 ppm (for the lowest concentrations), 1132.43, 12.29 and 4.64 ppm (for the highest concentrations). Carbon isotope ratios are presented relative to the Vienna Pee Dee belemnite standard and oxygen isotope ratios relative to the VSMOW standard. The Picarro water vapour laser was calibrated using two standard water samples of known isotopic values of -14.6 and -1.5‰ that spanned the range in  $\delta^{18}\text{O}$  of leaf chamber inlet and outlet air streams, following the procedure detailed in Simonin *et al.* (2013).

Mesophyll conductance to CO<sub>2</sub> ( $g_m$ ;  $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ ) was estimated using the online carbon isotope discrimination ( $\Delta^{13}\text{C}$ ) method and oxygen isotope discrimination ( $\Delta^{18}\text{O}$ ) method as described in Chapter 3. CO<sub>2</sub>-H<sub>2</sub>O equilibrium was assumed to occur at the chloroplast surface, as suggested by Gillon and Yakir (2000), and the chloroplast membrane conductance ( $g_{\text{cm}}$ ) was calculated from the combined measurements of  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  as described in Chapter 3.

### 5.2.3. Leaf hydraulic conductance

Leaf hydraulic conductance ( $K_{\text{leaf}}$ ;  $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ ) was determined using the evaporative flux method (Sack *et al.*, 2002, Brodribb & Holbrook, 2003) calculated based on Ohm's Law;

$$K_{\text{leaf}} = E / (\Psi_{\text{stem}} - \Psi_{\text{leaf}})$$

where  $E$  is the transpiration rate in  $\text{mmol m}^{-2} \text{s}^{-1}$ ,  $\Psi_{\text{stem}}$  is the stem water potential in MPa and  $\Psi_{\text{leaf}}$  is the leaf water potential in MPa. When a steady transpiration rate was achieved at the conclusion of the gas exchange measurements, sample leaves were cut at the stem, removed from the leaf chamber and immediately wrapped in plastic film to measure  $\Psi_{\text{leaf}}$  using a Scholander pressure chamber (115, Soil Moisture Equipment, Santa Barbara, CA, USA).  $\Psi_{\text{stem}}$  was determined as the average water potential of the leaves immediately above and below the sample leaf. Leaves for stem water potential measurement were wrapped in plastic film and aluminium foil the night before measurement, and water potential was measured using the above-mentioned pressure chamber.

### 5.2.4. Leaf anatomy

Five youngest fully expanded leaves from each genotype and growth treatment were used to measure leaf anatomy. Leaf samples were cut to 2 by 3 mm size, avoiding the major vein. Samples were fixed in 5 ml of buffer containing 2.5% glutaraldehyde, 3% formaldehyde, 0.01% Triton X and 2.5% sucrose using 50mM phosphate buffer solution, pH 7.2, and vacuum infiltrated overnight for a period of 18 hours. Samples were then washed twice for 10 minutes each in the phosphate buffer, and then post-fixed in 1% osmium tetroxide for 2 hours. Fixed sections were rinsed three times for 10 minutes each in sterile water, and dehydrated in an ethanol series (15, 30, 50, 75, 80, 90, 95 and 100%) for 10 min each, followed by two further rinses in 100% ethanol. Samples were then embedded in an LR White Resin (London Resin Co., London, UK) series (diluted in ethanol), first in 20% LR White overnight, then in 40, 60 and 80% LR White for 2 hours each, followed by three 24-h periods in 100% LR White. Leaf sections were then placed in embedding moulds filled with LR White and oven baked at 60°C overnight in an oxygen free environment.

Embedded samples were sectioned at 0.5  $\mu\text{m}$  on an ultramicrotome and imaged at 40 $\times$  magnification on a light microscope (DM6000B, Leica Microsystems, Hesse, Germany) at the University of Sydney, the Australian Centre for Microscopy and Microanalysis in Camden NSW, Australia. A stitched image (using Adobe Photoshop; Adobe Systems Inc., San Jose, CA, USA), containing at least five vascular bundles, was obtained for each of three samples per leaf and analysed using ImageJ software (NIH, Bethesda, MD, USA). Leaf thickness, fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{\text{ias}}$ ), mesophyll surface area exposed to intercellular airspace ( $S_{\text{mes}}$ ), chloroplast surface area exposed to intercellular airspace ( $S_{\text{c}}$ ) were estimated as described in Evans *et al.* (1994) using a curvature correction factor of 1.43.

### **5.2.5. Statistical analysis**

Leaves measured from each growth treatment were considered individuals and not replicates to avoid pseudo-replication (since growth treatments were not replicated). Relationships between the parameters were graphed for the individual leaves within each genotype using GraphPad Prism (Version 6.07, GraphPad Software Inc). Linear relationships and correlations were considered statistically significant when  $p < 0.05$ .

## **5.3. Results**

### **5.3.1. Do genotypes differ in the degree to which leaf anatomy determines leaf internal conductances?**

Leaves from each growth treatment were considered individuals and the genotype averages of the measured variables and ANOVA are presented in Table 5.1 to demonstrate the general effects of the growth treatments on those variables. Growth environment significantly affected leaf anatomical properties including leaf thickness, surface area of mesophyll ( $S_{\text{mes}}$ ) and chloroplast exposed to the intercellular air spaces; ( $S_{\text{c}}$ ) but did not alter the volume fraction of intercellular air space ( $f_{\text{ias}}$ ) (Table 5.1). Overall, there was small but significant genotypic variability in leaf anatomy, nevertheless all the genotypes responded similarly to the growth environments (Table 5.1). There was a general trend of lower leaf thickness,  $S_{\text{mes}}$  and  $S_{\text{c}}$  at 101 Pa/200 PPFD and 60.4 Pa/200 PPFD compared to the leaf anatomy at 101 Pa/600 PPFD and 60.4 Pa/600 PPFD. There was also a considerable variability in leaf anatomical parameters between

the individual leaves within each genotype and growth conditions. I observed positive correlation between leaf thickness and  $S_{mes}$  for all the genotypes, except for 220d; between leaf thickness and  $S_c$ , except for PBA Warda and 220d; and between  $S_{mes}$  and  $S_c$  for all the genotypes.

Similarly, leaf internal conductances including mesophyll conductance to  $CO_2$  measured from the carbon isotope method ( $\Delta^{13}C-g_m$ ), mesophyll conductance measured from the oxygen isotope method ( $\Delta^{18}O-g_m$ ), chloroplast membrane conductance ( $g_{cm}$ ) as well as leaf hydraulic conductance ( $K_{leaf}$ ) showed significant variability among the growth environments (Table 5.1). Genotypes did not differ significantly for any of the leaf internal conductances across the growth conditions (Table 5.1). All the genotypes responded similarly to the growth conditions for the mesophyll conductance, with the highest measured values at 60.4 Pa/600 PPFD compared to other growth environmental conditions. Variation in  $K_{leaf}$  among the growth environment was significant for the genotypes PBA Warda and 220d.

Genotypes differed in the relationships between environmentally driven changes in leaf anatomy and leaf internal conductances (Figure 5.1, Figure 5.2, Figure 5.3, Figure 5.4), with some genotypes showing weak but significant relationships while other genotypes showed no relationships. Across growth environments, leaf thickness was positively related to  $\Delta^{13}C-g_m$  (Figure 5.1), to  $\Delta^{18}O-g_m$  (Figure 5.2) and to  $g_{cm}$  (Figure 5.3) for PBA Rana and Cairo, but the relationships were not significant for other genotypes. Similarly,  $S_{mes}$  and  $S_c$  were positively related to  $\Delta^{13}C-g_m$ ,  $\Delta^{18}O-g_m$  and  $g_{cm}$  only for Cairo, while  $g_m$  was not linked to  $S_{mes}$  or  $S_c$  in other genotypes.  $f_{ias}$  ranged from 0.4 to 0.8 but was not an important factor in explaining variation in  $g_m$ . None of the genotypes showed any relationships between leaf thickness,  $f_{ias}$  or  $S_c$  with  $K_{leaf}$  (Figure 5.4). However, the relationship between  $S_{mes}$  and  $K_{leaf}$  was significant for PBA Warda and 220d, but not for other genotypes. Overall, the present study showed that these environmentally driven leaf anatomical traits did not determine mesophyll conductance or leaf hydraulic conductance, except in some genotypes. Moreover, leaf anatomy was not the major factor determining  $g_m$  or  $K_{leaf}$  in the genotypes which showed positive relationships between leaf anatomical traits and the internal conductances.



### 5.3.2. Do genotypes differ in the importance of leaf anatomy on the relationships between $g_m$ and $K_{leaf}$ ?

There was no relationship between  $\Delta^{13}C-g_m$  and  $K_{leaf}$  or between  $\Delta^{18}O-g_m$  and  $K_{leaf}$  across the growth conditions for each genotype (Figure 5.5) or all the genotypes combined.  $\Delta^{13}C-g_m$  and  $K_{leaf}$  or  $\Delta^{18}O-g_m$  and  $K_{leaf}$  were also not correlated within a growth environment. Changes in  $\Delta^{13}C-g_m$  or  $\Delta^{18}O-g_m$  due to differing growth environments were not paralleled by changes in  $K_{leaf}$  across the genotypes. Variability in  $K_{leaf}$  among the growth treatments was much smaller than the variability in  $\Delta^{13}C-g_m$  or  $\Delta^{18}O-g_m$ .

### 5.3.3. Are the components of $g_m$ closely correlated?

Most of the  $\Delta^{18}O-g_m$  measurements were significantly higher than  $\Delta^{13}C-g_m$  measurements and the  $\Delta^{13}C-g_m$  and  $\Delta^{18}O-g_m$  were positively correlated (Figure 5.6). Assuming the  $CO_2-H_2O$  equilibrium occurred at the chloroplast surface,  $\Delta^{18}O-g_m$  relates to cell wall and plasma membrane conductance and thus, chloroplast membrane conductance ( $g_{cm}$ ) was determined as described in Chapter 3.  $\Delta^{18}O-g_m$  values were then plotted against  $g_{cm}$  across the growth environment for each genotype. In general, there was a positive correlation between  $\Delta^{18}O-g_m$  and  $g_{cm}$  measurements but the data points were highly scattered around the 1:1 line (Figure 5.6). The linear regression slopes and intercepts were not significantly different between the genotypes, with the pooled slope=0.78 and the pooled intercept=0.47.

### 5.3.4. The relationships between $g_m$ and other leaf gas exchange parameters

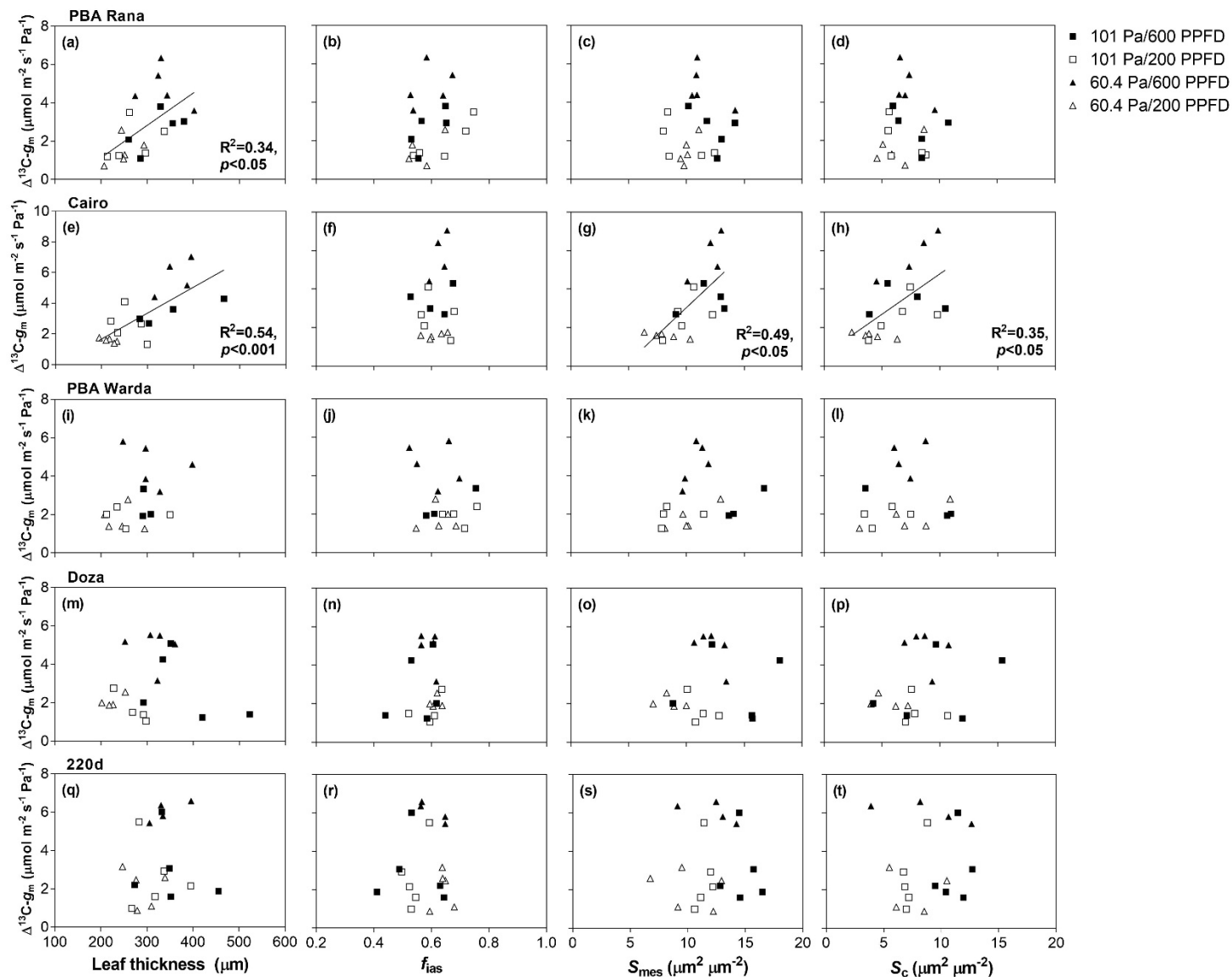
Growth environments significantly affected photosynthetic rate ( $A$ ,  $p<0.001$ ), stomatal conductance to water vapour ( $g_{sw}$ ,  $p<0.001$ ) and leaf intrinsic water use efficiency ( $A/g_{sw}$ ,  $p<0.001$ ), see Table 5.1.  $\Delta^{13}C-g_m$  was positively related to  $A$  ( $p<0.001$ , Figure 5.7A) and  $g_{sw}$  ( $p<0.001$ , Figure 5.7B) but not related to  $A/g_{sw}$  (Figure 5.7C) across the growth conditions and genotypes.  $K_{leaf}$  was very weakly correlated to  $A$  across growth conditions and genotypes ( $R^2 = 0.12$ ,  $p=0.02$ ), while the correlation was better at 60.4 Pa/600 PPFD environment ( $R^2 = 0.44$ ,  $p<0.001$ ).

**Table 5.1** Growth environment average (SE) of leaf gas exchange and leaf anatomical traits

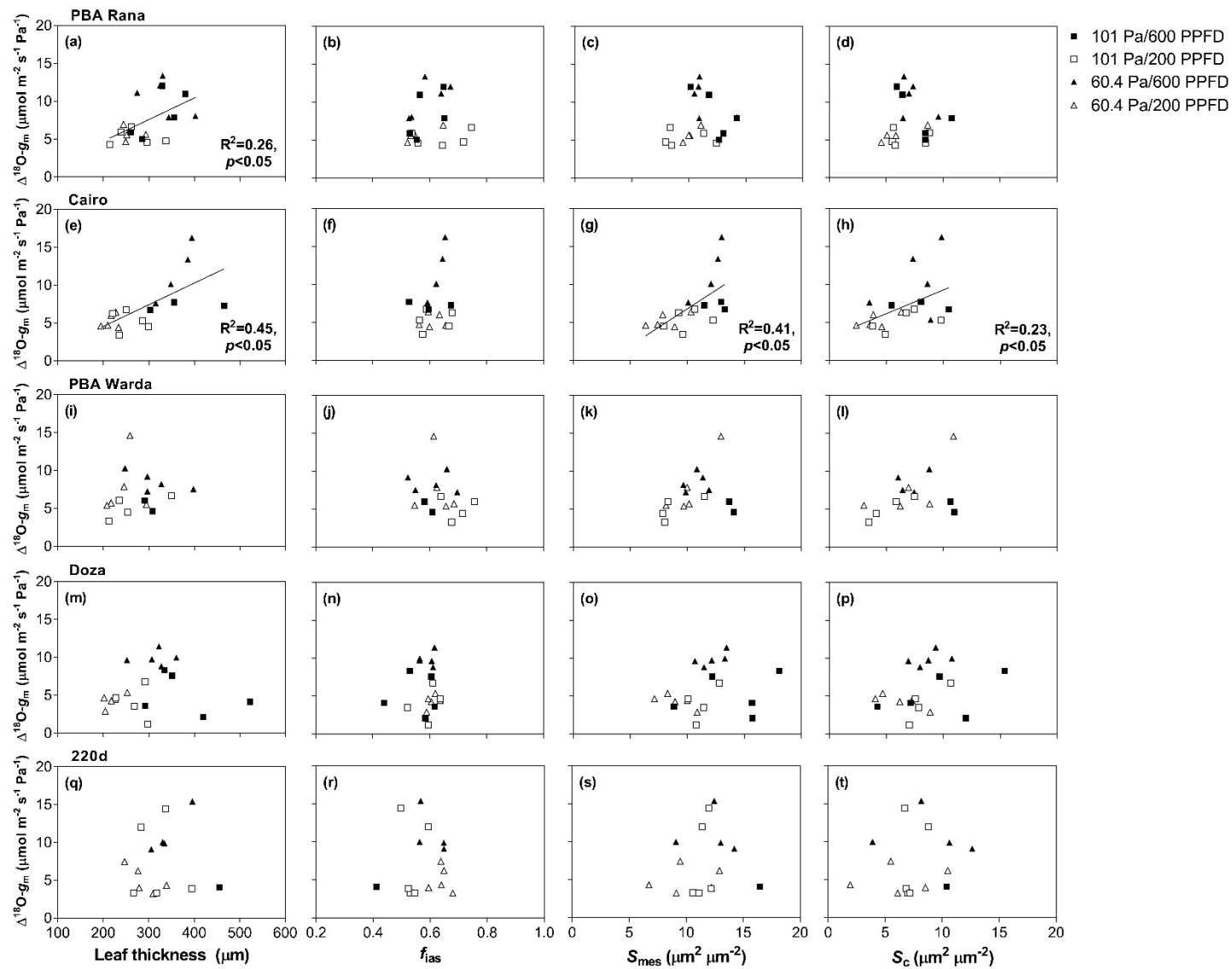
Growth environments	$A$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$g_{\text{sw}}$ ( $\text{mol m}^{-2} \text{s}^{-1}$ )	$A/g_{\text{sw}}$ ( $\mu\text{mol mol}^{-1}$ )	$\Delta^{13}\text{C}g_{\text{m}^{-2}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ )	$\Delta^{18}\text{O}g_{\text{m}^{-2}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ )	$g_{\text{cm}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1} \text{Pa}^{-1}$ )	$K_{\text{leaf}}$ ( $\text{mmol m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ )	leaf thickness ( $\mu\text{m}$ )	$f_{\text{ias}}$	$S_{\text{mes}}$ ( $\mu\text{m}^2 \mu\text{m}^{-2}$ )	$S_{\text{c}}$ ( $\mu\text{m}^2 \mu\text{m}^{-2}$ )
101 Pa/600 PPFD	22.8 (0.6)	0.4 (0.03)	59 (4)	2.8 (0.3)	6.3 (0.6)	5.1 (0.8)	22.6 (0.9)	344 (13)	0.6 (0.01)	13.3 (0.5)	8.6 (0.6)
101 Pa/200 PPFD	11.3 (0.9)	0.4 (0.02)	27 (2)	2.2 (0.2)	4.6 (0.3)	4.2 (0.5)	18.6 (0.7)	279 (9)	0.6 (0.01)	10.3 (0.3)	6.8 (0.3)
60.4 Pa/600 PPFD	22.7 (0.8)	0.6 (0.03)	41 (2)	5.0 (0.2)	10 (0.5)	10.8 (0.8)	19.9 (0.9)	330 (9)	0.6 (0.01)	11.8 (0.3)	8.0 (0.4)
60.4 Pa/200 PPFD	9.3 (0.2)	0.4 (0.03)	24 (2)	1.8 (0.1)	5.6 (0.5)	2.9 (0.3)	19.3 (0.7)	244 (7)	0.6 (0.01)	9.5 (0.3)	6.1 (0.5)
<b>ANOVA</b>											
Growth environments	***	***	***	***	***	***	**	***	ns	***	***
Genotypes	ns	ns	ns	ns	ns	ns	ns	(0.06)	*	*	*
Growth environments $\times$ genotypes	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Note:* Within a genotype, leaves from each treatment are considered individuals and not replicates. Photosynthetic rate ( $A$ ), stomatal conductance ( $g_{\text{sw}}$ ), leaf intrinsic water use efficiency ( $A/g_{\text{sw}}$ ), mesophyll conductance estimated from carbon isotope method ( $\Delta^{13}\text{C}g_{\text{m}^{-2}}$ ) and oxygen isotope method ( $\Delta^{18}\text{O}g_{\text{m}^{-2}}$ ), chloroplast membrane conductance ( $g_{\text{cm}}$ ), leaf hydraulic conductance ( $K_{\text{leaf}}$ ), fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{\text{ias}}$ ), Surface area of mesophyll ( $S_{\text{mes}}$ ) and chloroplast exposed to intercellular air space ( $S_{\text{c}}$ ). (n=15-20)

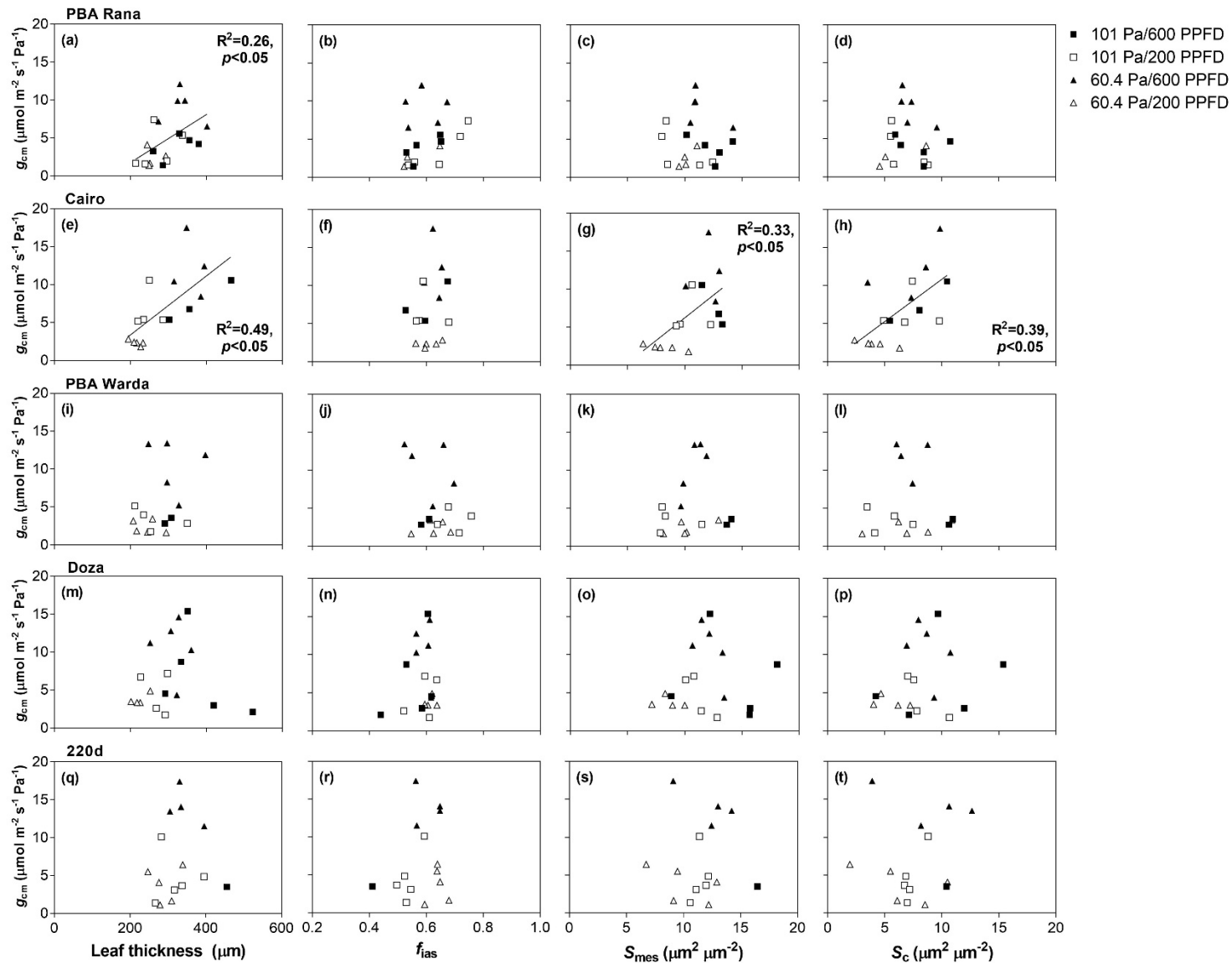
\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .



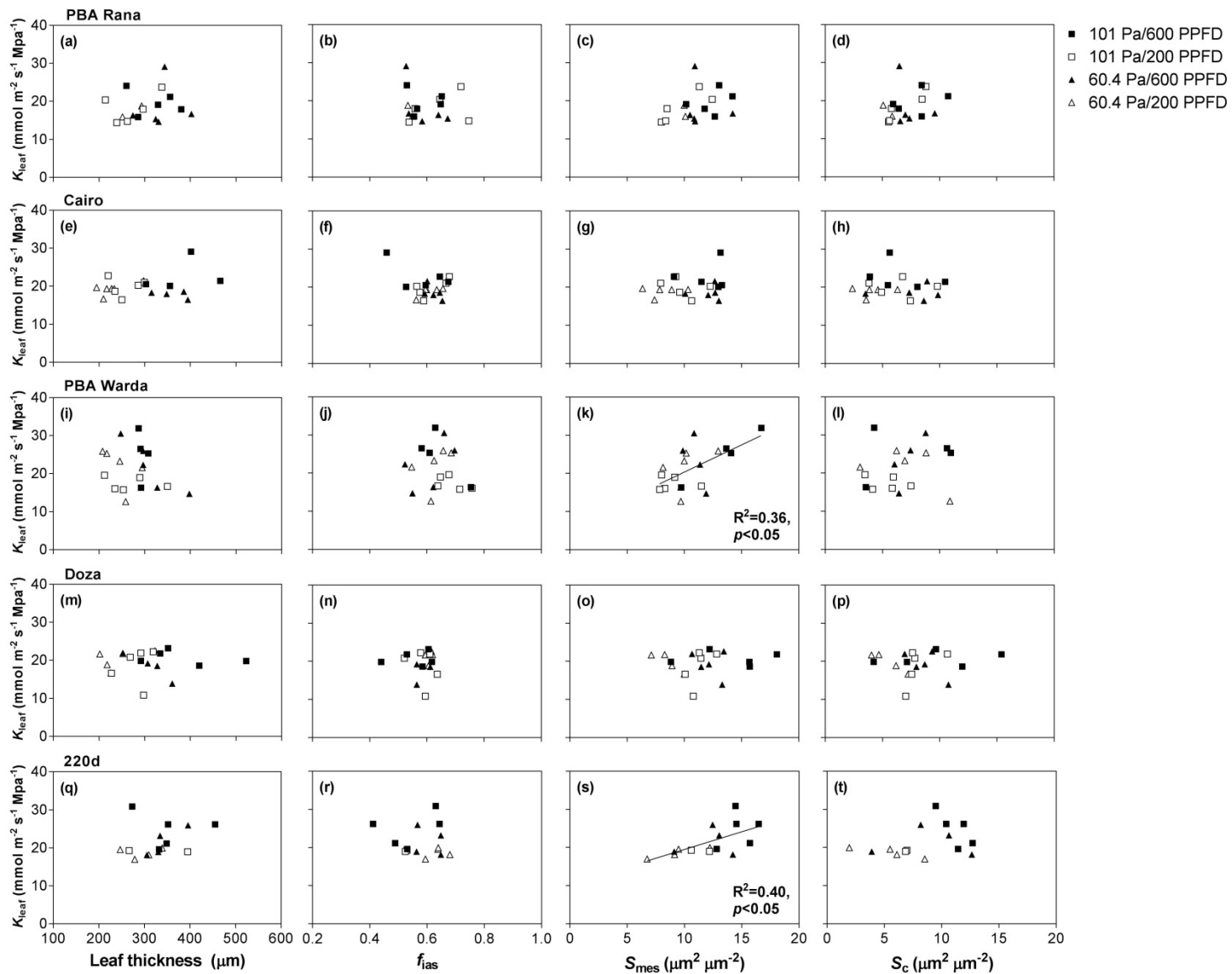
**Figure 5.1** Effects of leaf thickness, fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{ias}$ ), mesophyll cell surface area ( $S_{mes}$ ) and chloroplast surface area exposed to intercellular air space ( $S_c$ ) on mesophyll conductance to  $\text{CO}_2$  ( $\Delta^{13}\text{C-g}_m$ ) for individual leaves of five faba bean genotypes grown under differing environments ( $n = 15-20$ ). The solid lines indicate a significant linear regression.



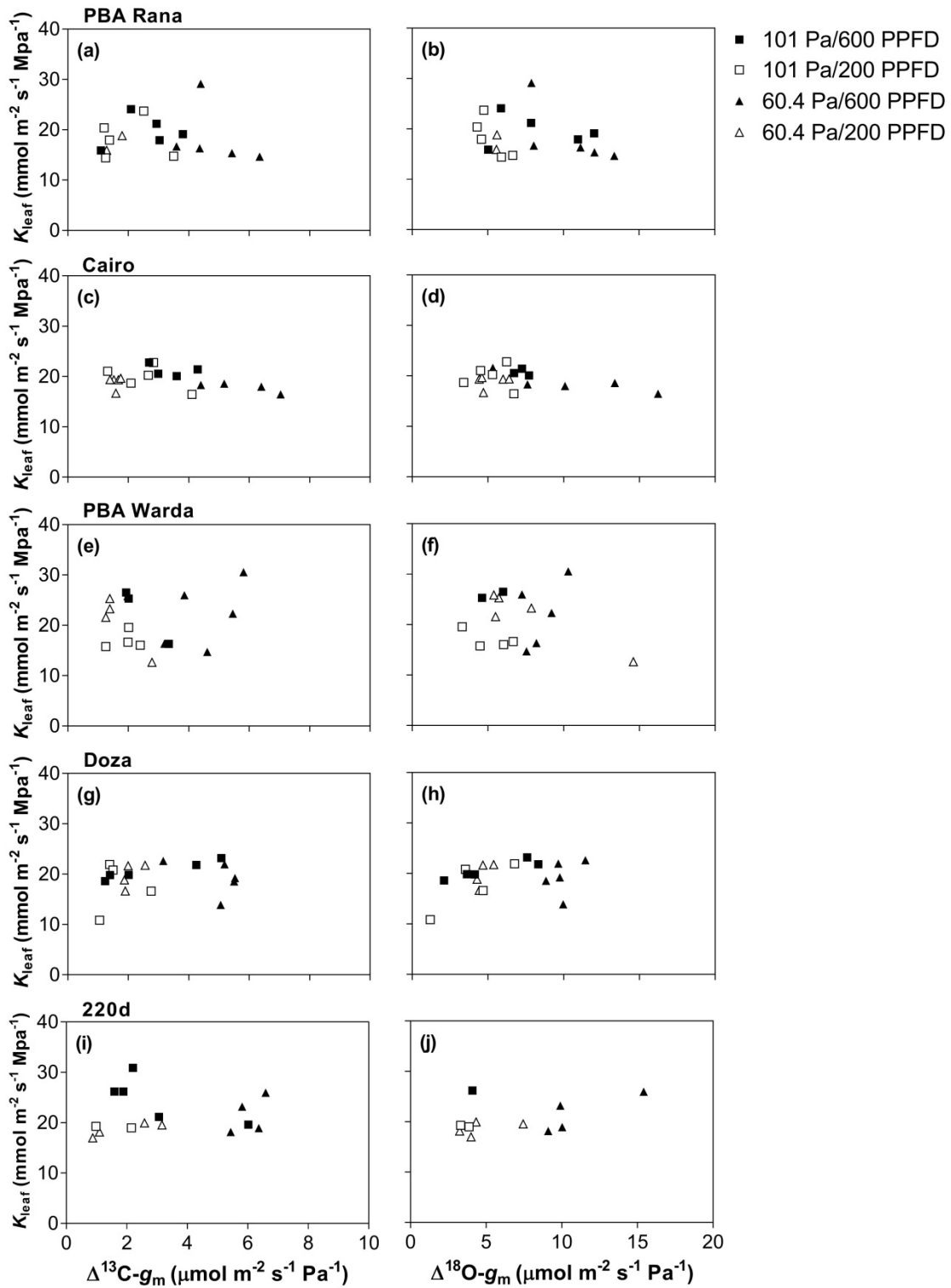
**Figure 5.2** Effects of leaf thickness, fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{ias}$ ), mesophyll cell surface area ( $S_{mes}$ ) and chloroplast surface area exposed to intercellular air space ( $S_c$ ) on mesophyll conductance estimated from oxygen isotope method ( $\Delta^{18}\text{Og}_m$ ) for individual leaves of five faba bean genotypes grown under differing environments (n = 15-20). The solid lines indicate a significant linear regression.



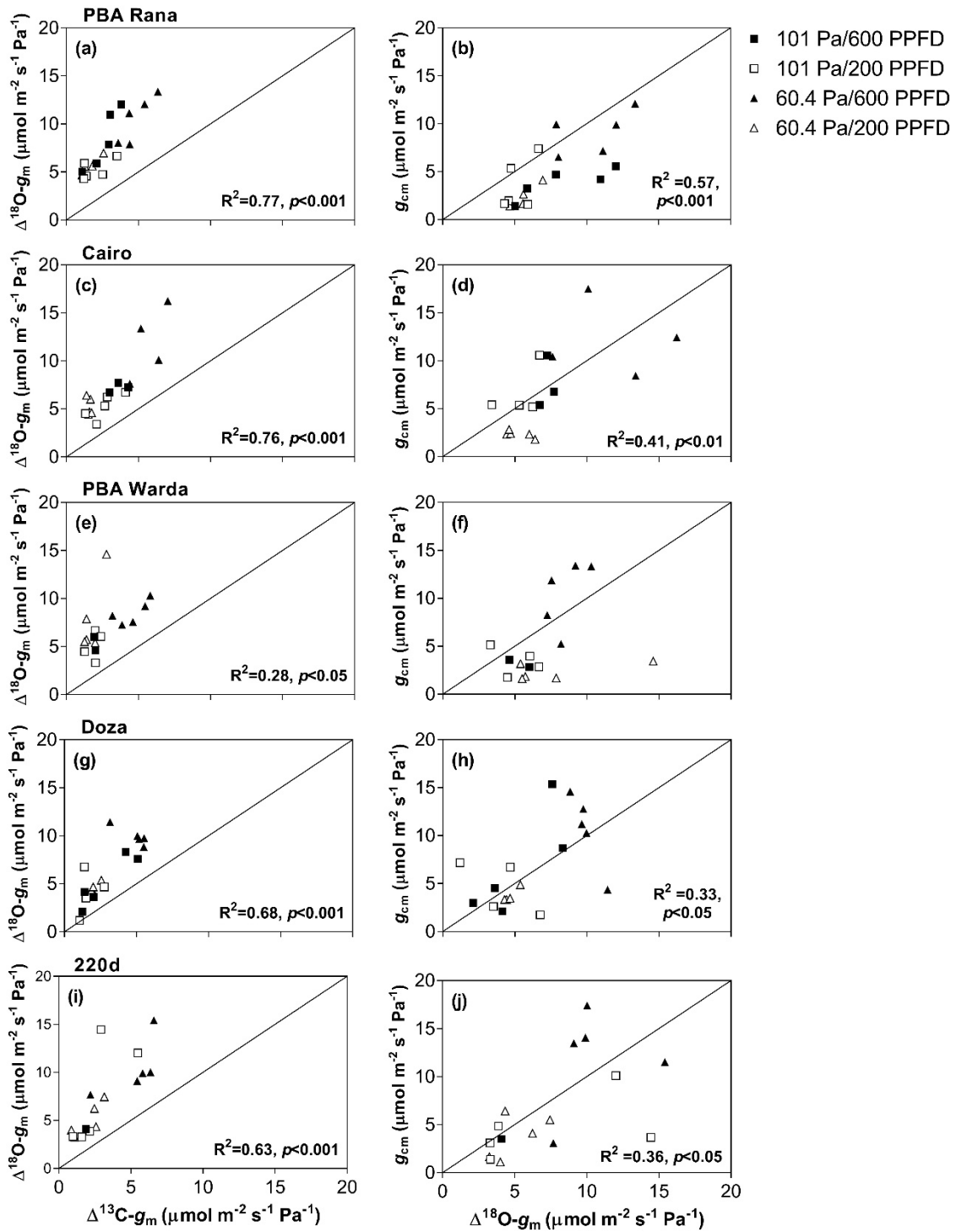
**Figure 5.3** Effects of leaf thickness, fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{ias}$ ), mesophyll cell surface area ( $S_{mes}$ ) and chloroplast surface area exposed to intercellular air space ( $S_c$ ) on chloroplast membrane conductance ( $g_{cm}$ ) for individual leaves of five faba bean genotypes grown under differing environments ( $n = 15-20$ ). The solid lines indicate significant linear regressions.



**Figure 5.4** Effects of leaf thickness, fraction of leaf mesophyll volume occupied by intercellular air space ( $f_{ias}$ ), mesophyll cell surface area ( $S_{mes}$ ) and chloroplast surface area exposed to intercellular air space ( $S_c$ ) on leaf hydraulic conductance ( $K_{leaf}$ ) for individual leaves of five faba bean genotypes grown under differing environments ( $n = 15-20$ ). The solid lines indicate significant linear regressions.

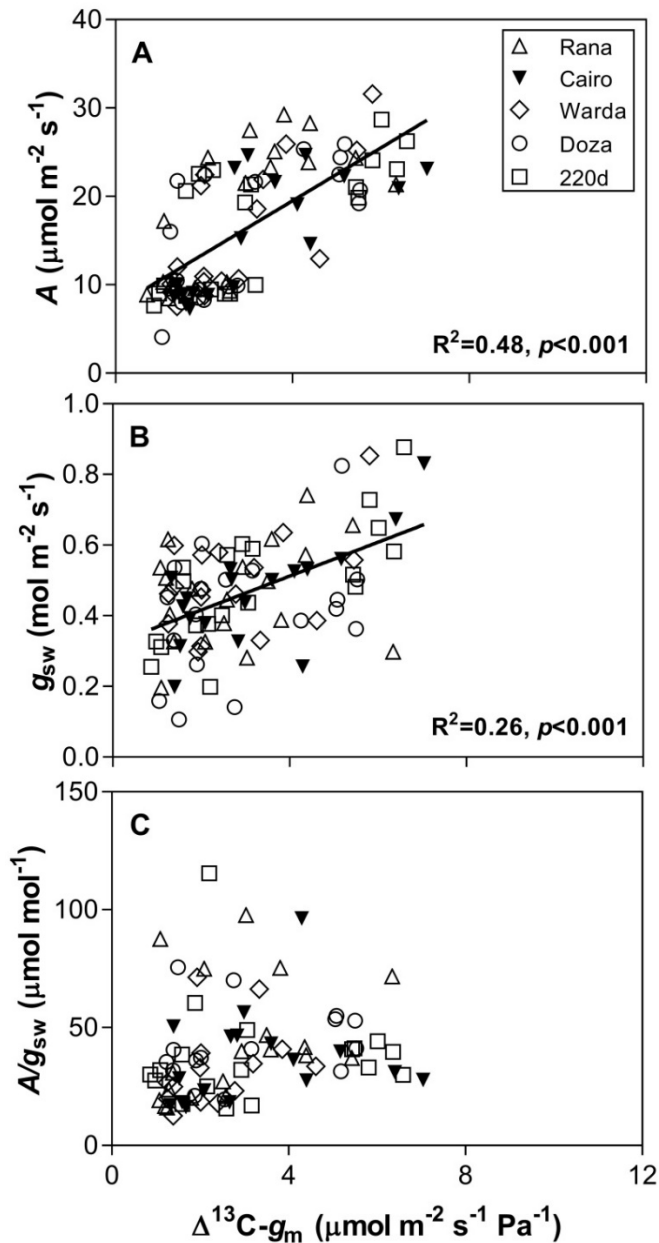


**Figure 5.5** Relationship between mesophyll conductance estimated from carbon isotope method ( $\Delta^{13}C-g_m$ ) and leaf hydraulic conductance ( $K_{leaf}$ ; a, c, e, g, i) and between mesophyll conductance estimated from oxygen isotope method ( $\Delta^{18}O-g_m$ ) and  $K_{leaf}$  (b, d, f, h, j) for individual leaves of five faba bean genotypes grown under differing environments (n = 15-20).



**Figure 5.6** Mesophyll conductance calculated using the  $\Delta^{13}\text{C}$  method ( $\Delta^{13}\text{C-g}_m$ ) plotted against mesophyll conductance calculated using the  $\Delta^{18}\text{O}$  method ( $\Delta^{18}\text{O-g}_m$ ; a, c, e, g, i) and chloroplastic membrane conductance ( $g_{cm}$ ; b, d, f, h, j) for five genotypes of faba bean. The solid line represents 1:1 line.





**Figure 5.7** The relationships of mesophyll conductance to CO<sub>2</sub> ( $\Delta^{13}\text{C-g}_m$ ) with photosynthetic rate ( $A$ , A), stomatal conductance to water vapour ( $g_{sw}$ ; B) and leaf intrinsic water use efficiency ( $A/g_{sw}$ ; C).

## 5.4. Discussion

### 5.4.1. Genotypes differed in the degree to which leaf anatomy influenced leaf internal conductances

In the present study, five genotypes of faba bean were grown under four different environmental conditions, created by varying CO<sub>2</sub> partial pressure and irradiance of the controlled growth rooms, in order to generate variation in leaf anatomy, physiology and biochemistry between the treatments. For each genotype, leaves measured from each growth

condition were considered individuals and not replicates to avoid pseudo-replication. I observed significant variability in leaf anatomical traits, mesophyll conductance to CO<sub>2</sub> and leaf hydraulic conductance among the growth environmental conditions. There is substantial evidence for adaptive and acclimation responses of  $g_m$  to growth environments such as irradiance, CO<sub>2</sub> partial pressure, water stress, salinity (Flexas *et al.*, 2008, Flexas *et al.*, 2012b). However, the mechanisms that regulate these variations are poorly understood.  $g_m$  is determined by a series of physical barriers between the intercellular air space and chloroplast stroma, and thus leaf anatomical traits are expected to partly explain the variation in  $g_m$  (Evans *et al.*, 2009). In the present study, genotypes differed in the extent to which leaf anatomy determines  $g_m$ . Leaf thickness,  $S_{mes}$  and  $S_c$  were significantly related to  $\Delta^{13}C-g_m$  and its components,  $\Delta^{18}O-g_m$  and  $g_{cm}$  across the growth environments for some genotypes but not for other genotypes.  $g_m$  has been found to correlate with leaf anatomical traits in several studies (Evans *et al.*, 1994, Scafaro *et al.*, 2011, Terashima *et al.*, 2011, Tholen & Zhu, 2011, Peguero-Pina *et al.*, 2012, Tosens *et al.*, 2012a, Xiong *et al.*, 2015a, Peguero-Pina *et al.*, 2016, Xiong *et al.*, 2017), but not in other studies (Evans & Vellen, 1996, Hanba *et al.*, 2001, Hanba *et al.*, 2002, Hanba *et al.*, 2004, Miyazawa *et al.*, 2008, Tomás *et al.*, 2014). The capacity to acclimate to different growth environments through changes in anatomical and physiological leaf traits may vary between plant species (Fini *et al.*, 2016). In the present study,  $g_m$  was not correlated with  $f_{ias}$  in any genotype, as reported by other studies (Evans, 2009, Tomás *et al.*, 2013), although recently, a relationship between the two was found significant in *Oryza* cultivars (Xiong *et al.*, 2017).  $f_{ias}$  measurements were higher than reported in other species, i.e. leaves are generally not densely packed, so these factors might be relatively less important in faba bean.

The results of this study suggest that the studied leaf anatomical traits are not the major factor determining  $g_m$  or its components in faba bean under the experimental conditions. The observed relationships in some genotypes were statistically significant but weak, implying that those leaf traits were not the only determining factor for  $g_m$ . The observed response of  $g_m$  to the growth conditions in this study may be due to the variation in other leaf traits like cell wall thickness or chloroplast thickness as observed in other studies (Tomás *et al.*, 2013, Muir *et al.*, 2014, Peguero-Pina *et al.*, 2016). In the present study, leaf N content was significantly higher in 101 Pa/600 PPFD and 60.4 Pa/600 PPFD than other growth conditions, and might have played a role in  $g_m$  response to the growth environments. Results from some studies indicate that  $g_m$  is determined not by a single trait, but rather by trait covariation (Tosens *et*

*al.*, 2012b, Giuliani *et al.*, 2013, Muir *et al.*, 2014).  $g_m$  may also be affected by cell wall composition (Hanba *et al.*, 2002, Flexas & Diaz-Espejo, 2015), or some biochemical factors affecting membrane permeability to CO<sub>2</sub>.  $g_m$  response to the growth environments could also be due to changes in aquaporin expression and/or activity along plasma membrane and/or chloroplast membrane (Bernacchi *et al.*, 2002, Hanba *et al.*, 2004).

The degree that mesophyll anatomy influences  $K_{leaf}$  is currently debated (Buckley *et al.*, 2015, Sack *et al.*, 2015). In the current study, faba bean genotypes differed in the relationship between  $S_{mes}$  and  $K_{leaf}$ , with some genotypes showing a weak but positive relationship across the growth environments but no link between the two in other genotypes. Xiong *et al.* (2017) also found significant relationship  $S_{mes}$  and outside xylem component of  $K_{leaf}$  ( $K_{ox}$ ), and suggested that if the liquid water evaporates at the mesophyll surface along transport pathway, then an increase in the evaporating surface area,  $S_{mes}$ , will increase  $K_{ox}$ . Nevertheless, the site of evaporation is still not clear (Pieruschka *et al.*, 2010, Buckley *et al.*, 2017), and recent modelling shows that water may evaporate deep within the leaf (cells near the vascular bundles) or near the stomata, and the sites of evaporation may depend on the resistances of different pathways through the tissues (Rockwell *et al.*, 2014, Buckley, 2015, Scoffoni, 2015).

Leaf thickness,  $S_c$  and  $f_{ias}$  were not related to  $K_{leaf}$  in any genotype of faba bean.  $K_{leaf}$  is composed of xylem and outside xylem components, and thus depends on multiple leaf structural traits. Water flow outside xylem is poorly understood. Once in the bundle sheath, water may move apoplastically (through the cell walls), symplastically (through plasmodesmata), or transcellularly (through aquaporins in cell membranes) (Steudle *et al.*, 1993) or through the intercellular air spaces in vapour phase (Buckley, 2015). The relative contribution of the xylem or outside xylem components to the total  $K_{leaf}$  as well as the relative contribution of different modes of water transport to flow outside the bundle sheath might affect the response of  $K_{leaf}$  to leaf anatomy (Buckley, 2015, Buckley *et al.*, 2015, Sack *et al.*, 2015). In contrast to the current study, a few recent studies (Buckley *et al.*, 2015, Xiong *et al.*, 2017) have found significant effect of  $f_{ias}$  on  $K_{leaf}$ . A theoretical analysis by Buckley *et al.* (2015) found negative mechanistic effects of vertical pathlength (including leaf thickness) on the outside xylem hydraulic conductance, but no significant correlation between leaf thickness and simulated  $K_{ox}$  across 14 diverse species, as the covariation of other traits compensated leaf thickness effects. Brodribb and Jordan (2011) reported that leaf thickness

could not explain the reduction in  $K_{\text{leaf}}$  in shade leaves of *Nothofagus cunninghamii* tree. Other studies (Sack *et al.*, 2013, Xiong *et al.*, 2015b) showed positive relationships between leaf thickness and  $K_{\text{leaf}}$ .

$K_{\text{leaf}}$  has also been shown to be related to regulation of aquaporins (Cochard *et al.*, 2007, Baaziz *et al.*, 2012). However Voicu *et al.* (2009) did not find correlation between light-induced  $K_{\text{leaf}}$  and aquaporins in bur oak. They suggested that light may induce large ionic fluxes in the vascular bundles of the leaf (Shabala *et al.*, 2002), which might influence xylem hydraulic conductance. Pou *et al.* (2013b) suggested that the role of aquaporin and the water pathway within a leaf also depends on the intensity and duration of stress.

#### **5.4.2. Mesophyll conductance to CO<sub>2</sub> and leaf hydraulic conductance are not related in faba bean**

Flexas *et al.* (2013b), a review, observed a general positive relationship between  $g_m$  and  $K_{\text{leaf}}$  across species under non-limiting conditions, and suggested potential coordination between  $g_m$  and  $K_{\text{leaf}}$  based on the shared pathway through the mesophyll cell walls and plasma membranes. Few published studies, following the review, have shown contrasting results (Theroux-Rancourt *et al.*, 2014, Loucos *et al.*, 2017, Xiong *et al.*, 2017). Our results did not show any relationships between  $\Delta^{13}\text{C}-g_m$  or  $\Delta^{18}\text{O}-g_m$  and  $K_{\text{leaf}}$  across growth environments in faba bean genotypes. Environmentally driven leaf anatomy effects on  $\Delta^{13}\text{C}-g_m$  or  $\Delta^{18}\text{O}-g_m$  were not parallel to the anatomy effects on  $K_{\text{leaf}}$ . For example, in this study,  $S_{\text{mes}}$  was related to  $g_m$  only in Cairo but was related to  $K_{\text{leaf}}$  only in PBA Warda and 220d. Overall,  $g_m$  and  $K_{\text{leaf}}$  did not share any of the studied anatomical determinants within each genotype. In contrary, Xiong *et al.* (2017) found a positive correlation between  $g_m$  and  $K_{\text{leaf}}$  within *Oryza* genus across cultivars mediated by leaf anatomical and structural features. In their study, leaf anatomy including  $f_{\text{ias}}$ , cell wall thickness,  $S_{\text{mes}}$  and  $S_c$  were significantly related to both  $g_m$  and  $K_{\text{leaf}}$  (outside xylem). More recently, Loucos *et al.* (2017) found a weak correlation between  $\Delta^{13}\text{C}-g_m$  and  $K_{\text{leaf}}$  in cotton (single genotype) when variation is driven by anatomy created by differing growth conditions (varying CO<sub>2</sub> partial pressure and irradiance). Furthermore, Loucos *et al.* (2017) found a positive correlation between  $\Delta^{18}\text{O}-g_m$  and  $K_{\text{leaf}}$  under both short-term environmental variation and growth conditions, suggesting a coordination between  $g_m$  and  $K_{\text{leaf}}$  across cell wall and plasma membrane. In contrast, measurements of  $\Delta^{18}\text{O}-g_m$  in faba bean in the current study did not show a correlation between cell and plasma membrane conductance and  $K_{\text{leaf}}$ .

Although mesophyll conductance for water and CO<sub>2</sub> did not respond similarly to the growth conditions and  $g_m$  and  $K_{leaf}$  were not correlated in faba bean genotypes in this study, coupling between water and CO<sub>2</sub> pathways or regulation may occur in response to other environmental variables or in other species. Theroux-Rancourt *et al.* (2014) found a linear relationship between  $g_m$  and  $K_{leaf}$  as drought developed in poplar, but the timing of the changes in  $g_m$  and  $K_{leaf}$  were not coincident in that study, supporting the hypothesis of a partial hydraulic isolation of the mesophyll from the main transpiration pathway. Ferrio *et al.* (2012) demonstrated a strong linear relationship between  $g_m$  and  $K_{ox}$  and the effective path length ( $L$ ) for water transport from xylem vessels to the sites of evaporation in grapevines only when  $K_{leaf}$  fell below certain threshold point as a result of water stress, while  $g_m$  and  $K_{ox}$  were not related above this point. Therefore, in the latter two studies, we can see that  $g_m$  and  $K_{leaf}$  were not conclusively correlated.

The lack of correlation between  $g_m$  and  $K_{leaf}$  in this study may suggest an independent regulation of CO<sub>2</sub> and water in faba bean under the growth conditions. Aquaporins are the most abundant protein belonging to membrane intrinsic proteins family that facilitate transport of water as well as dissolved gases such as CO<sub>2</sub> (Maurel *et al.*, 2015, Groszmann *et al.*, 2017, Uehlein *et al.*, 2017). However, the role of aquaporins in membrane CO<sub>2</sub> transport is yet to be fully and conclusively determined. Recently, Zhao *et al.* (2017) measured water and CO<sub>2</sub> permeability ( $P_{os}$ ,  $P_{CO2}$ ) using stopped flow spectrofluorimetry on plasma membrane vesicles isolated from *Pisum sativum* and *Arabidopsis thaliana* leaves and found a weak positive correlation between  $P_{os}$  and  $P_{CO2}$ . They suggested that aquaporins may facilitate CO<sub>2</sub> transport across plasma membranes, but probably via a different pathway than water since inhibitors of  $P_{os}$  did not alter  $P_{CO2}$ . Recently, von Caemmerer and Evans (2015) confirmed that the temperature response of  $g_m$  differed greatly between species, emphasizing the lack of complete understanding of the mechanistic bases of  $g_m$  responses. Likewise, water movement pathways outside the xylem are complex and poorly understood, and potentially vary strongly across species due to anatomical variation including vein length per unit area and mesophyll anatomy (Sack & Holbrook, 2006, Buckley *et al.*, 2015). The location of the phase change between liquid and vapour is still not clear (Pieruschka *et al.*, 2010, Buckley *et al.*, 2017). The differences in results for  $g_m$  and  $K_{leaf}$  correlation between studies could also be due to the variation in the hydraulic conductance outside the xylem. Whether the lack of the relationship in our study is due to the isolation of the CO<sub>2</sub> and water pathway or due to the independent regulation of CO<sub>2</sub> and water (e.g. through aquaporins) is uncertain.

### 5.4.3. Are the components of $g_m$ closely correlated?

In the present study, total mesophyll conductance measured from the carbon isotope method ( $\Delta^{13}\text{C}-g_m$ ) was partitioned into conductance from the intercellular air space to the site of  $\text{CO}_2\text{-H}_2\text{O}$  isotopic equilibrium ( $\Delta^{18}\text{O}-g_m$ ) and conductance from  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium to the chloroplast stroma, using the two isotope methods (Barbour *et al.*, 2016b).  $\Delta^{18}\text{O}-g_m$  measurements were significantly higher than  $\Delta^{13}\text{C}-g_m$  measurements across genotypes and growth environments, indicating that  $\text{CO}_2\text{-H}_2\text{O}$  isotopic equilibration did not occur in the chloroplast and thus the partitioning technique is feasible in faba bean under the growth conditions. Assuming the  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium occurred at the chloroplast surface (as assumed by Gillon and Yakir (2000)), total mesophyll conductance was partitioned into cell wall and plasma membrane conductance ( $\Delta^{18}\text{O}-g_m$ ) and chloroplast membrane conductance ( $g_{cm}$ ). Previously, two studies (Barbour *et al.*, 2016b, Loucos *et al.*, 2017) had partitioned  $\Delta^{13}\text{C}-g_m$  into  $\Delta^{18}\text{O}-g_m$  and  $g_{cm}$  on different crop species. However, as Barbour *et al.* (2016b) highlighted, the interpretation of  $\Delta^{18}\text{O}-g_m$  should be made cautiously. CA has been localized to the chloroplast, the cytosol, the mitochondria and the plasma membrane in  $\text{C}_3$  plants (Fabre *et al.*, 2007), which means that  $\text{CO}_2\text{-H}_2\text{O}$  isotopic equilibration can occur at the plasma membrane and  $\Delta^{18}\text{O}-g_m$  could relate to conductance through the cell wall only. On the other hand, if CA activity within the leaf is mostly in the chloroplast, then  $\text{CO}_2$  would be fully equilibrated with chloroplastic water only, and the partitioning technique would not be feasible. Further, low carbonic anhydrase activity could result in incomplete equilibrium between  $\text{CO}_2$  and  $\text{H}_2\text{O}$  leading to an underestimation of  $\Delta^{18}\text{O}-g_m$  (Barbour *et al.*, 2016b).

When compared across growth environments, cell wall/plasma membrane conductance and chloroplast membrane conductance varied similarly with growth environmental changes across faba bean genotypes. In contrast, Loucos *et al.* (2017) observed higher sensitivity of chloroplast membrane conductance to growth environment in a single cotton genotype. Differences in the results between the studies might be due to species-related differences. In the current study,  $S_{mes}$  and  $S_c$  were weakly but significantly related to  $g_m$  components only in Cairo, while overall, variation in leaf anatomical traits did not explain the observed variation in  $g_m$  and its components, suggesting the  $g_m$  response to the growth environment might reflect the changes in both plasma membrane and chloroplast membrane permeability.

Simultaneous measurement of  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  is a promising area of research towards a better understanding of the relative magnitude of  $g_m$  determinants. There are very few studies examining the responsiveness of  $g_m$  components to different environmental condition.

More studies on the covariation of  $\Delta^{18}\text{O}$ - $g_m$  and  $g_{cm}$  on a range of species and short term and long term environmental conditions are needed, and assessment of the location and activity of carbonic anhydrase would assist in correctly interpreting the results.

#### **5.4.4. Water use efficiency under the growth conditions**

Improving water use efficiency has been a primary target for plant breeders and physiologists under the global climate change scenario. In line with these previous studies on elevated  $\text{CO}_2$  (Ainsworth & Rogers, 2007, Leakey *et al.*, 2009), I observed the highest photosynthetic rates and leaf WUE (due to low  $g_{sw}$ ) for plants grown at elevated  $\text{CO}_2$ /high irradiance environment followed by ambient  $\text{CO}_2$ /high irradiance conditions. There has been growing interest in  $g_m$  for increasing photosynthesis and leaf water use efficiency. I observed a positive relationship between photosynthetic rate and mesophyll conductance across the growth environments and genotypes, as reviewed in Flexas *et al.* (2008). In the present study,  $g_m$  was related to  $g_{sw}$  but not related to  $A/g_{sw}$ . Manipulation of aquaporins has been shown to result in increased  $g_m$  and  $A$ , but the concomitant increase in  $g_{sw}$  may cancel any improvement in  $A/g_{sw}$  (reviewed in Flexas *et al.* (2016)). Several studies have shown a correlation between  $g_m$  and  $g_{sw}$  (Flexas *et al.*, 2002, Flexas *et al.*, 2008, Centritto *et al.*, 2009, Barbour *et al.*, 2010, Galmés *et al.*, 2011, Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016), prompting suggestions of co-regulation (Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016) while some studies did not find a correlation between  $g_m$  and  $g_{sw}$  (Bunce, 2009, Jahan *et al.*, 2014). Several studies have highlighted that the combination of low  $g_{sw}$  and high  $g_m$  would produce high water-use efficiency (Barbour *et al.*, 2010, Buckley & Warren, 2014, Cano *et al.*, 2014, Flexas *et al.*, 2016). Our results suggest that  $g_m$  plays an important role in regulating photosynthetic capacity of faba beans under varying growth conditions. However, stomatal conductance has a stronger effect on leaf intrinsic water-use efficiency than mesophyll conductance under the growth conditions.

#### **5.5. Conclusions**

Mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ) and leaf hydraulic conductance ( $K_{leaf}$ ) are two important leaf variables that influence  $\text{CO}_2$  and water transport within the leaf respectively and thus have potential to improve plant productivity. Variation in  $g_m$  and  $K_{leaf}$  was measured simultaneously across faba bean genotypes grown and measured under differing conditions. Our results showed that leaf anatomy,  $g_m$  and  $K_{leaf}$  underwent modification in response to changes in growth environments but genotypes differed in the degree to which variation in

leaf anatomy explained variation in  $g_m$  or  $K_{leaf}$ . Further, the effects of environmentally driven leaf anatomy were not parallel between  $g_m$  and  $K_{leaf}$ . Nevertheless, the changes in leaf anatomical traits could not explain the observed variability in the leaf internal  $CO_2$  and  $H_2O$  conductance, which implies that other leaf traits including biochemical changes might be involved. As opposed to a review across species and published studies within a genus or species and different environments, I did not find any correlation between  $\Delta^{13}C$ - $g_m$  and  $K_{leaf}$  or between  $\Delta^{18}O$ -  $g_m$  and  $K_{leaf}$  across the growth conditions in faba bean genotypes. The differences in results for  $g_m$  and  $K_{leaf}$  correlation between studies could be related to species specific differences in  $g_m$  and  $K_{leaf}$  regulation. The lack of the relationships in our study might be due to the isolation of the  $CO_2$  and water pathways or due to the independent regulation of  $CO_2$  and water. Further research could focus on understanding the water pathways and its regulation, particularly outside xylem and on unravelling the mechanistic basis of  $g_m$  regulation (which can vary within and among species) to further understand if and to what extent  $CO_2$  and water coordinated within a leaf. Moreover,  $g_m$  was related to photosynthetic rates, but the co-regulation of  $g_m$  and  $g_{sw}$  might imply a trade-off between photosynthesis and WUE under the growth conditions.



## 6. Response of mesophyll conductance to abiotic factors in chickpeas

### 6.1. Introduction

Mesophyll conductance to CO<sub>2</sub> ( $g_m$ ), which regulates the diffusion of CO<sub>2</sub> from sub-stomatal cavities to the carboxylation site, is now recognized as a significant and variable limitation to photosynthesis (Flexas *et al.*, 2008, Flexas *et al.*, 2012a), and affects water and nitrogen use efficiencies (Barbour *et al.*, 2010, Buckley & Warren, 2014, Flexas *et al.*, 2016).  $g_m$  is a combination of gaseous diffusion through the intercellular airspaces and diffusion in the liquid phase through the mesophyll cell walls, plasma membrane, cytosol and chloroplast envelope to chloroplast stroma (Evans *et al.*, 2009) and thus may be determined by complex traits including leaf anatomical and biochemical properties. Understanding the  $g_m$  response to environmental and physiological changes is imperative for incorporating  $g_m$  into photosynthesis models (which previously regarded  $g_m$  as infinite) and thus to accurately predict the carboxylation rate (Niinemets *et al.*, 2009a). The effects of different environmental factors on  $g_m$  have been reported in several important crop species and are reviewed by Flexas *et al.* (2013).

Light is the driving force of photosynthesis, but light intensity and light quality vary widely in both time and space. It is therefore important to understand whether plants have the ability to dynamically regulate the CO<sub>2</sub> diffusion through the mesophyll in response to short-term changes in light intensity. Positive relationships between the  $g_m$  and light intensity have been observed in several species (Gorton *et al.*, 2003, Flexas *et al.*, 2007, Douthe *et al.*, 2011, Douthe *et al.*, 2012, Xiong *et al.*, 2015a) but not in others (Tazoe *et al.*, 2009, Yamori *et al.*, 2010). There has been speculation that rapid change in  $g_m$  with light intensity is a methodological artefact (Tholen *et al.*, 2012, Gu & Sun, 2014). The two most commonly used methods for estimating  $g_m$  are (i) gas exchange in combination with <sup>13</sup>C isotope discrimination (Evans *et al.*, 1986), and (ii) gas exchange in combination with chlorophyll fluorescence (Harley *et al.*, 1992). Both methods rely on models for the calculation of  $g_m$  and are sensitive to variation in the values of the model parameters (Pons *et al.*, 2009). Evans (2009) used a multilayer leaf model to suggest that an apparent dependence of  $g_m$  (calculated from fluorescence measurements) on irradiance or CO<sub>2</sub> may be produced if CO<sub>2</sub> concentration at the sites of carboxylation within chloroplasts ( $C_c$ ) varies with depth through the mesophyll. Douthe *et al.* (2012) reported that the  $g_m$  response to irradiance is unlikely to

be a computation artefact since using different values for the parameters of the discrimination model did not affect the relative response to irradiance in *Eucalyptus* species, except for  $^{13}\text{C}$ – $^{12}\text{C}$  fractionation during carboxylation (b).

The mechanism of  $g_m$  responses to rapid changes in environment is not yet clear but could be due to the changes in membrane permeability through  $\text{CO}_2$ -transporting aquaporin or carbonic anhydrase (Hanba *et al.*, 2004, Perez-Martin *et al.*, 2014) or changes in chloroplast size and position (Tholen *et al.*, 2008, Li *et al.*, 2012). Positional movements of chloroplasts are known to be influenced by blue light (Kagawa & Wada, 2002, Banaś *et al.*, 2012). Chloroplasts accumulate at illuminated cell areas under weak light while they move away from blue light under strong light. This avoidance response to blue light reduces chloroplast surface area facing intercellular air spaces ( $S_c$ ) and might thus reduce  $g_m$ . A few studies have examined the effect of light colour on  $g_m$  (Loreto *et al.*, 2009, Pallozzi *et al.*, 2013) and found that the exposure to blue light rapidly reduces  $g_m$ . However, they suggested that this  $g_m$  reduction was faster than any possible chloroplast redistribution and thus might be related to aquaporin.

Recent work has shown that  $g_m$  response to environmental factors can be genotype- and species-dependent. von Caemmerer and Evans (2015) demonstrated that short-term response of  $g_m$  to temperature differs greatly between species, with some species showing a strong response while others showing almost no change. Barbour and Kaiser (2016) found genotypic variation in the  $g_m$  response to nitrogen and water availability in wheat. Genotypic variation in  $g_m$  has been observed in other important crop species (Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014, Tomás *et al.*, 2014, Olsovska *et al.*, 2016). Moreover, plants might respond to multiple stresses differently to their response to an individual stress. In a study by Xiong *et al.* (2015a), rapid responses of  $g_m$  to changes of  $\text{CO}_2$  concentration, temperature and light intensity were affected by nitrogen supplements in rice, and  $g_m$  was more sensitive to these factors in plants with high nitrogen than with low nitrogen. Galle *et al.* (2009) showed that  $g_m$  response to drought in tobacco depends on the prevailing environmental conditions. Under prolonged water stress, they observed long-lasting decline of  $g_m$  even during the water stress acclimation period in outdoor plants in summer while  $g_m$  recovered completely in plants in the growth chamber. Thus, it is important to determine if the relationship between  $g_m$  and light intensity holds under different growth conditions and for different genotypes.

Generally, grain legumes have received less attention than cereals in studies of  $g_m$  regulation. Grain legumes are the primary affordable sources of proteins and minerals and thus play a key role in human nutrition, particularly in developing countries (Broughton *et al.*, 2003, Graham & Vance, 2003, Foyer *et al.*, 2016, Temba *et al.*, 2016). These crops also fix relatively large amounts of atmospheric nitrogen, playing an important role in enhancing the productivity and potential sustainability of farming systems (Evans *et al.*, 1991, Howieson *et al.*, 2000, Graham & Vance, 2003, Crews & Peoples, 2005, Peoples *et al.*, 2009, Foyer *et al.*, 2016). Chickpea (*Cicer arietinum* L.) is the second most important legume crop in the world and third most important pulse crop (Kaur *et al.*, 2016). The global demand for chickpea is projected to be 18.3 million tons in 2050 compared with a supply of 9.4 million tons in 2010 (Krishnamurthy *et al.*, 2013). Grain legumes including chickpea are mostly grown as intercrop with cereals, and so share the same environments. Legumes are sensitive to reduced light level and often suffer due to shading caused by the associated crop in the intercropping system (Akhter *et al.*, 2009). The collective yield losses due to abiotic stresses (6.4 million tonnes) are somewhat higher than due to biotic stresses (4.8 million tonnes), as estimated by Ryan (1997). Among the abiotic stresses, drought is almost ubiquitous to major chickpea growing regions. Drought leads to a 40-50% reduction in yield globally (Ahmad *et al.*, 2005). Moreover, approximately 90% of world's chickpea is grown rain-fed (Kumar & Abbo, 2001) and is cultivated mostly in the arid and semiarid regions of the world (Kashiwagi *et al.*, 2015). Yields can be improved by increasing water use efficiency, which is lower for chickpeas than for other cool season pulses such as field pea and faba bean (Knight, 2000). Water stress has often been shown to reduce  $g_m$  (Flexas *et al.*, 2008), but recent studies have shown that  $g_m$  is less sensitive than stomatal conductance ( $g_{sw}$ ), particularly during moderate water stress (Bunce, 2009, Flexas *et al.*, 2010, Theroux-Rancourt *et al.*, 2014). Chickpeas can derive their nitrogen requirements from symbiotic nitrogen fixation (Lodeiro *et al.*, 2000). However, nitrogen fixation has a higher energetic cost compared to soil mineral N uptake and assimilation (Andrews *et al.*, 2009). Lodeiro *et al.* (2000) compared drought tolerance in nitrogen-fixing and inorganic nitrogen-grown common beans but the response of  $g_m$  to nitrogen source has not been examined to date.

Here, I conducted two experiments on different genotypes of chickpeas to understand their responses to growth environment. The first experiment investigated the interactive effects of genotype and water availability on the response of  $g_m$  to short-term changes in light intensity and light quality. The second experiment examined the interactive effects of genotype and

nitrogen source (biologically fixed or inorganic-N-supplied) on the response of  $g_m$  to rapid changes in light intensity. I also assessed the influence of  $g_m$  on leaf intrinsic water-use efficiency in both experiments.

## **6.2. Materials and Methods**

### **6.2.1. Plant material and experimental arrangements**

#### ***Experiment 1: Water availability***

I used three chickpea genotypes: Amethyst (kabuli type), PBA Slasher and Sonali (desi type) for the first experiment. Seeds were obtained from the University of Sydney, I. A. Watson Grains Research Centre, Narrabri. Seeds were germinated in 7 L pots filled with commercial potting mix supplemented with slow release fertilizer (Osmocote Exact, Scotts, NSW, Australia). Plants were grown in a controlled-environment growth cabinet at the University of Sydney, Centre for Carbon, Water and Food (Camden, NSW, Australia). The growth cabinet was set to 25/17°C day/night temperature, 75% relative humidity, 700  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density at plant height and 14 h photoperiod. After emergence, the plants were thinned to two per pot and were well-watered until two watering treatments were imposed. The pots in each watering treatment (3 genotypes  $\times$  3 pots/6 replicate plants) were arranged in a completely randomized design. The watering treatment was imposed at 18 days after planting (DAP) when all the plants were at the vegetative stage: (i) one-half of the plants were kept well-watered by daily watering (WW); and (ii) the other-half were exposed to water stress (WS) by withholding water until the first sign of temporary leaf wilting point. Leaf water potential ( $\Psi_{\text{leaf}}$ ) of upper fully expanded leaves was measured to monitor the water-stress intensity using a Scholander pressure chamber (115, Soil Moisture Equipment, Santa Barbara, CA, USA) and following the precautions recommended by Turner (1988). The measurements were performed on lateral branches for each genotype.

At the temporary wilting point (7 days after the start of the water stress treatment), average leaf water potentials for WW and WS plants were -0.6 and -1.2 MPa respectively, i.e. the WS plants were moderately stressed. The weight of each WS pot at this point was designated as the target weight for the pot. The soil moisture content of the WS pots was maintained gravimetrically throughout the measurement period by weighing each pot daily at one hour after the start of the light period and adding water to replace water transpired and evaporated.

### ***Experiment 2: Nitrogen source***

The second experiment was carried out with three chickpea genotypes; Flip079C (kabuli type) and the two desi types used in the first experiment, PBA Slasher and Sonali. The study was conducted in a controlled growth room with environmental condition similar to Experiment 1 except photosynthetic photon flux density was  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$  at plant height. Plants were grown in 7 L pots, filled with washed river sand (N-free media) and lined with approximately 2.5 cm of gravel on the bottom of the pots. Five seeds were sown per pot and thinned to two seedlings per pot after 2 weeks. The two nitrogen source treatment was (i) inoculation with *Rhizobium* spp. without mineral N supply (N-fixing) and (ii) 2.5mM  $\text{NH}_4\text{NO}_3$  supplied (N-fed). One-half of the seeds of each genotype (N-fixing treatment) were inoculated with a peat-based Nodule N *Rhizobium* immediately before sowing, while the other half (N-fed) were uninoculated. The plants in both N treatments were provided with quarter-strength Herridge N-free mineral nutrient solution (Herridge, 1977):  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  250  $\mu\text{M}$ , KCL 250  $\mu\text{M}$ ,  $\text{KH}_2\text{PO}_4$  125  $\mu\text{M}$ ,  $\text{K}_2\text{HPO}_4$  125  $\mu\text{M}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  500  $\mu\text{M}$ , FeEDDHA 25  $\mu\text{M}$  and Trace Elements 25  $\mu\text{M}$  ( $\text{H}_3\text{BO}_3$  2.86  $\text{mg L}^{-1}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  1.81  $\text{mg L}^{-1}$ ,  $\text{ZnCl}_2$  0.11  $\text{mg L}^{-1}$ ;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  0.05  $\text{mg L}^{-1}$ ;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.025  $\text{mg L}^{-1}$ ).

For the first 10 days after planting,  $\text{KNO}_3$  0.5mM was included in the nutrient solution as “starter nitrogen” to help the plants establish until the inoculated plants were nodulated. Plants in both the treatments received the same nutrient solution during this period. All the pots were then flushed with pure water to wash away any nitrogen residues from the media. Thereafter,  $\text{NH}_4\text{NO}_3$  2.5mM was included in the nutrient solution only for the  $\text{NH}_4\text{NO}_3$ -fed plants while the  $\text{N}_2$ -fixing plants received the N-free solution. The pots in each N treatment (3 genotypes  $\times$  3pots/6 replicate plants) were placed on separate benches to avoid mixing of the throughfall waters and contamination of uninoculated pots. All the plants were watered with the nutrient solution in excess to avoid water stress at all times.

### **6.2.2. Simultaneous gas exchange and mesophyll conductance measurements**

#### ***Experiment 1:***

Leaf gas exchange and mesophyll conductance measurements were conducted 5 weeks after planting. A LI6400XT portable photosynthesis system (Li-Cor Biosciences, Lincoln, NE, USA), equipped with a custom-built leaf chamber of area  $38 \text{ cm}^2$  (Barbour *et al.*, 2007) and red-green-blue light source (Li6400 18A), was coupled to a Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) as

describe in Chapter 3. Two calibration cylinders were used to calibrate the TDL. Absolute values of  $^{12}\text{CO}_2$ ,  $^{13}\text{CO}_2$  and  $\text{C}^{18}\text{O}^{16}\text{O}$  were, respectively, 287.07 ppm, 3.28‰ and 1.18‰ for low concentrations and 477.57 ppm, 5.44‰ and 1.94‰ for high concentrations. The leaf chamber conditions were controlled to provide  $\text{CO}_2$  concentration of  $400 \mu\text{mol mol}^{-1}$ , flow rate of  $500 \mu\text{mol s}^{-1}$  and a leaf temperature of  $25^\circ\text{C}$  for all the measurements. For the leaf responses to rapidly changing light intensity and light colour, simultaneous leaf gas exchange and isotopic discrimination measurements were made in the order 950, 700 and  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ , sequentially under red light and then under blue light. The blue light had a peak emission at 457 nm, with a range from 424 to 524 nm, while the red light peak emission was centred at 636 nm, ranging from 584 to 661 nm. The leaves remained in the chamber for at least 15 minutes at each ‘light intensity-colour’ step. The measurements were made for both the well-watered and water-stressed plants. The uppermost fully expanded leaves of the primary branches were used for each set of measurements. For the leaves not covering the entire leaf chamber, leaf area within the chamber was calculated from the digitized images of the leaf using ImageJ (NIH, Bethesda, MD, USA) and the gas exchange variables were recalculated with the corrected leaf area. Mesophyll conductance was estimated with an online carbon isotope discrimination method using the experimental set-up as described in Chapter 3. Leaf water potential ( $\Psi_{\text{leaf}}$ ) was measured for all leaves immediately after gas exchange measurements.

### ***Experiment 2:***

Leaf gas exchange and mesophyll conductance measurements were performed as for Experiment 1, except that the light colour was set to 10% blue and 90% red. The rapid response of gas exchange and  $g_m$  was measured by varying the light intensity within the leaf chamber in the order 1000, 800, 600, 400,  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The measurements were made for plants in both N treatments. Along the entire set of measurements,  $\text{CO}_2$  concentration in the sample cell was maintained at  $400 \mu\text{mol mol}^{-1}$ , flow rate at  $500 \mu\text{mol s}^{-1}$  and leaf temperature at  $25^\circ\text{C}$ .

### **6.2.3. Crop traits**

For Experiment 2, the youngest fully expanded leaf samples were collected after the gas exchange measurements and were oven dried at  $65^\circ\text{C}$  for 72 hours. Samples were then ground to a fine powder and analysed for total N content (N%),  $^{15}\text{N}$  composition and  $^{13}\text{C}$  composition using isotope ratio mass spectrometry (Delta V, Thermo Fisher Scientific,

Bremen, Germany). The plants were harvested, cleaned of root media and roots were washed. Roots and nodules were separated and oven dried at 65 °C for 72 hours for measurement of dry weight. The proportion of N derived from N-fixation (%Ndfa) for the N-fed plants was determined using the  $\delta^{15}\text{N}$  Natural Abundance Method (Unkovich *et al.*, 2008).

$$\%Ndfa = \frac{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of } N_2 \text{ fixing legume}}{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of } N_2} \times \frac{100}{1}$$

where  $\delta^{15}\text{N}$  of  $N_2$  fixing legume represents the  $\delta^{15}\text{N}$  value of the non-inoculated legume supplied with  $\text{NH}_4\text{NO}_3$ , and  $\delta^{15}\text{N}$  of  $N_2$  is the  $\delta^{15}\text{N}$  value of the inoculated legume grown with atmospheric  $N_2$  as the sole source of N.  $\delta^{15}\text{N}$  of soil N ( $\text{NH}_4\text{NO}_3$  fertilizer supplied to N-fed plants) was estimated using isotope ratio mass spectrometry.

#### 6.2.4. Statistical analyses

Significant differences between values were assessed using general analysis of variance, as implemented by GenStat 14th edition (VSN International Ltd, London, UK), and means were compared using Fisher's Unprotected least significant difference test. Differences were considered statistically significant when  $p < 0.05$ . Regression lines were compared using a general linear regression procedure.

### 6.3. Results

#### 6.3.1. Response of leaf gas exchange and $g_m$ to growth and measurement conditions

##### *Experiment 1*

Net  $\text{CO}_2$  assimilation rate ( $A$ ) was significantly affected by dynamic changes in light intensity, light colour and by water availability (Table 6.1, Figure 6.1). The mean value of  $A$  significantly declined with the decrease in light intensity across genotypes, but I found significant interactive effects of light intensity by light colour ( $p < 0.05$ ) and by water stress ( $p < 0.001$ ). Sensitivity to light intensity was greater for the WW plants measured under red light than for the WS plants or when the plants were measured under blue light. Switching from red light to blue light reduced  $A$  at all light intensities in both WW and WS plants across genotypes. I observed genotypic variation in the response of  $A$  to water stress ( $p < 0.001$ ). Sonali was more responsive to water stress than the other two genotypes. In PBA Slasher, water stress reduced  $A$  only when measured under red light, while Amethyst was not affected

under any light environment. Light intensity and light colour did not affect stomatal conductance (Figure 6.1). However, WS plants had significantly lower  $g_{sw}$  than the WW plants across genotypes and the light treatments. Water stress lowered leaf water potential,  $\Psi_{leaf}$  ( $p < 0.001$ ). The average  $\Psi_{leaf}$  for WW and WS plants were -0.66 and -1.32 MPa respectively, but I did not find any differences between genotypes.

Light intensity, light colour and water availability significantly affected  $g_m$  (Figure 6.1).  $g_m$  declined with the decrease in light intensity at each light colour for both WW and WS plants across three genotypes (Table 6.1). When genotypes and treatments were analysed separately, the linear relationships between  $g_m$  and light intensity (regression fitted to the individual data) were significant at each light colour and water treatment except for the WS plants in PBA Slasher and Sonali when measured under blue light (data not shown). Blue light reduced  $g_m$  across the genotypes and water treatments (Table 6.1). However, when genotypes and treatments were separately analysed, the reduction of  $g_m$  under blue light was not significant at lower light intensities in Amethyst and for WS plants in Sonali (Figure 6.1).

Similarly, there was a significant interactive effect of genotype by water stress by light colour ( $p = 0.008$ ) for  $g_m$ . Water stress reduced  $g_m$  only in Sonali when measured under red light.  $g_m$  was unaffected by water availability under blue light in Sonali and under any light colour in the other two genotypes. Under WW conditions, significant differences among the genotypes were found for  $A$ ,  $g_{sw}$  and  $g_m$ , and Sonali had the highest  $A$ ,  $g_{sw}$  and  $g_m$  compared to the other genotypes. Under WS conditions, differences among the genotypes were non-significant for  $A$  and  $g_{sw}$ , while average  $g_m$  was the lowest for Sonali. PBA Slasher had the highest leaf intrinsic WUE ( $A/g_{sw}$ ) but the difference among the genotypes was statistically significant only under WS conditions. Overall, genotypes differed in their interactive response to water stress, light intensity and light colour, and Sonali was more sensitive to growth and measurement conditions.

## ***Experiment 2***

Photosynthetic rate ( $A$ ) was significantly affected by rapid changes in light intensity and by nitrogen source, and there was a significant light intensity by nitrogen source effect for  $A$ ,  $p = 0.001$  (Table 6.2, Figure 6.2). The mean value of  $A$  significantly declined with the decrease in light intensity across genotypes, but not when light was decreased from 1000 to 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . N-fixing plants had lower  $A$  than N-fed plants only at higher light intensities from 600



$\mu\text{mol m}^{-2} \text{s}^{-1}$  in Flip079C and from  $800 \mu\text{mol m}^{-2} \text{s}^{-1}$  in PBA Slasher and Sonali, and were unaffected at lower intensities. There was a genotype by light intensity effect for  $g_{\text{sw}}$  ( $p=0.014$ ).  $g_{\text{sw}}$  declined with decreasing light intensity for PBA Slasher and Flip079C but Sonali was unaffected. Overall average  $g_{\text{sw}}$  was higher for N-fixing plants than for N-fed plants but the differences were not significant for each genotype.

Light intensity and nitrogen source significantly affected  $g_{\text{m}}$ .  $g_{\text{m}}$  declined with a decrease in light intensity across the genotypes and nitrogen treatments (Table 6.2, Figure 6.2);  $g_{\text{m}}$  values at  $300 \mu\text{mol mol}^{-1}$  were significantly lower than the values at  $800$  or  $1000 \mu\text{mol mol}^{-1}$ . When individual genotypes were separately analyzed,  $g_{\text{m}}$  sensitivity to light intensity was altered by Nitrogen source in Flip079C and Sonali but not in PBA Slasher. The linear relationships between  $g_{\text{m}}$  and light intensity (regression fitted to the individual data) were significant for N-fed plants of each genotype (Flip079C:  $p<0.001$ , PBA Slasher:  $p<0.05$  Sonali:  $p<0.001$ ), while in N-fixing plants, the linear relationship between  $g_{\text{m}}$  and light intensity was significant only for PBA Slasher ( $p<0.001$ ) but not for the other two genotypes. Similarly, there was a significant interactive effect of genotype by nitrogen source ( $p<0.05$ ) for  $g_{\text{m}}$ . Nitrogen source did not affect  $g_{\text{m}}$  for PBA Slasher and Sonali across different light intensity. However, N-fixing Flip079C plants had significantly lower  $g_{\text{m}}$  values than N-fed Flip079C plants when data from all the light intensities were pooled together ( $p<0.05$ ) as well as at higher light intensities. Genotypes differ significantly in their photosynthetic capacity as in Experiment 1. Sonali had higher overall average  $A$ ,  $g_{\text{m}}$  and  $A/g_{\text{sw}}$  than the other genotypes and PBA Slasher had higher  $g_{\text{sw}}$ .

### 6.3.2. Correlations among $g_{\text{m}}$ , photosynthetic parameters and leaf N content

When genotype and treatment averages were calculated, both  $g_{\text{m}}$  and  $g_{\text{sw}}$  were positively related to  $A$ , and the correlation between  $A$  and  $g_{\text{m}}$  (Experiment 1:  $p<0.001$ ,  $R^2=0.82$  and Experiment 2:  $p<0.0001$ ,  $R^2=0.64$ ; Figure 6.3, Figure 6.4) was stronger than the correlation between  $A$  and  $g_{\text{sw}}$  (Experiment 1:  $p<0.0001$ ,  $R^2=0.43$  and Experiment 2:  $p<0.006$ ,  $R^2=0.24$ ). In Experiment 1, the regression lines were also separately fitted to the WW and WS plants.  $A$ - $g_{\text{m}}$  relationships for both WW ( $p<0.0001$ ,  $R^2=0.82$ ) and WS plants ( $p<0.0001$ ,  $R^2=0.85$ ) were stronger than  $A$ - $g_{\text{sw}}$  relationships for WW ( $p<0.01$ ,  $R^2=0.52$ ) and WS plants ( $p<0.001$ ,  $R^2=0.45$ ). In Experiment 2,  $A$  was more closely related to  $g_{\text{m}}$  ( $p<0.0001$ ,  $R^2=0.68$ ) than to  $g_{\text{sw}}$  ( $p<0.0192$ ,  $R^2=0.35$ ) for N-fed plants, while  $A$  was more strongly related to  $g_{\text{sw}}$  ( $p<0.0001$ ,  $R^2=0.73$ ) than to  $g_{\text{m}}$  ( $p<0.0014$ ,  $R^2=0.56$ ) for N-fixing plants. There was no relationship

between  $g_m$  and  $g_{sw}$  in either experiment ( $p>0.05$ ).  $g_m$  was positively correlated with  $A/g_{sw}$  for both WS ( $p<0.0001$ ,  $R^2=0.81$ ) and WW plants ( $p=0.0002$ ,  $R^2=0.59$ ), when genotype and light treatment averages were calculated.

Leaf N content (%N), calculated for Experiment 2, was affected by the nitrogen treatments ( $p<0.001$ ) and was significantly lower for N-fixing (4.6%) than for N-fed plants (6.5%). The relationships between %N and  $A$  were positive when all the data were pooled together ( $p<0.0001$ ,  $R^2=0.51$ ) or when averages were calculated for each nitrogen treatment ( $p=0.02$  for N-fed and  $p=0.002$  for N-fixing) (Figure 6.5). I did not find any relationship between  $g_m$  and %N (Figure 6.5).

### 6.3.3. Leaf carbon and nitrogen isotope composition

Leaf carbon ( $\delta^{13}C$ ) and nitrogen isotope ( $\delta^{15}N$ ) compositions were measured for Experiment 2. There was a significant interactive effect of genotype by nitrogen source for  $\delta^{13}C$  ( $p=0.015$ ). N-fixing plants had lower  $\delta^{13}C$  than N-fed plants for Flip079C and PBA Slasher but not for Sonali (Figure 6.6). Across the nitrogen treatments, genotype average  $\Delta^{13}C$  (assuming  $\delta^{13}C_{air}$  was  $-14\text{‰}$ ) in the leaf dry matter was not related to genotype averages of  $A/g_{sw}$ , the ratio of intercellular to ambient  $CO_2$  partial pressure ( $C_i/C_a$ ) and the ratio of chloroplastic to ambient  $CO_2$  partial pressure ( $C_c/C_a$ ) (Figure 6.6).

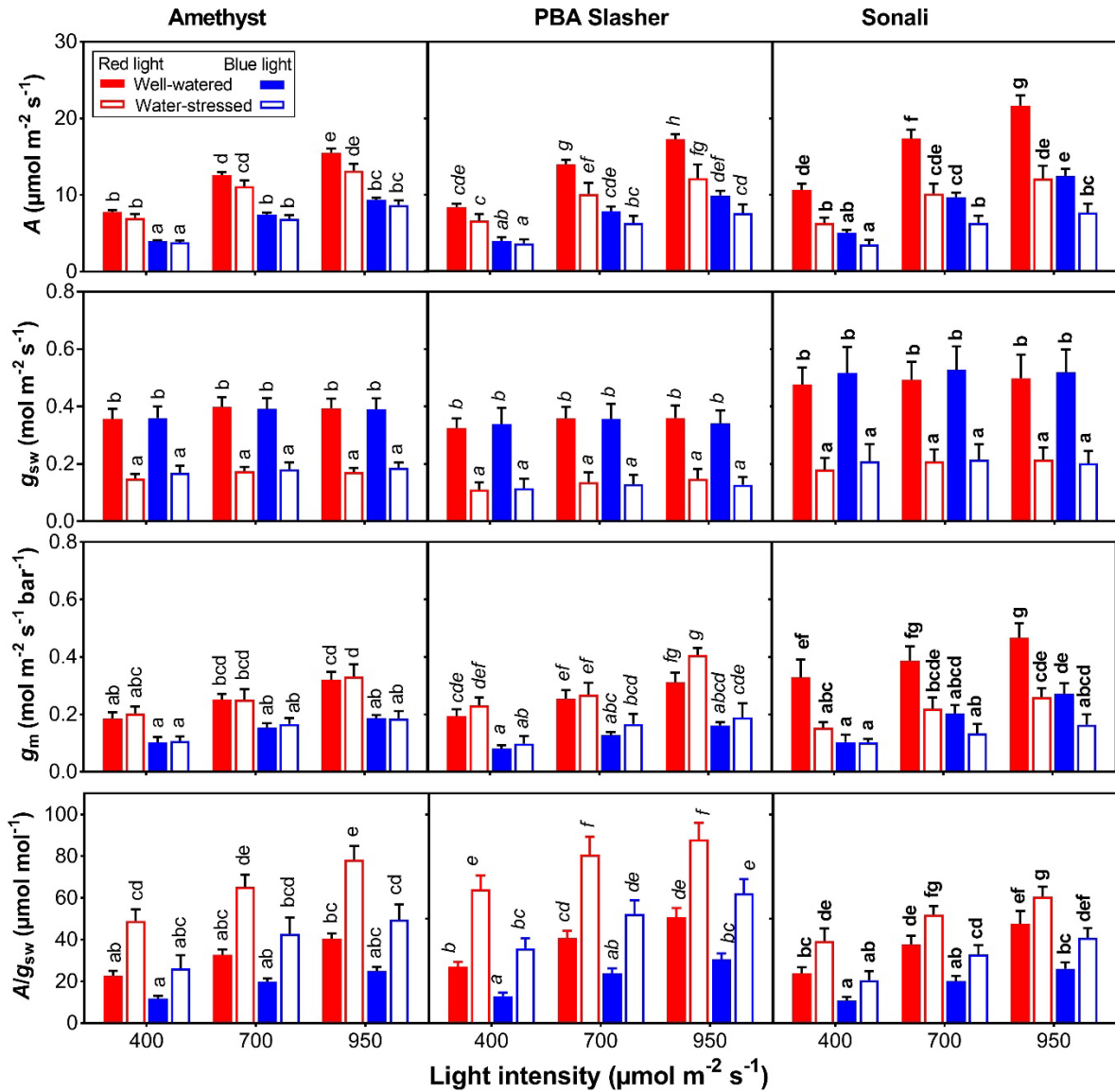
Leaf  $\delta^{15}N$  should be close to that of the leaf N source/s under ideal conditions (Ariz *et al.*, 2015). Leaves of N-fixing plants were depleted in  $^{15}N$  compared to N-fed leaves ( $p<0.001$ ;  $1.8\text{‰}$  N-fed and  $-1.8\text{‰}$  for N-fixing leaves) indicating that different nitrogen sources were used (Figure 6.7). The  $\delta^{15}N$  value of  $NH_4NO_3$  fertilizer supplied to N-fed plants was  $2.4\text{‰}$ . N-fed PBA Slasher and Sonali had  $\delta^{15}N$  values close to that of the fertilizer indicating negligible N derived from N-fixation (%Ndfa). %Ndfa for PBA Slasher and Sonali was 6.2% and 9.3% respectively. The  $\delta^{15}N$  value of N-fed Flip079C ( $1.3\text{‰}$ ) was lower than that of the fertilizer and so the proportion of N derived from N-fixation was higher, at 25%. There was no correlation between the leaf carbon and nitrogen isotope composition.

There was no significant nitrogen source or genotype effect for root dry weight but there was a significant nitrogen source by genotype effect for nodule weight ( $p=0.003$ ). N-fed plants were not inoculated but some nodulation was observed in these plants (Figure 6.7). However, the nodule size and number for N-fed plants was less than a tenth that in N-fixing

plants ( $p < 0.001$ ). In N-fed plants, PBA Slasher had greater nodule biomass than the other two genotypes but the nodule biomass did not differ between genotypes in N-fixing plants.

**Table 6.1** Effects of light intensity, light colour, water stress and genotypes on net photosynthetic rate ( $A$ ), stomatal conductance to water vapour ( $g_{sw}$ ) and mesophyll conductance to  $CO_2$  ( $g_m$ ).  
The degree of freedom (df) for light intensity=2, light colour=1, water stress=1 and genotypes=2.

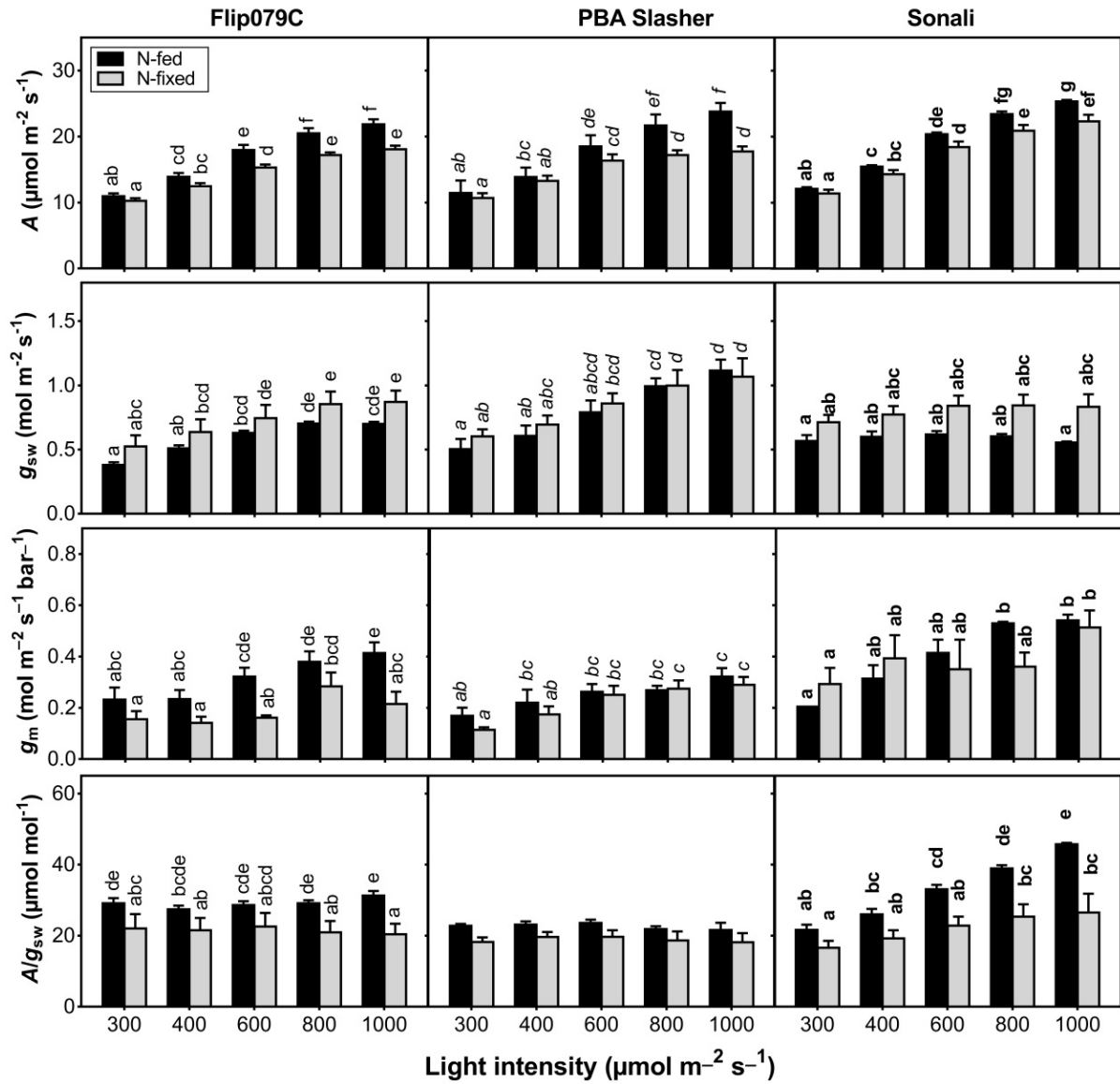
		$A$	$g_{sw}$	$g_m$
Light intensity	F	205.78	NS	41.43
	$p$	<.001	NS	<.001
Light colour	F	365.35	NS	157.79
	$p$	<.001	NS	<.001
Water stress	F	120.97	250.92	5.96
	$p$	<.001	<.001	0.016
Genotypes	F	10.7	20.32	3.18
	$p$	<.001	<.001	0.044
Light intensity $\times$ light colour	F	6.19	NS	NS
	$p$	0.003	NS	NS
Light intensity $\times$ water stress	F	8.64	NS	NS
	$p$	<.001	NS	NS
Light colour $\times$ water stress	F	20.02	NS	2.61
	$p$	<.001	NS	(0.10)
Light intensity $\times$ genotypes	F	NS	NS	NS
	$p$	NS	NS	NS
Light colour $\times$ genotypes	F	NS	NS	NS
	$p$	NS	NS	NS
Water stress $\times$ genotypes	F	21.57	3.62	22.72
	$p$	<.001	0.029	<.001
Light colour $\times$ water stress $\times$ genotypes	F	2.31	NS	4.92
	$p$	(0.1)	NS	0.008



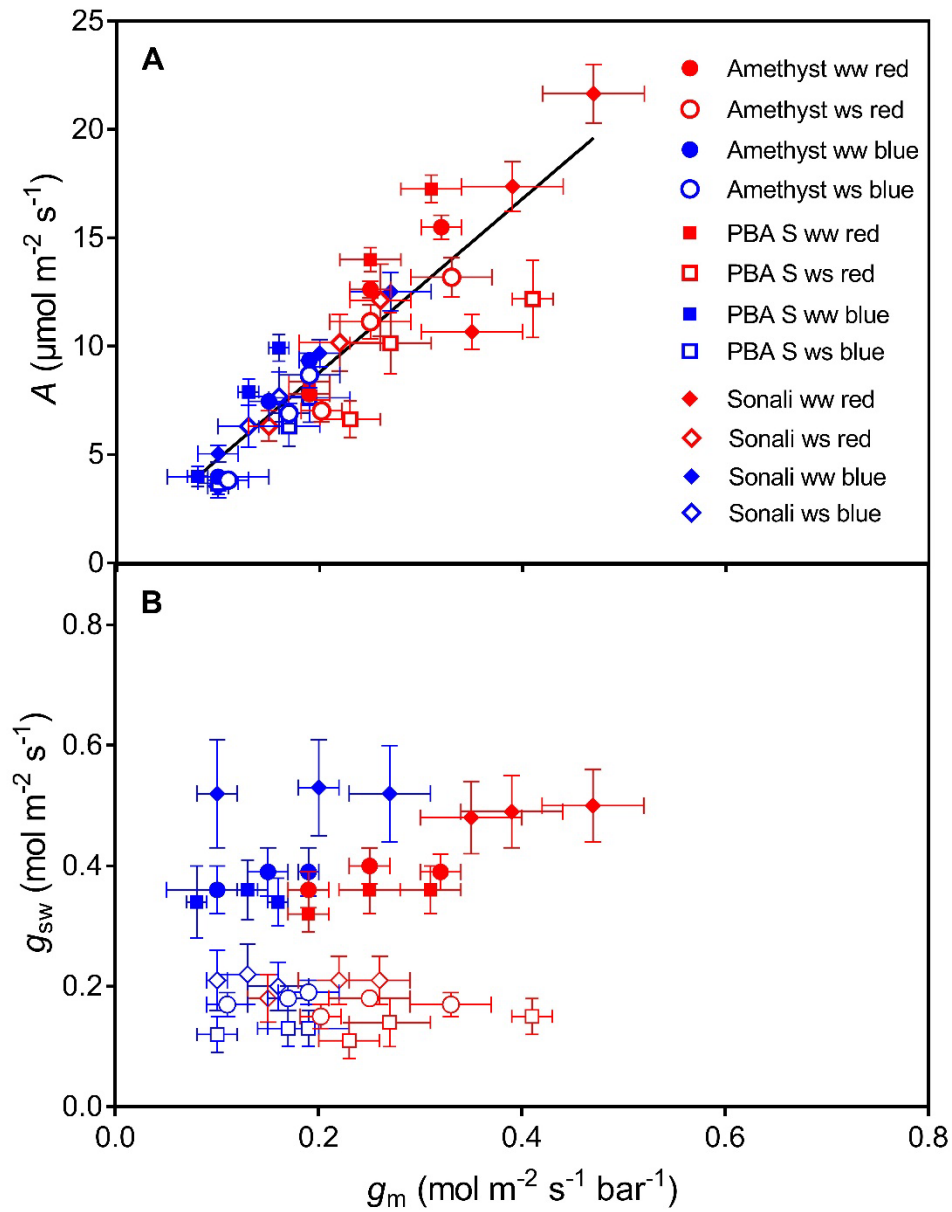
**Figure 6.1** Leaf gas exchange of three chickpea genotypes grown under well-watered or water-stressed conditions and measured under varying light intensities and light colour. Means and s.e. are shown (n=5-6). Letters indicate significant differences ( $p < 0.05$ ) between the treatments within each genotypes.

**Table 6.2** Effects of light intensity, nitrogen source and genotypes on net photosynthetic rate ( $A$ ), stomatal conductance to water vapour ( $g_{sw}$ ) and mesophyll conductance to  $CO_2$  ( $g_m$ )  
The degree of freedom (df) for light intensity=4, nitrogen source=1 and genotypes=2.

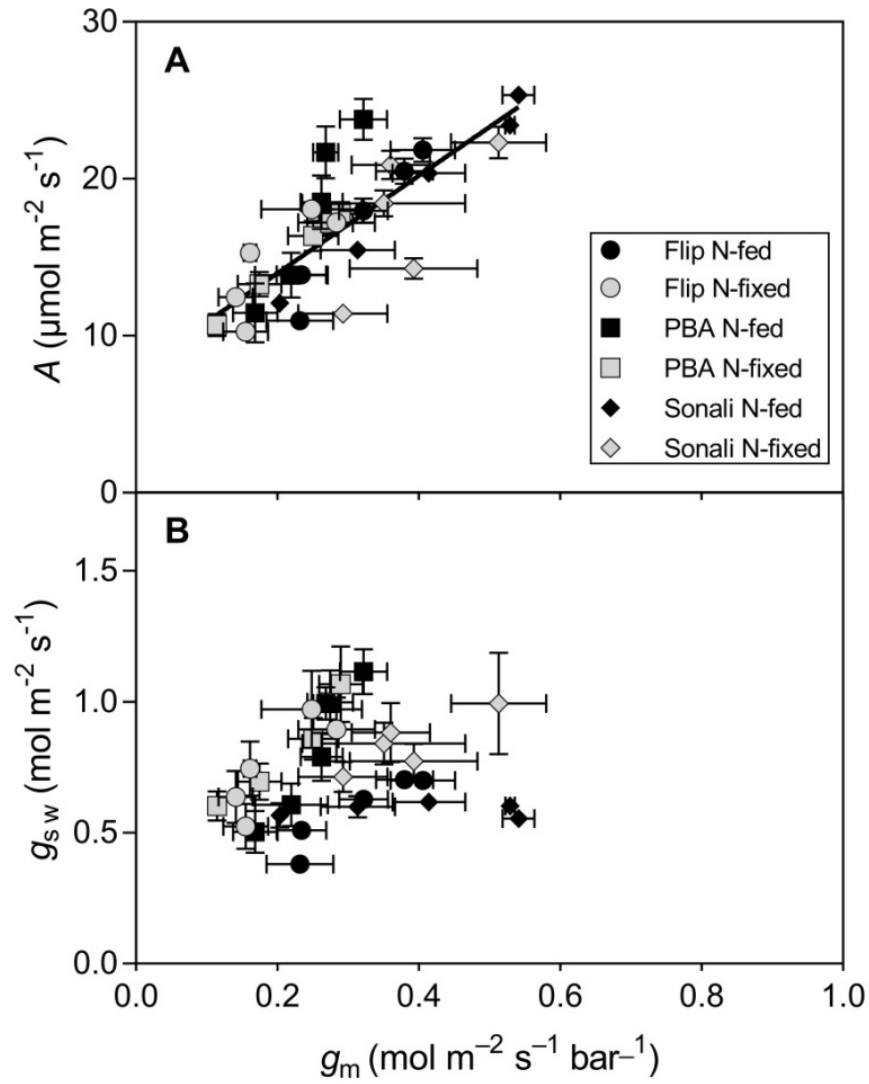
		$A$	$g_{sw}$	$g_m$
Light intensity	F	160.16	15.71	15.76
	$p$	<.001	<.001	<.001
Nitrogen source	F	61.28	19.99	12.97
	$p$	<.001	<.001	<.001
Genotypes	F	23.04	8.88	30.71
	$p$	<.001	<.001	<.001
Light intensity $\times$ nitrogen source	F	4.94	NS	NS
	$p$	0.001	NS	NS
Light intensity $\times$ genotypes	F	NS	2.55	NS
	$p$	NS	0.014	NS
Nitrogen source $\times$ genotypes	F	NS	2.77	3.62
	$p$	NS	(0.067)	0.03



**Figure 6.2** Leaf gas exchange of three chickpea genotypes grown under two nitrogen source treatments and measured under different light intensities. Means and s.e. are shown ( $n=5-6$ ). Letters indicate significant differences ( $p < 0.05$ ) between the treatments within each genotypes.

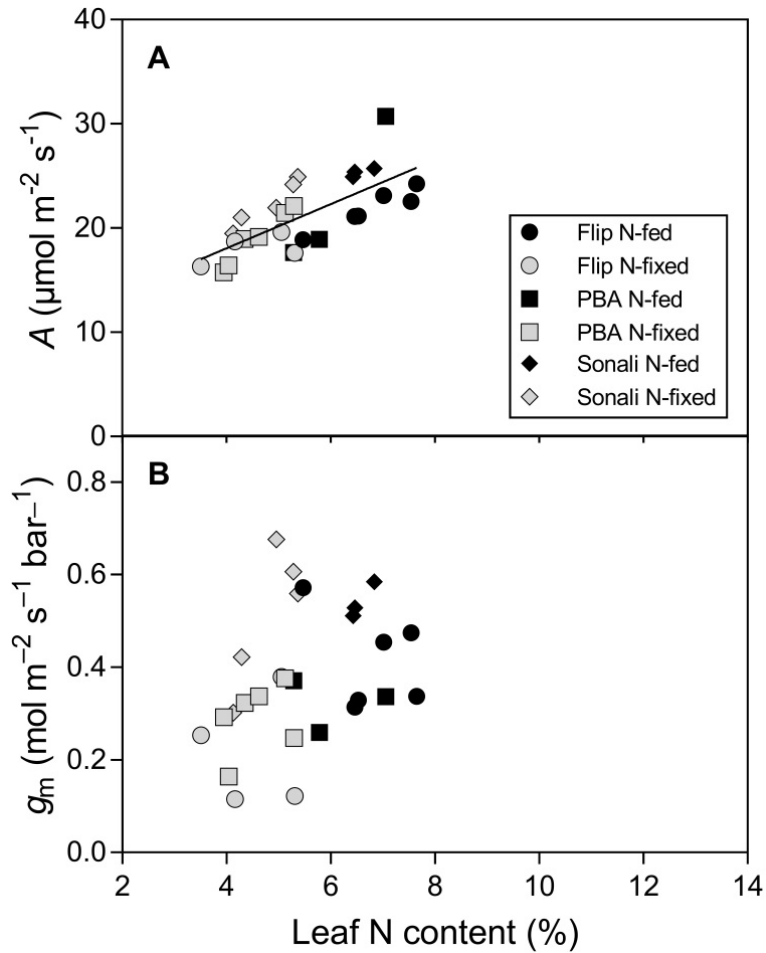


**Figure 6.3** The relationships between  $g_m$  and photosynthetic rate ( $A$ ; A) and stomatal conductance ( $g_{sw}$ ; B) for three chickpea genotypes grown under well-watered or water-stressed conditions and measured under varying light intensities and light colour. Values are means  $\pm$  s.e. at each light intensity,  $n = 5-6$ . The solid line in plot A indicates a significant linear regression ( $p \leq 0.001$ ,  $R^2 = 0.82$ ).

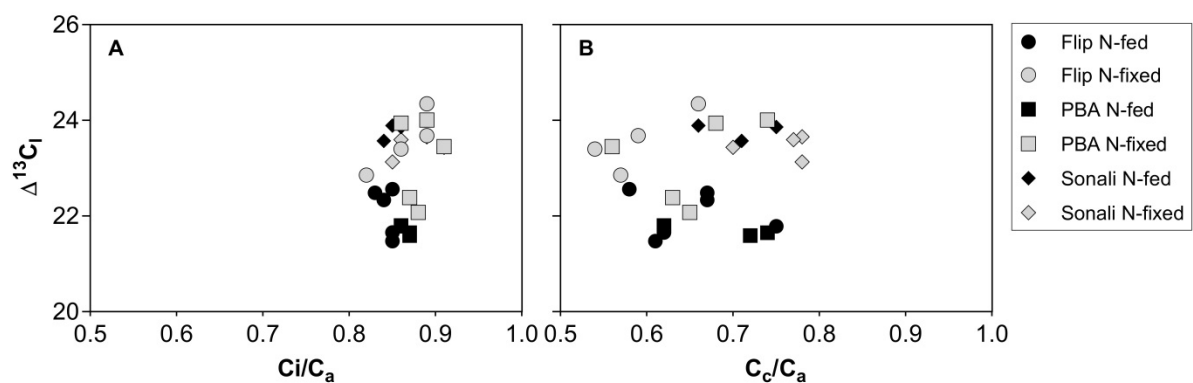


**Figure 6.4** The relationships between  $g_m$  and photosynthetic rate ( $A$ ; A) and stomatal conductance ( $g_{sw}$ ; B) for three chickpea genotypes grown under two nitrogen source treatments and measured under different light intensities. Values are means  $\pm$  s.e at each light intensity,  $n = 3-6$ . The solid line in plot A indicates a significant linear regression ( $p < 0.0001$ ,  $R^2 = 0.64$ ).

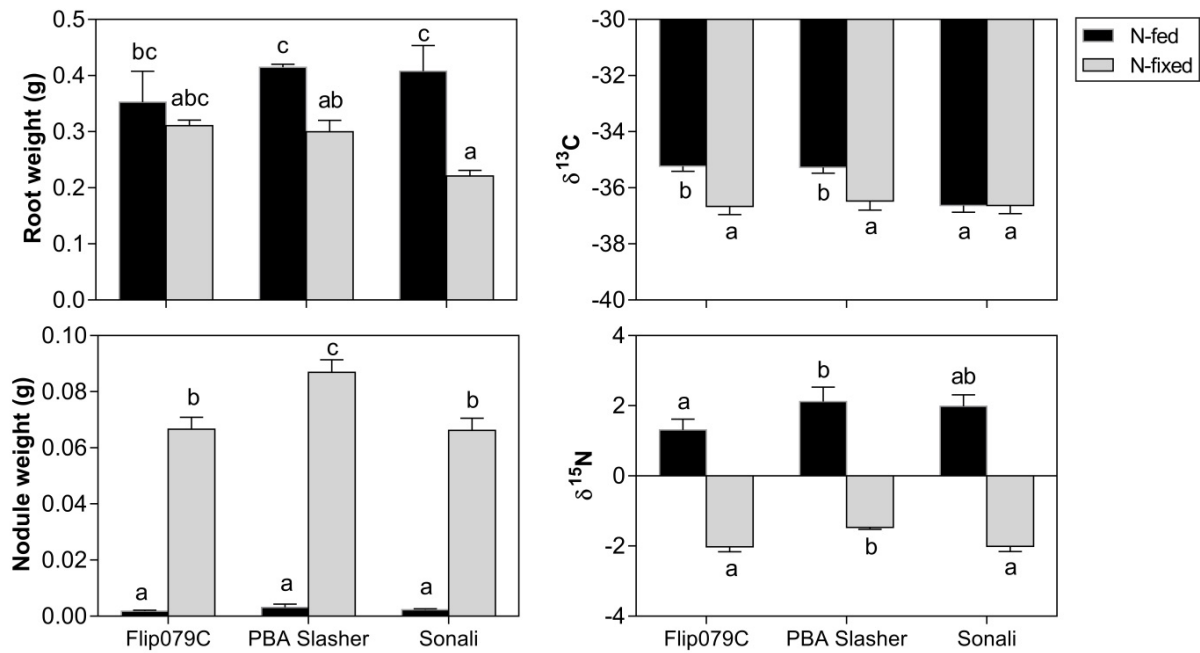




**Figure 6.5** Relationships between leaf N content and photosynthetic rate ( $A$ ; A) and mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ; B), measured at  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, for three chickpea genotypes grown under two nitrogen source treatments. The solid line in plot A indicates a significant linear regression ( $p < 0.0001$ ,  $R^2 = 0.51$ ).



**Figure 6.6** Relationship between  $\Delta^{13}\text{C}_1$  in leaf dry matter and the instantaneous ratios  $C_i/C_a$  (A) and  $C_c/C_a$  (B) in three chickpea genotypes grown under two nitrogen source treatments. Gas exchange parameters were measured at  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.  $n = 3-5$ .



**Figure 6.7** Crop traits of three chickpea genotypes grown under two nitrogen source treatments. Means and s.e. are shown (n=5-6). Letters indicate significant differences ( $p < 0.05$ ) between the treatments.

## 6.4. Discussion

### 6.4.1. Genotypes differ in their $g_m$ response to water availability and nitrogen source

Two experiments were conducted to investigate the interactive effects of genotype and growth environment on the response of  $g_m$  to measurement light intensity in chickpea. In the first experiment, the plants were either well-watered or water-stressed while in the second experiment, the plants were either  $\text{NH}_4\text{NO}_3$ -fed or biologically-fixing N. Two genotypes, Sonali and PBA Slasher, were common between the two experiments. Although the absolute value varied between the experiments, Sonali had the highest  $g_m$  value under optimum growth conditions in both the experiments. Overall,  $g_{sw}$  was higher for the plants in Experiment 2 than those in Experiment 1, perhaps due to the lower growth light level.

In the first study, moderate water stress was imposed on four-week old plants for few days. Restricted  $\text{CO}_2$  diffusion across the leaves, due to reduced stomatal and mesophyll conductance, has been found to be one of the major causes of photosynthesis reduction under moderate water stress, while the responses of these two conductances have been found to depend on the intensity and the duration of the stress (Grassi & Magnani, 2005, Flexas *et al.*, 2008, Centritto *et al.*, 2009, Loreto *et al.*, 2009, Flexas *et al.*, 2012b, Olsovska *et al.*, 2016). Water stress resulted in a reduction in  $g_{sw}$  by more than 2-fold in all the genotypes in this

study, while genotypes differed in the responses of  $g_m$  and  $A$  to water stress. Water stress reduced  $g_m$  only in Sonali and by 1.7-fold. Water stress has often been shown to reduce  $g_m$  as reviewed by Flexas *et al.* (2008). Recently,  $g_m$  has been found to be less sensitive than  $g_{sw}$  to limited water availability, particularly during moderate water stress (Grassi & Magnani, 2005, Perez-Martin *et al.*, 2014, Theroux-Rancourt *et al.*, 2014). In the study by Bunce (2009), water stress did not affect  $g_m$  in soybean (*Glycine max* L. Merr.) at a stress level, which reduced  $g_{sw}$  by about 80%, though  $g_m$  decreased substantially under severe stress. Barbour and Kaiser (2016) did not find a significant effect of drought on  $g_m$  in wheat (*Triticum aestivum* L.) genotypes. On the other hand, Olsovska *et al.* (2016) observed that moderate water stress conditions reduced  $g_m$  twice as much as  $g_{sw}$  in four winter wheat genotypes from different origins. These differences in the  $g_m$  responses between the studies could be due to the intensity and/or duration of stress or be related to species/genotype specific differences. I found significant genotypic variation for  $g_m$  in both well-watered and water-stressed plants, as reported previously in several publications (Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014, Tomás *et al.*, 2014, Olsovska *et al.*, 2016). However, the variability was not similar between the two water treatments. Sonali had the highest  $g_m$  compared to the other genotypes in WW conditions, while  $g_m$  was the lowest for Sonali when water-stressed, suggesting that Sonali is more sensitive to water stress conditions.  $g_m$  for the other two chickpea genotypes were not affected by water stress. I also observed stronger correlation between  $A$  and  $g_m$  than between  $A$  and  $g_{sw}$  in both WW and WS plants.

The responses of  $g_m$  to water stress could be due to changes in their leaf anatomical properties.  $g_m$  has been found to be strongly correlated with cell wall thickness ( $T_{cw}$ ) and chloroplast surface area exposed to intercellular air spaces per unit of leaf area ( $S_c$ ) which can affect effective diffusion path length and area for CO<sub>2</sub> diffusion (Evans *et al.*, 1994, Terashima *et al.*, 2005, Tholen *et al.*, 2008, Terashima *et al.*, 2011, Tomás *et al.*, 2013, Peguero-Pina *et al.*, 2016). Water stress has been shown to reduce  $S_c$  and increase  $T_{cw}$  resulting in reduced  $g_m$  in *Populus tremula* (Tosens *et al.*, 2012a). However,  $g_m$  variability between cultivars was not associated with leaf anatomical variability in other studies including *Acer* (Hanba *et al.*, 2002), wheat (Evans & Vellen, 1996) and grapevine (Tomás *et al.*, 2014). Tomás *et al.* (2014) did not find any effect of water treatments on the distribution of chloroplasts in grapevines (*Vitis vinifera* L.) and speculated that the observed variations of  $g_m$  might be the result of genotype-dependent and water stress-induced differences in cell and chloroplast membrane permeability.

There are studies suggesting that aquaporins (AQPs), which can facilitate CO<sub>2</sub> transport across the plasma and chloroplast membranes (Terashima & Ono, 2002, Uehlein *et al.*, 2003, Kaldenhoff, 2012, Mori *et al.*, 2014, Maurel *et al.*, 2015), might be involved in  $g_m$  regulation under water stress (Miyazawa *et al.*, 2008, Perez-Martin *et al.*, 2014, Han *et al.*, 2016). Deokar and Tar'an (2016) performed a comprehensive genome-wide analysis of the chickpea aquaporin (CaAQPs) gene family and suggested a potential role of some CaAQPs in drought stress response in chickpea. A strong correlation has also been found between  $g_{sw}$  and some particular AQPs during water stress and recovery in grapevines (Pou *et al.*, 2013b). The response of aquaporin gene expression to water stress can vary considerably depending on the duration and intensity of stress (Tyerman *et al.*, 2002, Galmés *et al.*, 2007), and expression of different AQPs in response to drought stress may differ between genotypes (Almeida-Rodriguez *et al.*, 2010). Recently, Zhao *et al.* (2017) found a weak positive correlation between water and CO<sub>2</sub> permeability and thus suggested that aquaporins may facilitate CO<sub>2</sub> transport across plasma membranes, but probably via a different pathway than water. These complex patterns of AQP expression complicate our understanding of the potential relationship between aquaporin and mesophyll conductance to CO<sub>2</sub>. In addition, carbonic anhydrases (CAs), which consist of a large family of proteins located in multiple sites within plant cells (Fabre *et al.*, 2007), may have a role in the regulation of  $g_m$  through the establishment of the dynamic equilibrium between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> (Tholen & Zhu, 2011, Flexas *et al.*, 2012a), but their role could be species dependent (Gillon & Yakir, 2000). However, the data are limited and contradictory. Perez-Martin *et al.* (2014) found that CA expression had a small but significant effect on  $g_m$  in olive (*Olea europaea*) under water-stress conditions, while Han *et al.* (2016) showed that expression of CA may not be important in the regulation of  $g_m$  under drought pretreatment conditions in cotton (*Gossypium hirsutum* L.). Recently, CA has been shown to interact with AQP regulating stomatal closure in response to internal leaf CO<sub>2</sub> concentrations (Wang *et al.*, 2016). The coupling of CA and aquaporin could enhance  $g_m$  by creating a CO<sub>2</sub> concentration gradient adjacent to the chloroplast membranes (Groszmann *et al.*, 2017).

The second experiment showed that the genotypes differed in their  $g_m$  response to nitrogen source. The cultivar Flip079C had higher  $g_m$  when fertilized with nitrogen than when nitrogen was fixed by Rhizobium inocula. Nitrogen source did not affect  $g_m$  for PBA Slasher and Sonali. There are no published studies on variability of  $g_m$  between N-fixing and inorganic N-fed legumes, nevertheless, reduced nitrogen availability has been shown to

reduce  $g_m$  in several species (Warren, 2004, Bown *et al.*, 2009, Li *et al.*, 2012, Xiong *et al.*, 2015a). Positive correlations between  $g_m$  and leaf N content have been observed in non-N-fixing species (Li *et al.*, 2009, Yamori *et al.*, 2010, Xiong *et al.*, 2015a). I found higher  $A$  (at higher light intensities) and leaf N content in N-fed plants than in N-fixing plants for all three genotypes. Lodeiro *et al.* (2000) compared drought tolerance in nitrogen-fixing and inorganic nitrogen-grown common beans and found that growth and N content were significantly higher in  $\text{NH}_4\text{-NO}_3$  sufficient beans than in N-fixing beans. I found a significant positive correlation between  $A$  and leaf N content for both N-fed and N-fixing chickpeas, as reported in many other studies (Evans, 1989, Reich *et al.*, 1994, Li *et al.*, 2009, Yamori *et al.*, 2010), due to the dependence of photosynthesis on nitrogenous compounds. But recently, Adams *et al.* (2016) reported that  $A$  and  $g_{sw}$  are not correlated to nitrogen per leaf area for most N-fixing plants. I did not find any relationship between leaf N content and  $g_m$  across the genotypes, although both  $g_m$  and leaf N content were higher for N-fed Flip079C than for the N-fixing plants. Warren (2004) found a large positive response of photosynthesis to nutrient supply, whereas nutrient supply had a small and inconsistent effect on  $g_m$  and  $g_{sw}$ . N- $g_m$  relationships were generally weak when different studies were compared. Leaf N content explained only 11% of  $g_m$  variability in wheat genotypes in the study by Barbour and Kaiser (2016).

The mechanism of  $g_m$  regulation under different nitrogen sources is unclear. There are no published studies to date on the response of  $g_m$  to N-fixing versus  $\text{NH}_4\text{NO}_3$ -fed (non-inoculated) plants. Increased  $g_m$  in response to higher nitrogen availability has been shown to be strongly correlated to increases in  $S_c$  in rice (Xiong *et al.*, 2015a). Chloroplast downsizing has also been found to play a role in the regulation of  $g_m$  in response to drought and reduced nitrogen availability (Li *et al.*, 2012). Leaf ultra-structural properties of the genotypes were not examined in this study, and future work should investigate genotypic variation in leaf anatomy to understand the regulation of  $g_m$  in response to these growth conditions. As for the biochemical component of  $g_m$ , Warren (2004) suggested that a correlation between nutrient supply and leaf contents of carbonic anhydrase and/or aquaporins seems unlikely since carbonic anhydrase and aquaporins have a very low N cost. On the other hand, several studies have shown that aquaporin gene expression in the root system (Clarkson *et al.*, 2000, Guo *et al.*, 2007, Ishikawa-Sakurai *et al.*, 2014, Ren *et al.*, 2015) or in the stem xylem (Hacke *et al.*, 2010) is affected by nitrogen supply and/or nitrogen forms in the medium. Li *et al.* (2009) speculated that chloroplast PIPs might be regulated by different N status in rice leaves.

Whether mesophyll conductance is limited by nitrogen investment in one or more enzymes or membrane proteins remains to be investigated further.

Despite a lack of clear understanding of the underlying mechanisms of  $g_m$  regulation under water stress or different nitrogen sources, genetic variability of  $g_m$  observed in this study offers a potential target for improving photosynthetic rate and WUE simultaneously within crop breeding programs.

#### **6.4.2. $g_m$ increases with increasing light intensity**

In experiment 1, leaves were measured under red or blue light, while in experiment 2, a red-blue light source was used to measure the effect of light intensities. There was a decline in  $A$  and  $g_m$  with decreasing light intensity for all the genotypes in both experiments, as has been previously reported (Flexas *et al.*, 2008, 2011, Douthe *et al.*, 2012). In both of my experiments,  $g_m$  was significantly different only between the highest and the lowest light intensity (with an average change of  $\approx 40\%$  between 950 and 400  $\mu\text{mol mol}^{-1}$  in the first experiment, and an average change of  $\approx 48\%$  between 1000 and 300  $\mu\text{mol mol}^{-1}$  in the second experiment). The sensitivity of the light response in our study was different from that observed by Douthe *et al.* (2011) and Douthe *et al.* (2012) in Eucalyptus species. They found a positive relationship between  $g_m$  and light intensity at low light intensities (i.e. when light intensity was lowered from 600 or 500 to 200  $\mu\text{mol mol}^{-1}$ ) but constant at higher light intensities. However, some studies did not find a dependence of  $g_m$  on measurement light intensity (Tazoe *et al.*, 2009, Yamori *et al.*, 2010). The dissimilarity in results may be related to species-specific differences and the range in light intensity used.

The mechanism of  $g_m$  responses to dynamic changes in light environment is not yet clear. Rapid responses of  $g_m$  to environmental factors have been attributed to carbonic anhydrase and aquaporins. *Nicotiana tabacum* aquaporin NtAQP1 has been reported to be directly involved in mesophyll conductance to  $\text{CO}_2$  (Flexas *et al.*, 2006, Uehlein *et al.*, 2008) and light-regulation sites were found on the promoter sequence of NtAQP1 (Siefritz *et al.*, 2004). In *Juglans regia*, transcript abundance of two aquaporin isoforms was substantially up-regulated by light (associated with light-induced leaf hydraulic conductance), and this light effect occurred in the short term (within minutes) (Cochard *et al.*, 2007, Baaziz *et al.*, 2012).

The negative effect of blue light on  $A$  and  $g_m$  in our study was in accordance with previous studies in *Nicotiana tabacum*, *Platanus orientalis* (Loreto *et al.*, 2009) and *Populus × canadensis* and *Quercus ilex* (Pallozzi *et al.*, 2013).  $g_m$  was measured from chlorophyll fluorescence-based method in these two studies and Loreto *et al.* (2009) demonstrated that  $g_m$  response to blue light is real although approximately half of the observed effect of blue light on  $g_m$  might be due to experimental artefacts. Nevertheless, the two methods, which rely on substantially different assumptions, but produce similar results, support the observed response of  $g_m$  to light. The reduction of  $g_m$  under blue light could be related to chloroplast movement away from blue light, the avoidance response, to avoid photodamage to the photosynthetic machinery. The phototropin photoreceptor, phot2 mediates blue-light-induced chloroplast movement in most green plant species (Kagawa & Wada, 2002, Suetsugu & Wada, 2012). The avoidance response would result in lower  $S_c$  under high blue light, as reported by Tholen *et al.* (2008) in *Arabidopsis thaliana*.  $S_c$  has been found to be positively correlated with  $g_m$  (Evans *et al.*, 1994, Tholen *et al.*, 2008, Peguero-Pina *et al.*, 2016). Nevertheless, Gorton *et al.* (2003) did not find any effect of chloroplast movement on the liquid-phase  $CO_2$  diffusion in leaves of *Alocasia brisbanensis*. They used pulsed photoacoustics to measure oxygen diffusion times as a proxy for  $CO_2$  diffusion in leaf cells. Another study showed that the rapid reduction of  $g_m$  under blue light in *Nicotiana* and *Platanus* leaves was faster than any possible chloroplast movements and the response was still observed after the chloroplast movement inhibition (Loreto *et al.*, 2009). The response of  $g_m$  to blue light might have been caused by as yet unknown factors affecting aquaporin-facilitated  $CO_2$  diffusion in the mesophyll (Kaldenhoff, 2012).

#### **6.4.3. Genotypes differ in the interactive response of $g_m$ to light colour, intensity and water availability**

In the present study, there was a significant interactive effect of genotype by water stress by light colour for  $g_m$ . Water stress reduced  $g_m$  only in Sonali and the reduction was significant only when measured under red light. The linear relationships between  $g_m$  and light intensity were significant at each light colour and water treatments except for the water-stressed PBA Slasher and Sonali when measured under blue light. Previous studies have also found that genotypes and species may vary in their response to environmental conditions. Recently, Barbour and Kaiser (2016) observed that wheat genotypes differ in the interactive response of  $g_m$  to N and water availability. von Caemmerer and Evans (2015) observed that the temperature response of  $g_m$  differed greatly between species, and proposed that variation in

the  $g_m$  response may be due to variation in the activation energy for membrane permeability to  $\text{CO}_2$  (suggesting the involvement of fast biochemical components like aquaporins in the regulation of  $g_m$ ) and the effective pathlength for liquid phase diffusion (referring mostly to the cell wall thickness). Pallozzi *et al.* (2013) found the negative impact of blue light on  $g_m$  depends on the light intensity and the effect was significant only at higher light intensities i.e. when photosynthesis was no longer light-dependent. Diversity in the  $g_m$  response to environment emphasized the lack of complete understanding of the mechanistic bases of  $g_m$  responses. Our results further suggest that caution must be taken when including  $g_m$  as a trait in legume breeding programs and that screening should be done under a range of growth environments.

#### **6.4.4. Genotypic differences in $g_m$ sensitivity to light intensity is altered by N source**

$g_m$  response to light intensity was found to be affected by nitrogen source in Flip079C and Sonali but not in PBA Slasher. In Flip079C and Sonali,  $g_m$  responded to light intensity only in N-fed plants but not in N-fixing plants. However, the response of  $A$  to light intensity was significant for both N-fed and N-fixing plants in all three genotypes. Xiong *et al.* (2015a) observed that the  $g_m$  response to light intensity differed with N supplement, with  $g_m$  increasing with light in high Nitrogen leaves while remaining unaffected in low Nitrogen leaves, suggesting that N may play a role in the  $g_m$  rapid response. I am unable to explain, at this point in time, the basis for the observed genotypic variability in the  $g_m$  response in this study, nevertheless, the results are interesting and future studies should investigate the genotypic differences in the leaf anatomy or the integrated roles of aquaporin in carbon and nitrogen assimilation (Maurel *et al.*, 2008).

#### **6.4.5. $g_m$ and leaf water use efficiency**

The current study on chickpea grown under different water availability and nitrogen source did not find any relationship between  $g_m$  and  $g_{sw}$ . Several studies have shown a correlation between  $g_m$  and  $g_{sw}$  under optimum and water-stressed conditions (Flexas *et al.*, 2002, Flexas *et al.*, 2008, Barbour *et al.*, 2010, Perez-Martin *et al.*, 2014, Olsovska *et al.*, 2016). On the other hand, other studies have found no correlation between  $g_m$  and  $g_{sw}$  (Bunce, 2009, Jahan *et al.*, 2014). Co-regulation between  $g_m$  and  $g_{sw}$  might differ among species and the intensity of water stress, as suggested by Warren (2008b). Lack of corregulation between  $g_m$  and  $g_{sw}$  would be advantageous from the perspective of leaf water-use efficiency (Barbour *et al.*, 2010, Buckley & Warren, 2014, Cano *et al.*, 2014, Flexas *et al.*, 2016). Recently, Moualeu-



Ngangue *et al.* (2016), using a modelling approach for leaves of *Cucumis sativus* plants grown under non-stress conditions, suggested that increasing mesophyll CO<sub>2</sub> conductance might be more likely to increase daily WUE than increasing stomatal density and stomatal reaction speed to light. In the present study, I found a positive correlation between  $g_m$  and  $A/g_{sw}$  for both WW and WS plants (stronger correlation for WS plants), and  $g_m$  seemed to have a stronger effect on leaf water use efficiency than  $g_{sw}$ .

## 6.5. Conclusion

Plants actively regulate CO<sub>2</sub> assimilation as well as CO<sub>2</sub> diffusion through the mesophyll with short-term changes in light intensity or light quality. However, genotypes differed in their interactive response to water stress, light intensity and colour, and the genotypic differences in the rapid response of  $g_m$  to light intensity were affected by N source. This is the first work to examine the response of  $g_m$  to N-fixing versus NH<sub>4</sub>NO<sub>3</sub>-fed (non-inoculated) plants.  $g_m$  response to N sources differed between chickpea genotypes. Positive relationships between  $g_m$  and  $A$  and between  $g_m$  and  $A/g_{sw}$  (relationship with  $A/g_{sw}$  stronger in water-stressed plants than in well-watered plants) and the lack of correlation between  $g_m$  and  $g_{sw}$  in our study suggests that increasing  $g_m$  can provide an opportunity for simultaneous increases in photosynthesis and water use efficiency in water-limited environments. Our results imply the tendency for  $g_m$  to compensate for the reductions in  $g_{sw}$  due to water stress in these chickpea genotypes. Genotypic variability in the  $g_m$  response, observed in this study, is useful information for crop improvement through  $g_m$ .

## 7. Variability in the temperature response of mesophyll conductance

### 7.1. Introduction

Photosynthesis is limited by the conductance of CO<sub>2</sub> transfer from the atmosphere to the sub-stomatal cavities via stomata (stomatal conductance) and the conductance of CO<sub>2</sub> transfer from the sub-stomatal cavities to the sites of carboxylation inside the chloroplast stroma (mesophyll conductance to CO<sub>2</sub>,  $g_m$ ) in C<sub>3</sub> plants (Farquhar & Sharkey, 1982, Evans *et al.*, 2009, Flexas *et al.*, 2012a). After diffusing through stomata, CO<sub>2</sub> molecules must travel through intercellular air spaces, then dissolve into liquid water and pass through the cell wall, plasma membrane, cytosol and the chloroplast envelope to finally reach the chloroplast stroma. These resistors between the sub-stomatal cavities and the stroma, including the liquid and membrane diffusion paths, contribute to the total mesophyll conductance (Bernacchi *et al.*, 2002, Evans & von Caemmerer, 2013, von Caemmerer & Evans, 2015).  $g_m$  has been shown to vary widely within and among species and in response to environmental conditions, such as water and nutrient availability, growth irradiance, CO<sub>2</sub> concentration and temperature (Flexas *et al.*, 2008, Scafaro *et al.*, 2011, Evans & von Caemmerer, 2013, von Caemmerer & Evans, 2015, Xiong *et al.*, 2015a). The mechanisms of  $g_m$  regulation are poorly understood. The observed variability of  $g_m$  has been associated with leaf anatomical properties, particularly chloroplast surface area facing intercellular air spaces, mesophyll cell wall thickness and membrane permeability to CO<sub>2</sub> (Evans *et al.*, 1994, Evans *et al.*, 2009, Scafaro *et al.*, 2011, Tholen & Zhu, 2011, Tosens *et al.*, 2012a, Xiong *et al.*, 2015a).

Understanding the temperature response of  $g_m$  will improve our understanding of  $g_m$  regulation including the roles of cell wall, membrane and aquaporin in determining  $g_m$ , as highlighted by previous work (Bernacchi *et al.*, 2002, Evans & von Caemmerer, 2013, Walker *et al.*, 2013, von Caemmerer & Evans, 2015). It is important to understand the  $g_m$  response to temperature for photosynthesis models to accurately determine Rubisco kinetics (Bernacchi *et al.*, 2002), as well as for improving the ability to manipulate  $g_m$  to increase photosynthesis and photosynthetic WUE in C<sub>3</sub> plants (Barbour *et al.*, 2010, Flexas *et al.*, 2013a, Flexas *et al.*, 2016). However, results of previous studies that examined the dependence of  $g_m$  on temperature have shown a wide range of responses to increased temperature, including exponential increases in  $g_m$  until 35°C followed by a decline at higher temperatures in *Nicotiana tabacum* (Bernacchi *et al.*, 2002), increased  $g_m$  from 10 to 35°C

but constant from 30 to 35°C in *Eucalyptus regnans* (Warren, 2008a), increased  $g_m$  from 5 to 20°C and constant from 20 to 40°C in *Alocasia brisbanensis* (Gorton *et al.*, 2003), constant  $g_m$  from 20 to 35°C in *Quercus canariensis* (Warren & Dreyer, 2006) and *Spinacia oleracea* (Yamori *et al.*, 2006) and constant  $g_m$  from 28 to 38°C in *Eperua grandiflora* (Pons & Welschen, 2003).

Moreover, von Caemmerer and Evans (2015) reported considerable variation in the temperature response of  $g_m$  between species. They used a simplified two-component model described in Evans and von Caemmerer (2013) to suggest that the observed variation between species may be due to variation in both the activation energy for membrane permeability to CO<sub>2</sub> (probably due to aquaporins) and the effective pathlength for liquid phase diffusion (due to cell wall thickness). Walker *et al.* (2013) also found a positive response of temperature between 15 to 35°C on  $g_m$  in *N. tabacum*, but no significant response in *Arabidopsis thaliana*, and suggested that aqueous diffusion dominates  $g_m$  in *A. thaliana*, while membrane transport processes play a large role in *N. tabacum*. The  $g_m$  response to other aspects of the environment has been shown to differ between genotypes within a single species. Barbour and Kaiser (2016) reported a genotypic variability in the  $g_m$  response to nitrogen and water availability in wheat. Because of the wide range of temperature sensitivities of  $g_m$  observed in previous studies indicating the complex temperature response, I was interested to see how  $g_m$  varies with temperature within and among cultivars.

Temperature dependence of  $g_m$  has also been found to be affected by other factors and the effect may depend on the species. Yamori *et al.* (2006) showed that the temperature dependence of  $g_m$  in *S. oleracea* was affected by the growth temperature, particularly at high temperatures. They showed that at high temperatures,  $g_m$  declined for the low temperature grown plants, but not for high temperature grown plants. However, acclimation to growth temperature was not observed in  $g_m$  in *E. regnans* (Warren, 2008a). Previous studies have found that  $g_m$  decreases as leaves age (Flexas *et al.*, 2008, Jahan *et al.*, 2014). Thus, it would be interesting and useful to see if the temperature response of  $g_m$  is affected by leaf age.

The mechanism of rapid variation in  $g_m$  with temperature is still unclear. Some studies (Holzinger *et al.*, 2007a, Buchner *et al.*, 2015) observed an increase in chloroplast protrusions (CPs) in leaf mesophyll cells in response to a dynamic increase in temperature. Chloroplast protrusions are broad peaked stroma-filled extensions of the chloroplast envelope that do not

contain thylakoids (diameter 3-5  $\mu\text{m}$ , length 3-5  $\mu\text{m}$ ) (Holzinger *et al.*, 2007a). Increases in temperature resulting in CPs will lead to surface extension of the chloroplast. Previous studies have shown positive correlation of  $g_m$  with chloroplast surface area facing intercellular air spaces,  $S_c$  (Flexas *et al.*, 2008, Terashima *et al.*, 2011, Tomás *et al.*, 2013), but there are also studies where  $g_m$  was not related to  $S_c$  (Hanba *et al.*, 2002, Hanba *et al.*, 2004). Increased chloroplast protrusions leading to increased  $S_c$  might explain the  $g_m$  response to temperature but has not been demonstrated with concurrent gas exchange measurements.

In this study, I compared the temperature response of  $g_m$  within and between different genotypes of soybean, and for a single genotype of common bean. I also examined if leaf age has any effect on the temperature response of  $g_m$  and if temperature induces chloroplast protrusions in soybean genotypes that can be related to the temperature response of  $g_m$ .

## 7.2. Materials and Methods

### 7.2.1. Plant material and growth conditions

Three genotypes of soybean (*Glycine max* (L.) Merr.); Snowy, DJakal and edamame (vegetable soybean harvested when the seeds are immature), and one genotype of common bean (*Phaseolus vulgaris* L.) were grown in a controlled-environment growth room at the University of Sydney, Centre for Carbon Water and Food (Camden, NSW, Australia). The growth room was set to a 16 h photoperiod, 27/19°C day/night temperature, 75% relative humidity, and 800  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density at plant height. Seeds were sown in 7 L pots filled with commercial potting mix amended with a slow release fertilizer (Osmocote Exact, Scotts, NSW, Australia). After emergence, the plants were thinned to two per pot and were well watered throughout the experiment. Five replicates of each genotype were used. In a second experiment, two genotypes (DJakal and edamame) of soybean (*Glycine max*) were grown in a controlled-environment growth room in the same environmental conditions with five replications. These plants were used to assess the effect of leaf age on the temperature response of  $g_m$ . *Arabidopsis thaliana* were grown in a controlled environment growth chamber at the University of Sydney, Biomedical Building (Eveleigh, NSW, Australia). The growth chamber was set to a 16 h photoperiod, 22°C day/night temperature, 40% relative humidity, 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density.

### 7.2.2. Estimating mesophyll conductance from the $\Delta^{13}\text{C}$ method

Leaf gas exchange and mesophyll conductance to  $\text{CO}_2$  ( $g_m$ ) were estimated using the online carbon discrimination ( $\Delta^{13}\text{C}$ ) method as described in Chapter 3. Measurements and regulation of leaf temperature were conducted using LI-6400XT portable photosynthesis system (Li6400; LiCor Biosciences, Lincoln, NE, USA) fitted with  $2 \times 6$  cm leaf chamber (Li6400 11) and red-green-blue light source (Li6400 18A). The LI-6400XT was connected to a Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) as described in Chapter 3. The leaf chamber conditions were controlled to provide a  $\text{CO}_2$  concentration of  $400 \mu\text{mol mol}^{-1}$  in the sample cell, a flow rate of  $300 \mu\text{mol s}^{-1}$  and a photosynthetic photon flux density (PPFD) of  $1300 \mu\text{mol m}^{-2} \text{s}^{-1}$  for all the measurements. In the first experiment comparing the temperature response within and among the genotypes, five youngest fully expanded leaves per genotype of soybean and common bean were measured eight weeks after planting. For the leaf age experiment in two soybean genotypes; DJakal and edamame, measurements were made on five youngest fully expanded leaves of 4-week old plants, and the same leaves were measured 7 days later under the same leaf environmental conditions. For both experiments, leaves were measured at increasing temperature from  $15^\circ\text{C}$  to  $35^\circ\text{C}$  with  $5^\circ\text{C}$  increase in steps.

### 7.2.3. Modelling temperature dependence of $g_m$

Temperature dependence of mesophyll conductance was modelled following von Caemmerer and Evans (2015) for individual leaves of three soybean genotypes and one genotype of common bean. Mesophyll conductance to  $\text{CO}_2$  is composed of the liquid ( $g_{\text{liq}}$ ) and membranes ( $g_{\text{mem}}$ ) components.  $g_{\text{liq}}$  includes diffusional conductance through cell wall, cytoplasm and chloroplast stroma and  $g_{\text{mem}}$  includes conductance through the plasma membrane and chloroplast membrane.

$$g_m = 1 / \left( \frac{1}{g_{\text{liq}}} + \frac{1}{g_{\text{mem}}} \right) \quad (1)$$

The liquid component of  $g_m$  ( $g_{\text{liq}}$ ) is given by:

$$g_{\text{liq}} = \rho H D / l, \quad (2)$$

where  $\rho$  is the molar density of water ( $\text{mol m}^{-3}$ ),  $H$  is the Henry coefficient for  $\text{CO}_2$  ( $\text{bar}^{-1}$ ),  $D$  is the diffusivity of  $\text{CO}_2$  in water ( $\text{m}^2 \text{s}^{-1}$ ) and  $l$  is the effective pathlength (m).

The solubility of CO<sub>2</sub> in water ( $\rho H$ ) decreases with increasing temperature while the diffusivity of CO<sub>2</sub> in water ( $D$ ) increases with temperature (Evans & von Caemmerer, 2013).

The temperature dependence of CO<sub>2</sub> diffusion across the membranes is assumed to be exponential:

$$g_{\text{mem}} = \rho H P_{(\text{mem}25)} \times e^{\{(T-25)E/(\mathcal{R}298(273+T))\}}, \quad (3)$$

where  $P_{(\text{mem}25)}$  is the combined membrane permeability to CO<sub>2</sub> at 25 °C (ms<sup>-1</sup>),  $E$  is the activation energy (kJ mol<sup>-1</sup>),  $\mathcal{R}$  is the ideal gas constant (8.314 J K<sup>-1</sup> mol<sup>-1</sup>) and  $T$  is the leaf temperature (°C).

$g_{\text{liq}}$  (Eqn 2) and  $g_{\text{mem}}$  (Eqn 3) are then multiplied by the chloroplast surface area facing intercellular airspace per unit leaf area ( $S_c$ ) to express  $g_m$  (Eqn 1) per unit leaf area (von Caemmerer & Evans, 2015).  $S_c$  and  $l$  were assumed to be 15 and 0.68µm for the soybean genotypes and common bean in our study as assumed by von Caemmerer and Evans (2015) for soybean.

The temperature responses of measured  $g_m$  were fitted to a three-parameter log normal function (Warren & Dreyer, 2006) when  $g_m$  showed an optimum temperature response.

$$g_{\text{mem}} = g_{\text{mem opt}} e^{\left(-\left(\frac{\ln(T/T_{\text{opt}})}{b}\right)^2/2\right)}, \quad (4)$$

where  $g_{\text{mem opt}}$  is the conductance across membranes at optimum temperature,  $T_{\text{opt}}$  is the optimum temperature (°C), and  $b$  is a scaling factor. The best fit was determined by allowing the model parameters,  $g_{\text{mem opt}}$ ,  $T_{\text{opt}}$  and  $b$  to be varied.  $g_{\text{mem}}$  (Eqn 4) was multiplied by  $S_c$ . The combination of  $g_{\text{liq}}$  (Eqn 2) and  $g_{\text{mem}}$  (Eqn 4) gives  $g_m$  (Eqn 1) at the optimal temperature.

#### **7.2.4. Assessment of chloroplast protrusions**

##### ***Plant material***

Three genotypes of soybean (Snowy, DJakal and edamame), which were investigated for the temperature response of  $g_m$  in this study, were used to assess the influence of temperature on the dynamic formation of chloroplast protrusions (CPs) in mesophyll cells. After the completion of gas exchange measurements, the youngest fully expanded leaves of the same

plants (i.e. those used for  $g_m$  measurements) were selected for the assessment of CPs. *Arabidopsis thaliana* (five replicates) was also examined in this study as the temperature-induced CPs has been observed in transgenic *A. thaliana* plants.

### ***Experimental set-up and image acquisition***

Leaf sections (approximately 30-100  $\mu\text{m}$  thickness) of the youngest leaves of soybean genotypes (DJakal and edamame) and *Arabidopsis thaliana* were prepared manually with a razor blade and collected in precooled ( $12^\circ\text{C}$ ) 0.1M phosphate-buffered saline in a glass bottom dish (MatTek, 35mm size, No. 1.5 thickness). The sections were investigated with a Leica SP5 II Confocal microscope (Leica Microsystem, Germany) with differential interference contrast (DIC) optics ( $63\times/\text{NA } 1.3 \text{ Gly}$ ) at the Australian Centre for Microscopy & Microanalysis (ACMM) at the University of Sydney, NSW, Australia. A cooling/heating stage insert (Temperable Insert P, PeCon) with a circular observation opening ( $\text{\O } 35 \text{ mm}$ ) was installed to the microscope, and the insert was levelled in the stage by 4 screws. The stage insert was connected to the circulating waterbath (Lauda Ecoline RE 206) with the tubes with self-sealing couplings. This setting allowed for the temperature control of the leaf sections through a cooling/heating stage based on a closed circuit with water via the circulating waterbath (the temperature was regulated at the waterbath). The microscope stage was covered with protective enclosure to protect the sample from light. For confocal imaging, a 488 nm laser was used for excitation, images were collected at 560nm - 760nm for autofluorescence of chlorophyll. DIC images were simultaneously collected.

The leaf sections were exposed to three different temperatures in succession ( $15$ ,  $25$  and  $35^\circ\text{C}$ ). 5–10 leaf sections were mounted in the pre-cooled microscope stage at the first temperature level of  $15^\circ\text{C}$ . The light was switched off until the next target temperature ( $25^\circ\text{C}$ ) was reached and also during the image acquisition and this procedure was repeated for the last target temperature ( $35^\circ\text{C}$ ). A thermocouple sensor continuously monitored the temperature of the stage in close vicinity to the leaf sections. The total time the leaf sections were at each temperature was 25 minutes (including the time to reach to the set temperature). For each leaf sample, the same cells were imaged at different focal planes to collect the XZ sections. Leica Application Suite Advanced Fluorescence Lite (LAS AF Version 2.6.0 build 7266, Leica Microsystems CMS GmbH) was used for capturing the z-series. 5-15 palisade cells of the three soybean genotypes (Snowy, DJakal and edamame) and *A. thaliana* were manually screened for the occurrence of chloroplast protrusions at each temperature.

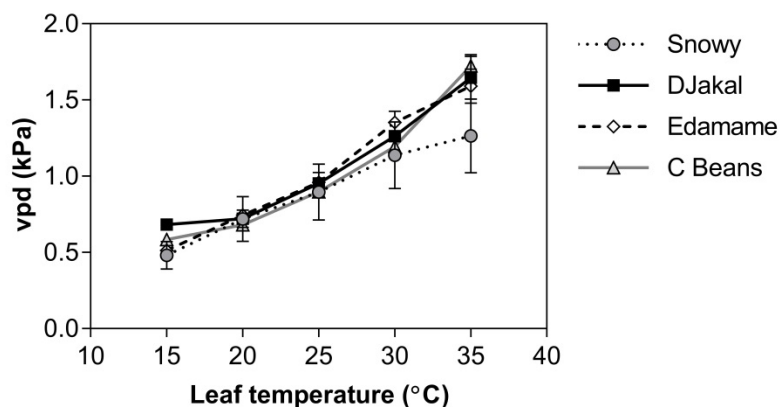
### 7.2.5. Statistical analysis

Relationships between leaf temperature, gas exchange and mesophyll conductance were graphed for both experiments using GraphPad Prism (Version 7, GraphPad Software Inc). Linear relationships of the average values for gas exchange and  $g_m$  over the temperature range were considered statistically significant when  $p < 0.05$ . Significant differences between the average values were assessed using analysis of variance, as implemented by GENSTAT 16th edn SP1 (VSN International Ltd, London, UK), and means were compared using Fisher's unprotected least significant difference test.

## 7.3. Results

### 7.3.1. Effect of leaf temperature on $g_m$

The temperature response of  $g_m$  was compared between three soybean genotypes: Snowy, Djakal and edamame and one genotype of common bean. There was an increase in leaf to air vapour pressure difference (vpd) with increasing temperature, with vpd ranging from 0.5 to 2.1 kPa across the genotypes (Figure 7.1). Relative humidity (RH) ranged from 85 to 60% over the temperature range across the genotypes. Due to the relatively small range in vpd and RH (above 60%) over the temperature, I consider that the responses observed in gas exchange and mesophyll conductance are predominantly due to changes in temperature. However, the effect of vpd on mesophyll conductance cannot be disregarded completely due to the observed covariance between vpd and temperature.



**Figure 7.1** Response of leaf to air vapour pressure difference (vpd) to variation in leaf temperature in soybean genotypes including Snowy, Djakal and edamame and common bean. Values are means  $\pm$  s.e.,  $n=5$ .

The mean value of photosynthetic rate ( $A$ ) at 25°C did not vary between soybean genotypes, but the mean  $A$  was higher for soybean genotypes than for common bean (Figure 7.2a). However, overall the absolute values of  $A$  were low at all temperatures for both the common bean and the soybean genotypes. In all three soybean genotypes, photosynthetic rate



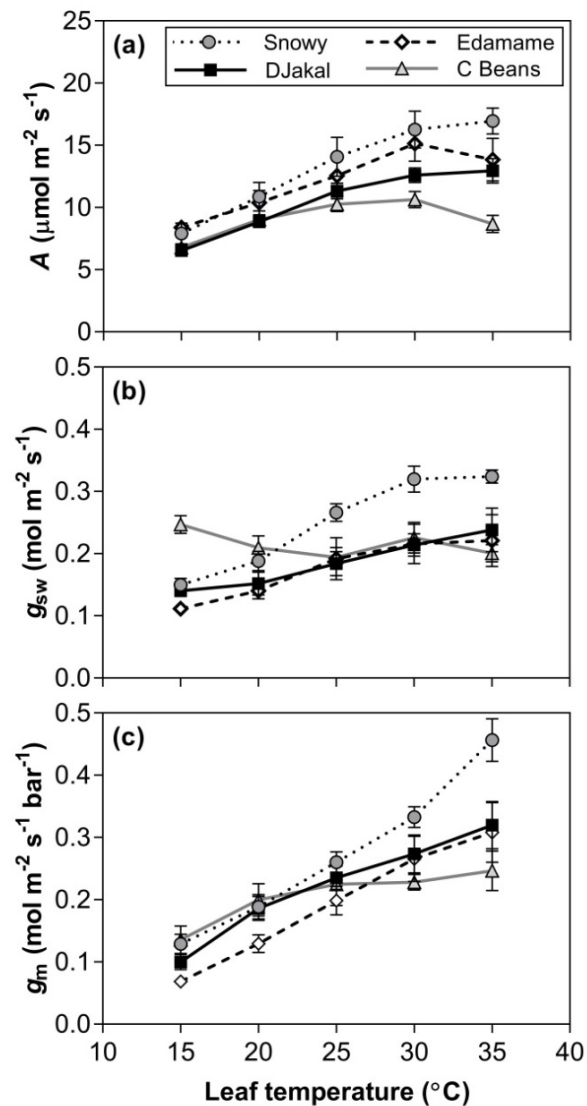
gradually increased from 15 to 25°C and either plateaued or declined at 30°C ( $p < 0.05$ , Figure 7.2a), but the increase in  $A$  over the temperature range was low (about 2-fold increase from 15 to 35°C). Common bean showed a shallower response to temperature for photosynthetic rate, and the change in  $A$  over the temperature range was not statistically significant (Figure 7.2a). Despite an increase in  $v_{pd}$  with temperature, the mean value of stomatal conductance ( $g_{sw}$ ) increased as temperature increased up to 30°C in Snowy. In DJakal and edamame, the increase in mean  $g_{sw}$  was significant only between 15°C and 30/35°C (Figure 7.2b).  $g_{sw}$  was unrelated to temperature in common bean (Figure 7.2b).

Across soybean genotypes, the mean values of  $g_m$  increased 3-4 fold between 15 and 35°C (Figure 7.2c). Snowy showed monotonic increase in mean values of  $g_m$  up to 35°C, and the standard error bars were not overlapping across the temperature range. Mean values of  $g_m$  increased with increasing temperature in DJakal and edamame, but the standard errors were larger and overlapping at higher temperature, between 25 and 35°C in DJakal and between 30 and 35°C in edamame (Figure 7.2c). Common bean showed less change in  $g_m$  over the temperature range (Figure 7.2c). In common bean,  $g_m$  increased about 1.5 fold from 15 to 20°C but was nearly temperature-independent from 20 to 35°C, with large and overlapping error bars (Figure 7.2c).

The regression line, fitted to measurements from individual leaves, was  $g_m = 0.02T - 0.13$  ( $R^2 = 0.85$ ,  $p < 0.0001$ ) for Snowy,  $g_m = 0.01T - 0.04$  ( $R^2 = 0.67$ ,  $p < 0.0001$ ) for DJakal,  $g_m = 0.01T - 0.11$  ( $R^2 = 0.69$ ,  $p < 0.0001$ ) for edamame, and  $g_m = 0.005T + 0.08$  ( $R^2 = 0.30$ ,  $p < 0.01$ ) for common bean. The slopes of the linear regression lines were not significantly different between soybean genotypes, but the differences in the intercepts were significant between soybean genotypes. The mean value of mesophyll conductance ( $g_m$ ) at 25°C did not vary within and across species, while the mean  $g_m$  of Snowy was significantly higher than that of DJakal and edamame at 35°C ( $p < 0.05$ ) and that of common bean at 30 and 35°C ( $p < 0.05$ , Figure 7.2c).

The temperature response of the photosynthetic rate was mostly similar among the replicates across the genotypes (Figure 7.7c) with few exceptions, e.g. some replicates showed shallower response than others. The optimum temperatures for  $g_m$  were not always similar to those of photosynthetic rate across the genotypes (Figure 7.7a-d,i-l). There was very little variation in the temperature response for  $g_{sw}$  among the replicates of Snowy and DJakal,

while the replicates of edamame did not follow any particular trend for  $g_{sw}$  over the temperature range. The  $g_m$  response to temperature was generally similar among replicates of Snowy (Figure 7.7i), while the response varied among replicates in DJakal (Figure 7.7j) and edamame (Figure 7.7k). All five replicates in DJakal and edamame showed linear increase in  $g_m$  from 15 to 25°C, then the replicates varied in response from 25 to 35°C, with some replicates showing an increase in  $g_m$  up to 35°C, while others showing a decline or plateauing  $g_m$  from 25 or 30°C (Figure 7.7j,k). In common bean, there was large variability among replicates, with no specific trend of  $g_m$  over the temperature range (Figure 7.7l).



**Figure 7.2** Response of photosynthetic rate ( $A$ ; a), stomatal conductance ( $g_{sw}$ ; b) and mesophyll conductance ( $g_m$ ; c) to variation in leaf temperature in soybean genotypes including Snowy, DJakal and edamame and common bean. Values are means  $\pm$  s.e.,  $n=5$ .

### 7.3.2. Effect of leaf temperature on chloroplast protrusions

DIC-autofluorescence merged images were used to assess the chlorophyll-free CPs in the mesophyll cells. Same cells were compared between the temperatures. Small movement in Z-axis was apparent during temperature increase from 15 to 35°C, thus for each sample, the XY images generated from a Z series for the three temperatures were thoroughly scanned to match the chloroplast position in the investigated cells at each temperature. 5-15 cells of the three soybean genotypes and *A. thaliana* were manually screened for the occurrence of chloroplast protrusions (CPs). Five leaves were used for each genotype. XY images (at a single focal plane) of mesophyll cells collected at three temperatures are shown for *A. thaliana* (Figure 7.3), Snowy (Figure 7.4), edamame (Figure 7.5) and DJakal (Figure 7.6). I did not find chloroplast protrusions at any temperature for soybean genotypes and *A. thaliana*, and thus their presence could not be confirmed in my observations.

### 7.3.3. Effect of leaf age on the temperature response of $g_m$

A second experiment was conducted to test the effect of leaf age on the temperature response of two soybean genotypes: DJakal and edamame. Gas exchange and  $g_m$  were measured on the youngest fully expanded leaves and then after 7 days on the same leaves under the same leaf environmental conditions. Photosynthetic rate showed a positive linear response to temperature for both the youngest fully expanded leaves and on the same leaves measured one week later ( $R^2=0.76$ ,  $p<0.001$  and  $R^2=0.70$ ,  $p<0.001$  respectively) in DJakal and ( $R^2=0.45$ ,  $p<0.001$  and  $R^2=0.35$ ,  $p<0.05$  respectively) in edamame. Leaf age did not alter the linear regression slopes but changed the intercept in both DJakal and edamame. Photosynthetic rates significantly declined with leaf age in DJakal ( $p<0.05$ ) and in edamame ( $p<0.05$ ). The individual leaves responded similarly to temperature for  $A$  for DJakal (Figure 7.8a.1,a.2) and for edamame (Figure 7.8b.1,b.2). The youngest fully expanded leaves of DJakal showed variability in temperature response for  $g_{sw}$  among the replicates, with some replicates showing increase in  $g_{sw}$  while the others were temperature-insensitive (Figure 7.8c.1). For the same leaves of DJakal, measured one week later,  $g_{sw}$  decreased from 15 to 20°C in all the replicates, but after 20°C the response varied among the replicates (Figure 7.8c.1).  $g_{sw}$  was not related to temperature regardless of the leaf age in edamame (Figure 7.8d.1, d.2).

The temperature response of the mean values of  $g_m$  was not significantly affected by leaf age in DJakal, with a linear increase with increasing temperature for both the youngest fully

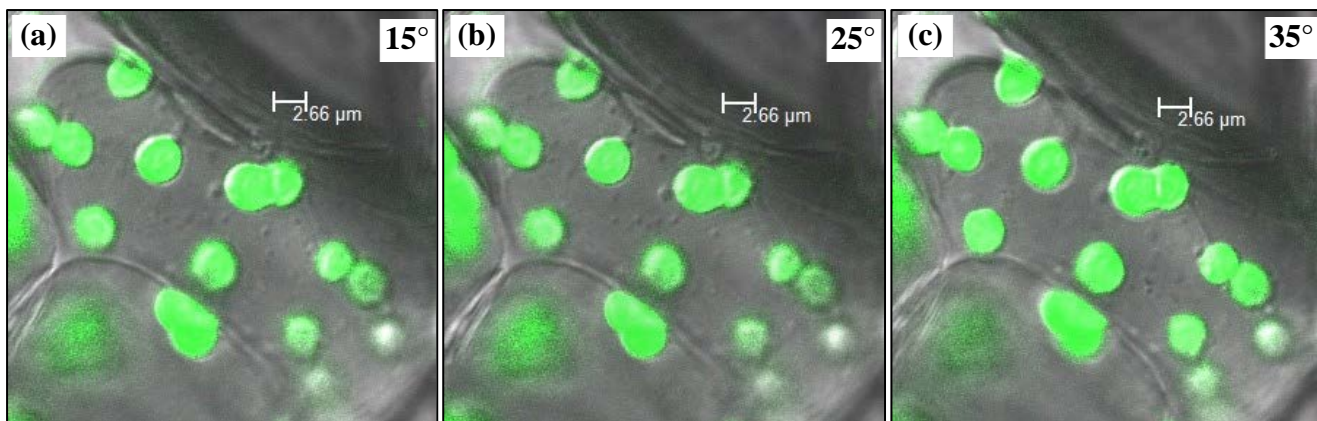
expanded leaves ( $R^2=0.71$ ,  $p<0.0001$ ) and when the same leaves were measured one week later ( $R^2=0.67$ ,  $p<0.0001$ ). However, in edamame, the linear relationship between  $g_m$  and temperature was weaker for leaves measured one week later ( $R^2=0.32$ ,  $p<0.01$ ) than for the same leaves when they had just finished expanding ( $R^2=0.63$ ,  $p<0.0001$ ). In DJakal, the individual leaves (of both ages) mostly showed similar increase in  $g_m$  from 15 to 25°C, but the  $g_m$  response varied considerably among the leaves from 25 to 35°C (Figure 7.8e.1, e.2). However, leaf age has little effect on the general shape of the response curve across the replicates in DJakal (Figure 7.8e.1, e.2). In the youngest fully expanded leaves of edamame, there was an increase in  $g_m$  from 15 to 25°C, but the  $g_m$  response varied among the replicates from 25 to 35°C. However, when the same leaves of edamame were measured one week later, they did not follow the trend of the younger leaves for  $g_m$  over the temperature range.  $g_m$  values of the older edamame leaves were mostly constant between 15 and 20°C.

#### 7.3.4. Modelling temperature dependence of $g_m$

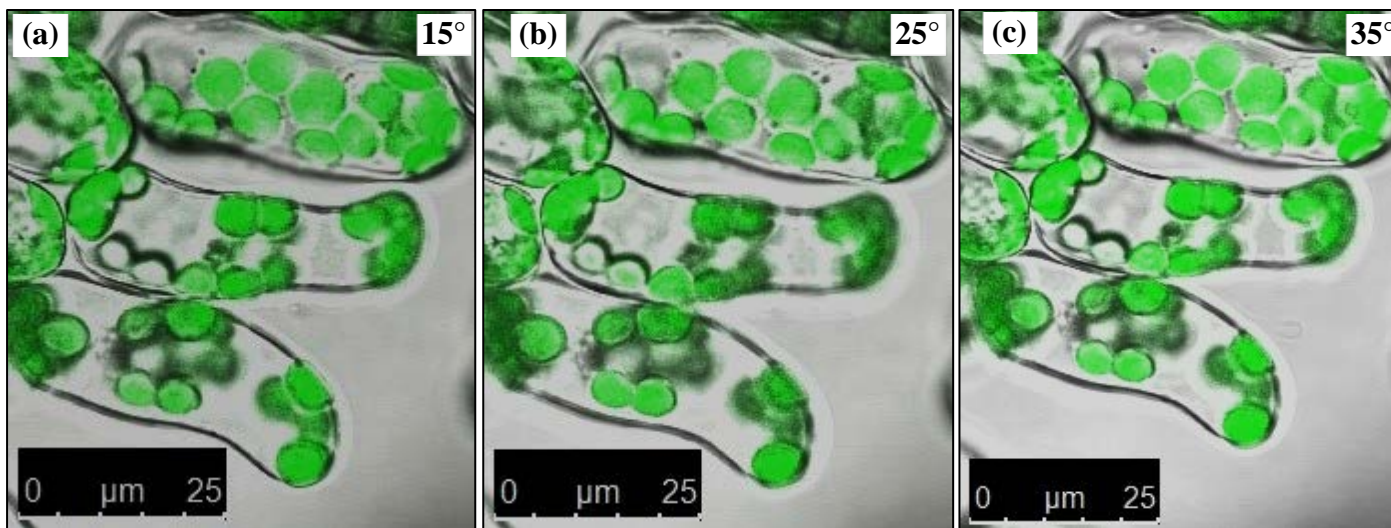
The measured values for the temperature response of  $g_m$  within and across the genotypes were fitted to either von Caemmerer and Evans (2015) exponential model or Warren and Dreyer (2006) optimum  $g_{mem}$  model as described in Materials and Methods section in this chapter. The von Caemmerer and Evans model assumed an exponential temperature dependence of  $g_{mem}$ , but could not simulate the observed temperature response for some leaves where  $g_m$  declined or plateaued at 25°C or higher temperatures, and thus the measured  $g_m$  values for those leaves were fitted to the model with optimum temperature for  $g_{mem}$ . The fitted parameters for the individual leaves in the first and the second experiment are given in Table 7.1 and Table 7.2 respectively. I used a value of 15 for chloroplast surface area facing intercellular airspace per unit leaf area ( $S_c$ ) and 0.68 $\mu$ m for effective pathlength ( $l$ ) for all the genotypes of soybean and for common bean, as assumed by von Caemmerer and Evans (2015) for soybean. It was necessary to vary  $P_{(mem25)}$ , which is the combined membrane permeability to CO<sub>2</sub> at 25°C, to achieve reasonable model fits for all the leaves.

Soybean genotypes had higher fitted membrane activation energy,  $E$  (60 to 85 kJ mol<sup>-1</sup>) compared to common bean (35 to 53 kJ mol<sup>-1</sup>, Table 7.1). The temperature response for the mean values of  $g_m$  in Snowy was well fitted to von Caemmerer and Evans (2015) model (exponential increase of  $g_{mem}$ ), while the responses for the mean values of  $g_m$  in edamame and DJakal were best fitted to model with temperature optima at 45°C (Table 7.1). Within soybean genotypes, the temperature response of all five replicates of Snowy could be fitted to

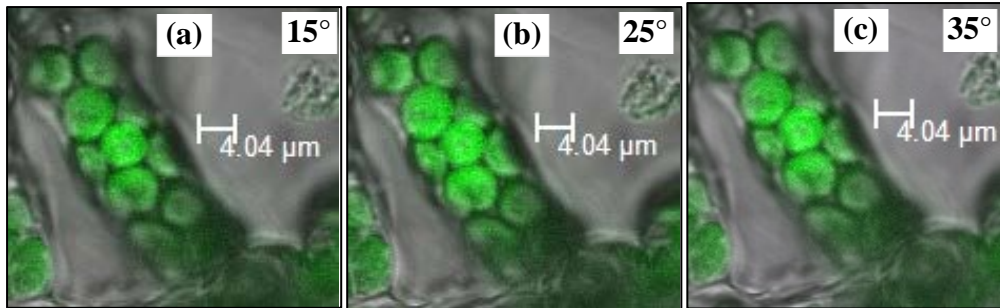
the von Caemmerer and Evans model, with the membrane activation energy ranging from 60 to 85 kJ mol<sup>-1</sup> among the replicates. However, for the youngest expanded leaves of edamame and DJakal, the best fit to the response of  $g_m$  for some replicate leaves was obtained with exponential temperature dependence of  $g_{mem}$ , but for other replicates, the best fit could be obtained with optimum temperature for  $g_{mem}$  around 30°C or higher. In Djakal, the fitted responses of  $g_m$  for a week old leaves were similar to those for the youngest expanded leaves (Table 7.2). In the older leaves of edamame, the fitted curve (exponential or optimum  $g_{mem}$ ) could not accurately reproduce the observed response but the residual sum of squares were always below 0.006. In the individual replicates of common bean, the fitted curve could not adequately include all  $g_m$  values over the temperature range. The best fit to average temperature response could be obtained with maximum  $g_m$  values at 35°C in common bean.



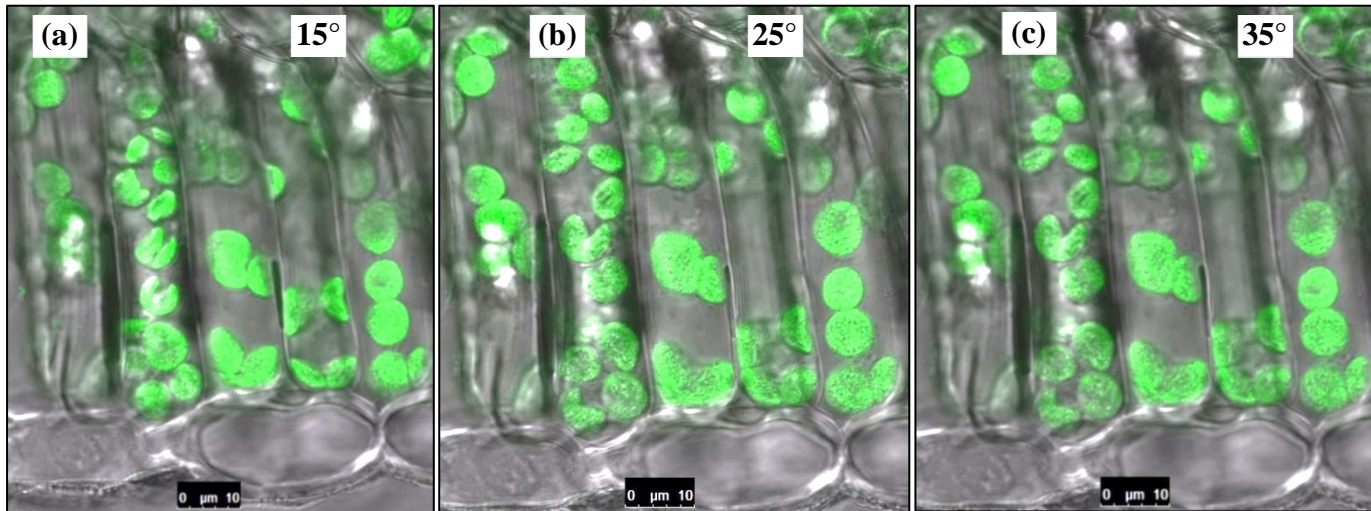
**Figure 7.3** Leaf mesophyll cell of *Arabidopsis thaliana* after exposure to three different temperature steps; 15°C (a), 25°C (b), and 35°C (c). Each image is autofluorescence-DIC merged XY image at a single focal plane. Shown is a typical mesophyll cell from 5 replicate samples from 5 individual plants.



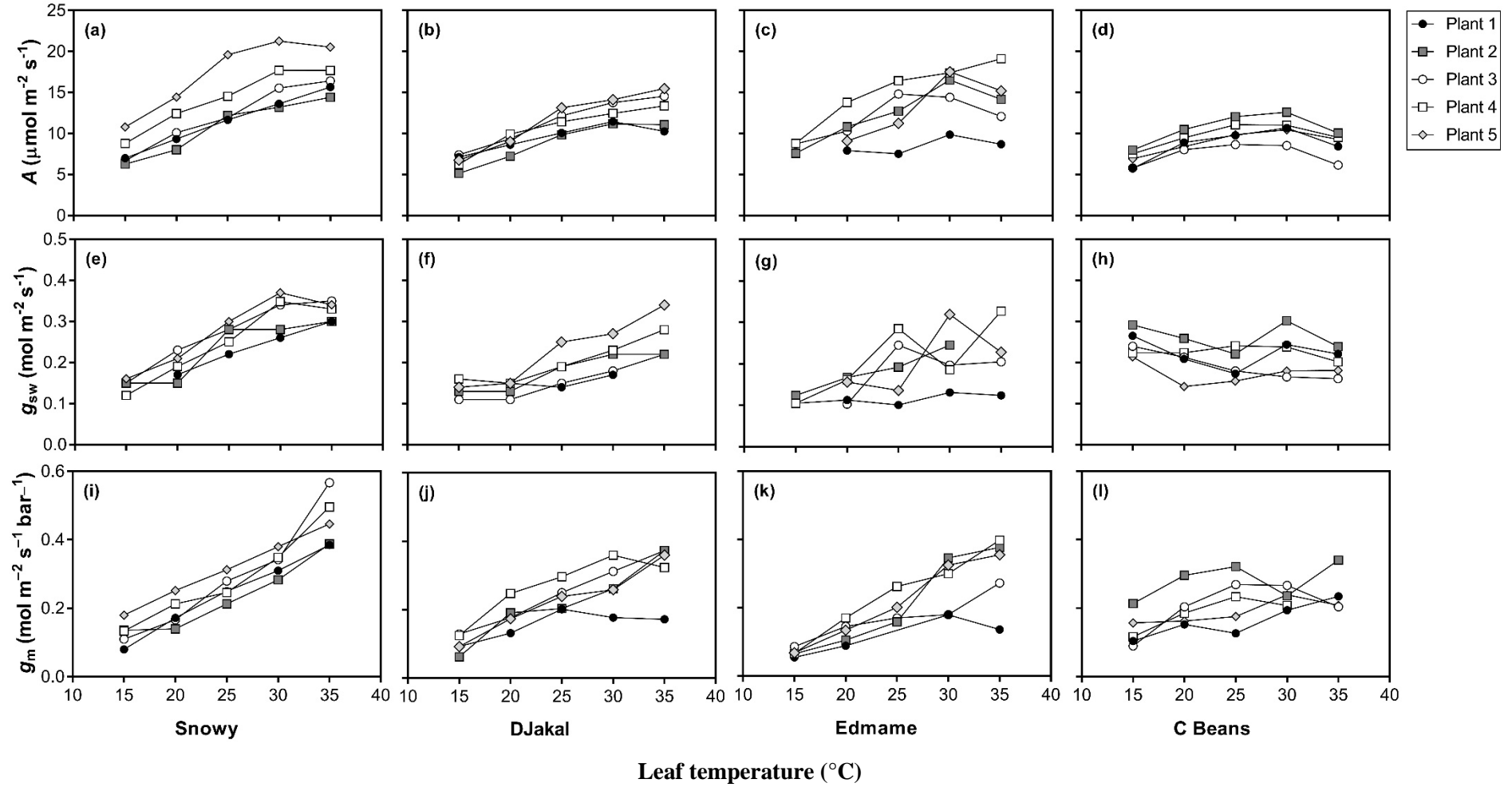
**Figure 7.4** Leaf mesophyll cells of soybean (Snowy genotype) after exposure to three different temperature steps; 15°C (a), 25°C (b), and 35°C (c). Each image is autofluorescence-DIC merged XY image at a single focal plane. Shown are typical mesophyll cells from 5 replicate samples from 5 individual plants.



**Figure 7.5** Leaf mesophyll cells of soybean (edamame genotype) after exposure to three different temperature steps; 15°C (a), 25°C (b), and 35°C (c). Each image is autofluorescence-DIC merged XY image at a single focal place. Shown is a typical mesophyll cell from 5 replicate samples from 5 individual plants.

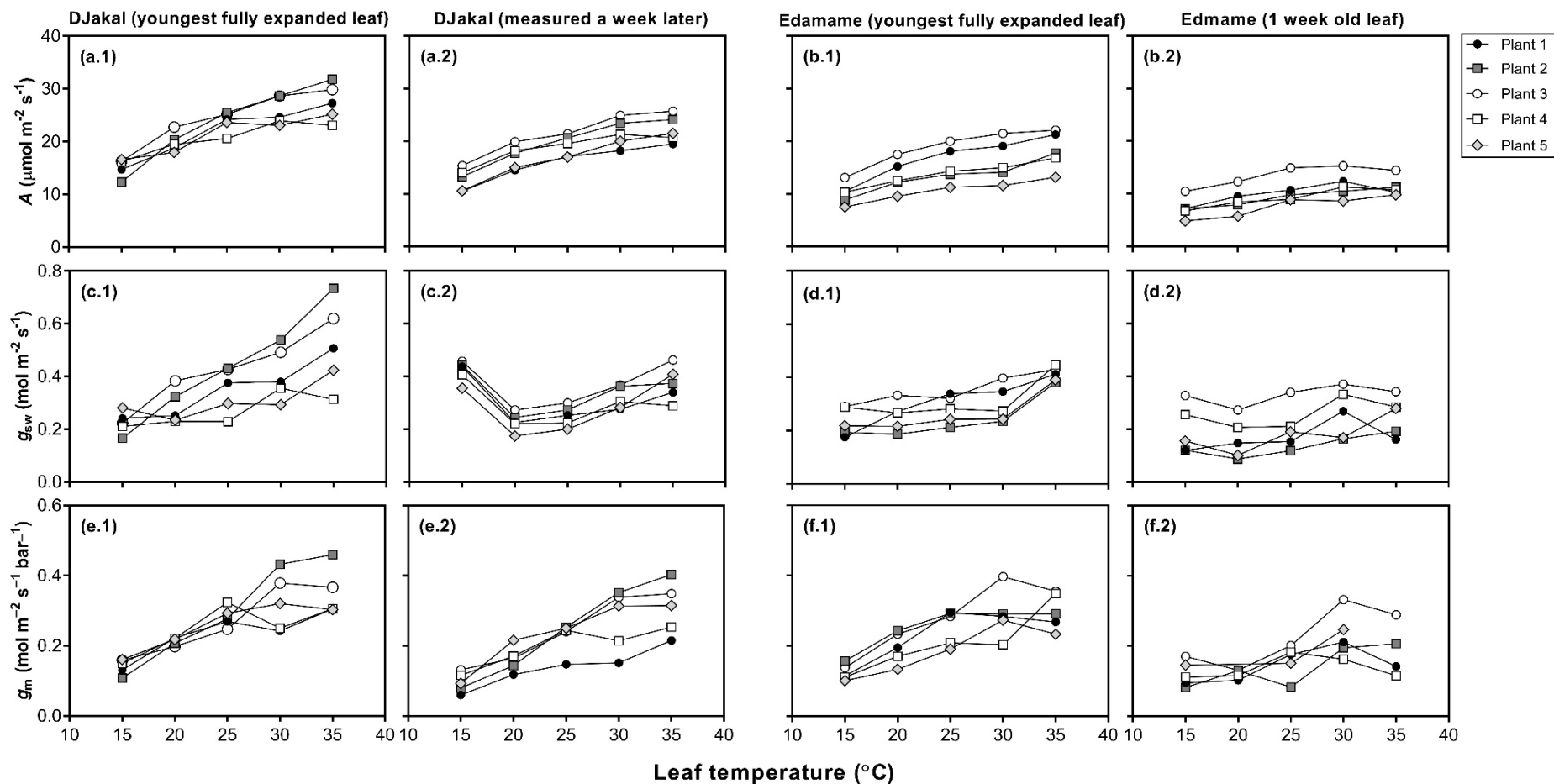


**Figure 7.6** Leaf mesophyll cells of soybean (DJakal genotype) after exposure to three different temperature steps; 15°C (a), 25°C (b), and 35°C (c). Each image is autofluorescence-DIC merged XY image at a single focal place. Shown are typical mesophyll cells from 5 replicate samples from 5 individual plants.



**Figure 7.7** Response of photosynthetic rate ( $A$ ; a to d), stomatal conductance ( $g_{sw}$ ; e to h) and mesophyll conductance ( $g_m$ ; i to l) to variation in leaf temperature for the individual replicate leaves of Snowy, DJakal and edamame (soybean genotypes) and common bean.





**Figure 7.8** Effects of leaf age on the temperature response of photosynthetic rate ( $A$ ; a.1 to b.2), stomatal conductance ( $g_{sw}$ ; c.1 to d.2) and mesophyll conductance ( $g_m$ ; e.1 to f.2) for the individual leaves ( $n=5$ ) of two soybean genotypes, DJakal and edamame. Measurements were made on the youngest fully expanded leaves and the same leaves were measured a week later.

**Table 7.1** Photosynthetic rate ( $A$ ), mesophyll conductance ( $g_m$ ) measured at 25°C and derived model parameter values fitted over the temperature range of 15-35°C for each replicate leaves of soybean genotypes and common bean.

	Reps	$A$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$g_m$ ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ )	$E$ ( $\text{kJ mol}^{-1}$ )	$P_{(\text{mem}25)}$	$T_{\text{opt}}$ ( $^{\circ}\text{C}$ )	$g_{\text{mem opt}}$	$b$	Sum of squares
Snowy	1	11.7	0.25	74.9	5.46E-04				0.002
	2	12.2	0.21	72.4	4.78E-04				0.002
	3	11.9	0.28	85.0	6.51E-04				0.003
	4	14.5	0.25	78.7	6.45E-04				0.002
	5	20.0	0.31	61.1	7.88E-04				0.002
	All reps	<b>14.1 ± 1.6</b>	<b>0.26 ± 0.02</b>	<b>77.1</b>	<b>6.36E-04</b>				<b>0.0003</b>
DJakal	1	10.1	0.20			28	0.014	0.474	0.0008
	2	9.8	0.20	76.1	4.78E-04				0.005
	3	12.1	0.25	66.4	5.78E-04				0.0003
	4	11.4	0.30			32	0.029	0.490	0.0008
	5	13.1	0.24	69.7	5.08E-04				0.002
	All reps	<b>11.3 ± 0.6</b>	<b>0.24 ± 0.02</b>			<b>45</b>	<b>0.029</b>	<b>0.663</b>	<b>0.0003</b>
Edamame	1	7.5				31	0.012	0.433	0.001
	2	12.7	0.16	82.7	4.84E-04				0.006
	3	14.8	0.17	61.6	3.71E-04				0.002
	4	16.4	0.26	78.5	5.42E-04				0.003
	5	11.2	0.20	82.1	4.75E-04				0.003
	All reps	<b>12.5 ± 1.5</b>	<b>0.20 ± 0.02</b>			<b>45</b>	<b>0.029</b>	<b>0.554</b>	<b>0.0002</b>
Common Beans	1	9.8	0.13	52.7	3.58E-04				0.002
	2	12.0	0.32			30	0.026	0.738	0.003
	3	8.6	0.27			27	0.022	0.368	0.0002
	4	11.0	0.23			26	0.018	0.425	0.0002
	5	9.8	0.18	35.0	4.36E-04				0.002
	All reps	<b>10.3 ± 0.6</b>	<b>0.22 ± 0.03</b>			<b>35</b>	<b>0.020</b>	<b>0.748</b>	<b>0.002</b>

Note: Surface area of chloroplast facing intercellular airspace per unit leaf area and effective pathlength was assumed to be 15 and 0.68  $\mu\text{m}$  as used by von Caemmerer and Evans (2015) in soybean

**Table 7.2** Photosynthetic rate ( $A$ ), mesophyll conductance ( $g_m$ ) measured at 25°C and derived model parameter values fitted over the temperature range of 15-35°C for the youngest fully expanded leaves and when the same leaves were measured a week later for DJakal and edamame

	Reps	$A$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$g_m$ ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ )	$E$ ( $\text{kJ mol}^{-1}$ )	$P_{(\text{mem}25)}$	$T_{\text{opt}}$ ( $^{\circ}\text{C}$ )	$g_{\text{mem opt}}$	$b$	Sum of squares
DJakal (the youngest fully expanded leaves)	1	24.2	0.27			36	0.023	0.690	0.002
	2	25.5	0.28	79.3	7.10E-04				0.005
	3	25.1	0.25	61.1	6.50E-04				0.004
	4	20.6	0.32			31	0.025	0.563	0.004
	5	23.6	0.29			31	0.026	0.588	0.0009
	All reps	<b>23.8 ± 0.9</b>	<b>0.28 ± 0.01</b>			<b>41</b>	<b>0.030</b>	<b>0.659</b>	<b>0.0002</b>
DJakal (same leaves measured a week later)	1	17.1	0.15			42	0.015	0.629	0.002
	2	20.6	0.25	82.3	5.54E-04				0.003
	3	21.4	0.24	65.1	5.68E-04				0.002
	4	19.6	0.24			33	0.019	0.617	0.001
	5	17.0	0.25			31	0.027	0.455	0.00007
	All reps	<b>19.6 ± 0.8</b>	<b>0.23 ± 0.02</b>			<b>46</b>	<b>0.027</b>	<b>0.650</b>	<b>0.0002</b>
Edamame (the youngest fully expanded leaves)	1	18.1	0.29			29	0.024	0.430	0.0008
	2	13.8	0.29			30	0.024	0.563	0.0001
	3	20.0	0.25			38	0.034	0.571	0.003
	4	14.3	0.21	65.7	4.67E-04				0.004
	5	11.3	0.19	55.8	4.21E-04				0.004
	All reps	<b>15.5 ± 1.6</b>	<b>0.25 ± 0.02</b>			<b>40</b>	<b>0.026</b>	<b>0.666</b>	<b>0.00005</b>
Edamame (same leaves measured a week later)	1	10.7	0.18			29	0.014	0.464	0.003
	2	9.8	0.08			39	0.016	0.659	0.00001
	3	14.9	0.21	55.6	5.23E-04				0.009
	4	9.0	0.18			25	0.012	0.493	0.002
	5	8.9	0.15	51.1	4.11E-04				0.002
	All reps	<b>10.7 ± 1.1</b>	<b>0.16 ± 0.02</b>	<b>47.1</b>	<b>3.65E-04</b>				<b>0.003</b>

## 7.4. Discussion

### 7.4.1. $g_m$ response to temperature varied within and among the genotypes

The temperature response of mesophyll conductance ( $g_m$ ) was studied for three genotypes of soybean and one genotype of common bean. Absolute values for gas exchange and  $g_m$  at 25°C for the three soybean genotypes were lower than the values reported in von Caemmerer and Evans (2015) for soybean. The lower values for photosynthetic rate in the present study for the first experiment might be due to the age of the plants at the time of measurements. The youngest fully expanded leaves of 8 week old plants were measured for the first experiment in this study. Loreto *et al.* (1994) reported 40% decline in net photosynthesis when wheat plants age from 30 to 45 days. In the leaf age experiment of this study, the measurements were made on 4 week old plants, and the youngest fully expanded leaves of DJakal had photosynthetic rates similar to that reported in von Caemmerer and Evans (2015) for soybean at 25°C. The values of  $A$  and  $g_m$  at 25°C, for the youngest fully expanded leaves of edamame in the second experiment of this study, were within the range reported for edamame genotypes in Tomeo and Rosenthal (2017).

In the present study, the temperature response of  $g_m$  varied between soybean and common bean. There was a 3-4 fold increase in  $g_m$  between 15 and 35°C in soybean genotypes, similar to the response observed by von Caemmerer and Evans (2015) for soybean between 15 and 40°C. Common bean showed less change in  $g_m$  over the temperature range, with 1.5 fold increases from 15 to 20°C but no clear differences in  $g_m$  for temperatures from 20 to 35°C. von Caemmerer and Evans (2015) also reported that the temperature response of  $g_m$  varied considerably between species, from 2-3 fold monotonic increase in  $g_m$  between 15 and 40 °C for some species to less than a 1.5 fold change for other species. In the current study, the temperature response of  $g_m$  (averaged for a genotype) was similar between the three genotypes of soybean, with no significant difference in the linear regression slope between the genotypes. However, there was a variation in the shape of the temperature response curve for  $g_m$  between the individual leaves within a genotype. Broadly, some leaves showed linear increase from 15 to 35°C while others had temperature optima at around 30°C. The temperature responses of  $g_m$  between 15 and 25°C were generally similar within a genotype in soybean, particularly when measured on the youngest fully expanded leaves. However, there was variation in  $g_m$  response to

temperature between 25 and 35°C within a genotype. In the current study, modelling the temperature dependence of  $g_m$  for individual leaves of each genotype also showed that there was variability in  $g_m$  response between the individual leaves within a genotype. Some leaves were best fitted to von Caemmerer and Evans (2015) exponential model, while the other leaves of the same genotype were best fitted to Warren and Dreyer (2006) optimum  $g_{mem}$  model.

Previous studies on the temperature dependence of  $g_m$  in several species have reported a wide range of responses including increases in  $g_m$  until 40°C, increase until 35°C and decline at higher temperatures, initial increase in  $g_m$  and then constant  $g_m$  from 30 to 35°C, constant from 28 to 38°C, constant from 20 to 35 or 40°C to almost no change over the temperature range (Bernacchi *et al.*, 2002, Gorton *et al.*, 2003, Pons & Welschen, 2003, Warren & Dreyer, 2006, Yamori *et al.*, 2006, Warren, 2008a, Evans & von Caemmerer, 2013, Walker *et al.*, 2013, von Caemmerer & Evans, 2015). The variability within a genotype in the present study together with the contrasting results from the previous studies on different species hints towards more diverse and complex CO<sub>2</sub> diffusion processes within a leaf. Moreover,  $g_m$  estimations from the current established methods are complicated and rely on models with several assumptions (Pons *et al.*, 2009). Variation in the temperature sensitivities of the model parameters and possibly different sensitivities of several other unidentified processes contributing to  $g_m$  might have caused a complex temperature response of  $g_m$ . The variability in the  $g_m$  response within a genotype may reflect the noise in measurements. However, at this point in time, I am unable to mechanistically explain the variability within a genotype observed in the present study. von Caemmerer and Evans (2015) suggested that the effective pathlength for liquid phase diffusion and  $g_{mem}$  (variation in the activation energy for membrane permeability to CO<sub>2</sub>) contributes to variation in the temperature response of  $g_m$  between species. The temperature dependence of the membrane phase component has not yet been completely defined; nonetheless,  $g_{mem}$  has been proposed to be determined by CO<sub>2</sub> diffusion through membranes via aquaporin-like enzymes (Flexas *et al.*, 2008, Evans *et al.*, 2009, Tholen & Zhu, 2011). Flexas and Diaz-Espejo (2015) published a commentary on von Caemmerer and Evans (2015) and highlighted three potential mechanisms for the rapid response of  $g_m$ : changes in cell wall properties (nature of chemical interactions between CO<sub>2</sub> and cell wall components), regulation of membrane properties (aquaporins) and reshaping and redistribution of chloroplasts (changes in  $S_c$ ). Although the chloroplast distribution

and thus  $S_c$  are known to be affected by rapid changes in blue light intensity (Tholen *et al.*, 2008), there is no confirmed evidence, to date, of the changes in  $S_c$  with temperature that can be related to  $g_m$ . Variation in  $g_m$  response in the current study suggested that  $g_m$  may be determined by multiple processes including aquaporins or other membrane enzymes or may reflect instrument noise in  $g_m$  measurements.

#### **7.4.2. Are chloroplast protrusions responsible for the $g_m$ response to temperature**

In the present study, the influence of dynamic change in temperature on the formation of chloroplast protrusions was examined in the mesophyll cells of three soybean genotypes and *A. thaliana*. I did not find clear evidence of chloroplast protrusions at any temperature in soybean genotypes or in *A. thaliana*. Holzinger *et al.* (2007a) observed an increase in chloroplast protrusions in mesophyll cells in *A. thaliana* in response to an increase in temperature. However, studies have shown that there is no significant temperature response of  $g_m$  in *A. thaliana* (Walker *et al.*, 2013, von Caemmerer & Evans, 2015). Therefore, the increase in chloroplast protrusions observed by Holzinger *et al.* (2007a) may not be related to a  $g_m$  response to temperature in *A. thaliana*. Moreover, Lütz (2010), in his review article, stated that chloroplast protrusions are not observed in all plant species and suggested that protrusions are mostly absent in *A. thaliana*. Chloroplast protrusions have been mostly observed in alpine and polar plants (Holzinger *et al.*, 2007b, Lütz, 2010, Buchner *et al.*, 2015, Moser *et al.*, 2015) although the occurrence of CPs are not exclusive to high mountain plants (Lütz, 2010). Two studies have observed salt-induced CPs in soybean (He *et al.*, 2014) and rice (Yamane *et al.*, 2012). Moser *et al.* (2015) also demonstrated that in *Ranunculus glacialis* L., chloroplast protrusions were not a result of heat or light stress but were most abundant under moderate temperature and non-stress irradiation conditions. Based on the lack of evidence of CPs in response to increasing temperature in the experiments described here, I conclude that CPs are not involved in the  $g_m$  response to temperature.

#### **7.4.3. Genotypes differ in the effect of leaf age on the temperature response of $g_m$**

The influence of leaf age on the temperature response of  $g_m$  was examined for DJakal and edamame. Genotypes differed in the effect of leaf age on  $g_m$  values. There was no significant effect of leaf age on  $g_m$  for DJakal, while for edamame, the older leaves had significantly

reduced  $g_m$  compared to younger leaves at 20, 25 and 35°C. The differing leaf age effect on  $g_m$  may be due to differing rates of plant growth or leaf development between the genotypes, and the effect of leaf age on  $g_m$  in DJakal might be significant if measurements had been made on more mature leaves. Nonetheless, leaf age reduced photosynthetic rates and stomatal conductance in both the genotypes. Previous studies have found that  $g_m$  decreases as leaves age (Flexas *et al.*, 2008, Jahan *et al.*, 2014, Barbour *et al.*, 2016b). Jahan *et al.* (2014) found a significant interactive effect of genotype by leaf age (5-8 days) on  $g_m$  in wheat, with reduced  $g_m$  for older leaves in one genotype but no change in the other genotype. Hanba *et al.* (2001) showed that variation in  $g_m$  with leaf age is partly related to  $S_c$  in deciduous trees, maple, alder and Japanese poplar. However, Barbour *et al.* (2016b) did not find relationships between  $g_m$  and  $S_c$  despite the reductions in both  $g_m$  and  $S_c$  in the older leaves in cotton and wheat.

Genotypes differed in the effect of leaf age on the temperature dependence of  $g_m$  in soybean.  $g_m$  response to temperature was similar between the youngest fully expanded leaves and when the same leaves were measured one week later in DJakal, while leaf age affected the temperature response  $g_m$  in edamame. The linear relationship between  $g_m$  and temperature was weaker for edamame leaves measured one week later than for the same leaves when they had just finished expanding. When the response of individual leaves of edamame was considered, the linear temperature response of  $g_m$  observed in the youngest fully expanded leaves disappeared in the same leaves measured one week later. The differing response could be attributed to differences in enzyme activities involved in CO<sub>2</sub> diffusion between the genotypes (not tested in the present study). The differing effect of leaf age on temperature response of  $g_m$  between genotypes could be one of the reasons for the conflicting results observed for the temperature response of  $g_m$  between studies with different species.

## **7.5. Conclusions**

The temperature response of mesophyll conductance to CO<sub>2</sub> was assessed in three genotypes of soybean and a single genotype of common bean.  $g_m$  response to temperature varied between soybean and common bean. All three soybean genotypes responded similarly to rapid increase in leaf temperature. However, I found diversity in the response among the individual leaves within each genotype. Some leaves showed linear increases in  $g_m$  with increasing temperature, some

leaves showed temperature optima at around 25°C or higher, while a small number of leaves showed no clear trend. I do not have an explanation for the variability in the response within genotypes. Results from the previous studies in different species have also shown diverse range of temperature response for  $g_m$ . Taken together, this suggests that the temperature response of  $g_m$  is much more complex and diverse, and several other processes with different temperature dependencies might be contributing to  $g_m$ , including aquaporins or other enzymes across membranes. The variability in the  $g_m$  response within a genotype in this study may also be related to noise in the  $g_m$  measurements, which rely on models with several assumptions. Furthermore, I found that the temperature response of  $g_m$  can be affected by leaf age in some genotypes. The results of the current study implied that assessing the general temperature response of  $g_m$  could be improved by including a higher number of replicates and ensuring that all leaves are of the same developmental age at the time of the measurements. Sensitivity analysis of the model parameters to a range of measurement conditions should be considered in future studies.



## 8. General Discussion

Conductance to CO<sub>2</sub> diffusion from substomatal cavities to sites of carboxylation in chloroplasts (mesophyll conductance;  $g_m$ ) has been recognized as a significant and variable limitation to photosynthesis;  $A$  (Flexas *et al.*, 2008), and a potential target for improving leaf intrinsic water use efficiency;  $A/g_{sw}$  (Barbour *et al.*, 2010).  $g_m$  has been extensively studied in the past few decades. However, there are still significant unknowns that researchers have been grappling with, namely (a) how  $g_m$  varies within species and in response to dynamic or growth environments, (b) what are the potential determinants of  $g_m$ , and (c) can we simultaneously improve  $A$  and  $A/g_{sw}$  by increasing  $g_m$ ? Recently, there also has been a growing interest in understanding the relationship between leaf internal conductances to carbon and water i.e. the relationship between mesophyll conductance to CO<sub>2</sub> and leaf hydraulic conductance ( $K_{leaf}$ ). This thesis aimed to address these questions and contribute to the current body of work on understanding  $g_m$  regulation. The studies are focussed on grain legumes because there has been an increasing realization of the importance of grain legumes in human health and sustainable cropping systems, and  $g_m$  may have significant unexploited potential for genetic improvements in legumes. However, information on  $g_m$  is limited for grain legumes compared to cereal crops. This thesis addressed the questions listed in Table 8.1, and found several important results (Table 8.1) through series of experiments. This discussion chapter synthesizes the results from the previous chapters to provide the overall conclusions of this thesis.

**Table 8.1** Summary of the thesis questions and the major experimental findings.

Thesis Questions	Major findings
1. Does variation in $g_m$ exist within and among legume species under non-limiting environments?	<ul style="list-style-type: none"> <li>• <math>g_m</math> varied between species.</li> <li>• Genotypes varied in <math>\Delta^{13}\text{C}-g_m</math> in faba bean, garden pea and field pea.</li> <li>• Genotypes did not vary in <math>\Delta^{18}\text{O}-g_m</math>.</li> </ul>
2. Does genetic variation exist for $g_m$ in response to growth environments?	<ul style="list-style-type: none"> <li>• Genotypes differed in their <math>g_m</math> response to water availability.</li> <li>• Genotypes differed in the response of <math>g_m</math> to nitrogen source.</li> </ul>
3. Does $g_m$ respond to short-term environmental changes, and is there an interactive response of $g_m$ between short- and long-term environmental changes?	<ul style="list-style-type: none"> <li>• <math>g_m</math> showed a positive linear response to light intensity.</li> <li>• Blue light reduced <math>g_m</math>.</li> <li>• Genotypes differed in the interactive response of <math>g_m</math> to light intensity, light quality and water stress.</li> <li>• Genotypes differed in the interactive response of <math>g_m</math> to light intensity and nitrogen source.</li> <li>• Temperature response of <math>g_m</math> varied between species.</li> <li>• Genotypes differed in the degree to which leaf age influenced the temperature response of <math>g_m</math>.</li> </ul>
4. To what degree does leaf anatomy influence $g_m$ ?	<ul style="list-style-type: none"> <li>• Genotypes differed in the degree to which leaf anatomy influenced <math>g_m</math>.</li> <li>• Overall, the environmentally driven leaf anatomical traits were not the major factor determining <math>g_m</math>.</li> </ul>
5. How variable is chloroplast membrane conductance?	<ul style="list-style-type: none"> <li>• Chloroplast membrane conductance (<math>g_{cm}</math>) varied between legume species</li> </ul>

	<ul style="list-style-type: none"> <li>• Genotypes varied in <math>g_{cm}</math> in garden pea and field pea</li> <li>• <math>g_{cm}</math> varied with growth environments</li> </ul>
6. Are $g_m$ and $K_{leaf}$ correlated due to leaf anatomy across genotypes and growth environments?	<ul style="list-style-type: none"> <li>• <math>g_m</math> and <math>K_{leaf}</math> are not correlated for five faba bean genotypes grown under four environments.</li> </ul>
7. How well does $g_m$ scale with $A$ and $A/g_{sw}$ under various environments for different species?	<ul style="list-style-type: none"> <li>• The closeness of <math>g_m</math> and <math>A/g_{sw}</math> relationships depended on species and environmental conditions.</li> <li>• <math>g_m</math> was significantly, though not strongly, related to <math>A/g_{sw}</math> in field pea under non-limiting environments.</li> <li>• <math>g_m</math> was significantly related to <math>A/g_{sw}</math> in chickpea, particularly under water-limiting environments.</li> </ul>
8. Methodological issues/measurement sensitivity	<ul style="list-style-type: none"> <li>• There was a large variability in the short-term temperature response of <math>g_m</math> between individual leaves within a single genotype.</li> </ul>

## 8.1. Synthesis of the results

### 8.1.1. $g_m$ variation within and among legume species under ideal conditions

Chapter 4 investigated the variability in  $g_m$  within and among important legume species; common bean, faba bean, garden pea and field pea, grown and measured under non-limiting environments. I observed genotypic variability in  $\Delta^{13}C$ - $g_m$  in faba bean, garden pea and field pea but not in common bean. This is not a novel finding as previous studies have also shown  $g_m$  variability among species (reviewed in Flexas *et al.* (2008) or within a species, but notably in cereals (Barbour *et al.*, 2010, Gu *et al.*, 2012, Jahan *et al.*, 2014, Xiong *et al.*, 2017) and in a few other crops like *Castanea sativa* (Lauteri *et al.*, 1997), and *Vitis vinifera* (Tomás *et al.*, 2014) and recently among soybean (edamame) genotypes (Tomeo & Rosenthal, 2017). But the information

on  $g_m$  variability in legumes was limited before this study, making it difficult to include  $g_m$  in legume crop improvement programs or in photosynthesis models.

More importantly, chapter 4 also looked at the variability in  $g_m$  components determined experimentally by partitioning  $g_m$  into cell wall and plasma membrane conductance (measured from the oxygen isotope method;  $\Delta^{18}\text{O}-g_m$ ) and chloroplast membrane conductance (calculated from the  $g_m$  estimates from carbon and oxygen isotope methods;  $g_{cm}$ ). I did not find genetic variation in  $\Delta^{18}\text{O}-g_m$  across the species investigated here. This suggests that the observed genetic variation in  $g_m$  may be related to the variation in chloroplast membrane conductance.  $g_{cm}$  also varied between legume species. Partitioning  $g_m$  also showed that the resistance to diffusion lies mostly in cell wall and plasma membrane in faba bean, while the resistance is divided nearly equally between the cell wall/plasma membrane and the chloroplast membrane for most of the genotypes in common beans, garden peas, and field peas. However, as discussed in chapter 4 and in Barbour *et al.* (2016b), the interpretation of the  $\Delta^{18}\text{O}-g_m$  should be made cautiously as  $\Delta^{18}\text{O}-g_m$  relates to the conductance to  $\text{CO}_2$  from intercellular air spaces to the location of  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium, which further depends on the location and activity of carbonic anhydrase (CA).  $\Delta^{18}\text{O}-g_m$  was higher than  $\Delta^{13}\text{C}-g_m$  for most of the grain legume genotypes in this study suggesting that  $\text{CO}_2\text{-H}_2\text{O}$  equilibrium occurred outside the chloroplast and the equilibrium was assumed to be at the chloroplast surface in this study, as proposed by Gillon and Yakir (2000). Nevertheless, CA isoforms have been localized to most intracellular compartments (Fabre *et al.*, 2007, Dimario *et al.*, 2017). If the  $\text{CO}_2\text{-H}_2\text{O}$  equilibration had occurred at the plasma membrane, then  $\Delta^{18}\text{O}-g_m$  would have related to conductance through the cell wall only, in which case the observed variation in total mesophyll conductance might relate to variability in plasma membrane permeability as well as chloroplast membrane permeability.

Other chapters in this thesis also covered  $g_m$  variability within or among other legume species. The genetic variation observed in faba bean (between PBA Rana and Cairo) grown and measured under ideal conditions (chapter 4) disappeared when they were grown and measured under differing environmental conditions (chapter 5). Chapter 6 showed that  $g_m$  varies between chickpea genotypes grown under different water availability and nitrogen sources. Chapter 7,

which studied the temperature response of  $g_m$ , showed that common bean and soybean genotypes had similar  $g_m$  values at 25°C.

### **8.1.2. $g_m$ response to growth environment**

Different legume species and genotypes grown and measured under different environmental conditions provided a surprising diversity in  $g_m$  response to environments. Chapter 5 examined the effect of differing growth environments on  $g_m$  and its components, cell wall plus plasma membrane conductance ( $\Delta^{18}\text{O}-g_m$ ) and chloroplast membrane conductance ( $g_{cm}$ ) in five genotypes of faba bean. Different growth environments were created by varying only  $\text{CO}_2$  and light but other parameters like temperature and humidity, though set to match, might have varied a little between the environments. Hence, the variations in the measured parameters including  $g_m$  were the general effects of the growth environments and not the individual effect of  $\text{CO}_2$  or light.  $g_m$  was significantly affected by the growth environment. Both the components of  $g_m$  (cell wall plus plasma membrane conductance and chloroplast membrane conductance) varied similarly, with the highest values at ambient  $\text{CO}_2$  plus high light environment compared to other growth environmental conditions. In contrast, Loucos *et al.* (2017) observed higher sensitivity of chloroplast membrane conductance to growth environment in a single cotton genotype.

Chapter 6 covered two experiments that investigated the response of  $g_m$  to measurement (light intensity and light quality) and growth conditions (water availability in experiment 1 and nitrogen source in experiment 2) in different genotypes of chickpea. Water stress decreased  $g_m$  only in Sonali and the reduction was only significant when measured under red light. The genotypes responded differently to water availability. Sonali was the most sensitive to water stress, with the highest  $g_m$  of the chickpea genotypes under well-watered conditions but the lowest when water-stressed. Variability in the  $g_m$  response to water stress has been shown previously to be related to species differences and the intensity and/or duration of stress (Grassi & Magnani, 2005, Perez-Martin *et al.*, 2014, Theroux-Rancourt *et al.*, 2014). Our results further suggest that genotypes of a single species may vary in  $g_m$  response to water stress. This result is particularly important when including  $g_m$  as a trait in breeding programs for water-limiting environments.

Similar to water availability, genotypes differed in the sensitivity of  $g_m$  to nitrogen source. Flip079C had higher  $g_m$  when fertilized with  $\text{NH}_4\text{NO}_3$  (non-inoculated) than when nitrogen was fixed by *Rhizobium* inoculate. In contrast, the genotypes responded similarly to nitrogen source in terms of photosynthetic rate and leaf N content. N-  $g_m$  relationships have generally been found to be weak (Warren, 2004, Barbour & Kaiser, 2016). This is the first work to examine the response of  $g_m$  to N-fixing versus N-fed legumes. It is not clear how nitrogen source could affect  $g_m$  in some genotypes but not in others. The proportion of N derived from N-fixation is lower in chickpeas than in other cool-season legumes, with similar values for all the genotypes studied here (Dr. C. Blessings, the University of Sydney, personal communication, 07 November, 2016). Preferential nitrogen assimilation by either one of these routes in different genotypes might lead to differences in modifications in leaf architecture or in the influence of N on other physiological or biochemical factors contributing to  $g_m$ , giving rise to differences in the  $g_m$  response. Nevertheless, the result is interesting in the context of increasing recognition of the legume based farming systems and thus needs further consideration.

### **8.1.3. $g_m$ response to short-term environmental changes, and the interactive response of $g_m$ between short- and long-term environmental changes**

$g_m$  was also found to respond to short-term changes in environmental conditions, and the rapid responses of  $g_m$  were affected by other environmental conditions. Rapid changes in light quality (red light or blue light) and light intensity showed significant effects on  $g_m$  (chapter 6, experiment 1). Blue light reduced  $g_m$  when measured at different light intensities for both well-watered and water-stressed plants but the reduction was not significant for water-stressed plants in Sonali, and at lower light intensities in Amethyst.  $g_m$  increased linearly with light intensity across light quality and water treatments except for the water-stressed PBA Slasher and Sonali measured under blue light. Similarly, genotype differences in  $g_m$  sensitivity to light intensity were altered by N source (N-fed or N-fixing) in chapter 6, experiment 2.  $g_m$  values showed linear increases with rapid changes in leaf temperature across soybean genotypes, while  $g_m$  in common bean was less sensitive over the temperature range (chapter 7). Soybean genotypes differed in the degree to which leaf age influenced the temperature response of  $g_m$ . In DJakal, the temperature responses of  $g_m$  were similar for the youngest fully expanded leaves ( $R^2=0.71$ ) and when the same leaves were measured a week later ( $R^2=0.64$ ). However, in edamame, the linear

relationship between  $g_m$  and temperature was weaker for leaves measured one week later ( $R^2=0.31$ ) than for the same leaves when they had just finished expanding ( $R^2=0.64$ ). When the response of individual leaves of edamame was considered, the linear response to temperature observed in the youngest fully expanded leaves disappeared in the same leaves measured one week later.

Previous studies have shown differences between species and genotypes in the sensitivity of  $g_m$  to environmental conditions. For example, von Caemmerer and Evans (2015) observed that the temperature response of  $g_m$  differed greatly between species, and Barbour and Kaiser (2016) reported genetic variation in the interactive response of  $g_m$  to N and water availability in wheat. The present results with different legumes species and genotypes under various growth environments are statistically remarkable, and they contribute to the general knowledge of the variability in  $g_m$ , showing the  $g_m$  response to environments is much more variable than previously recognized. These results have important implications for photosynthesis model parameterization and scaling, as the gas exchange responses to temperature, light,  $[CO_2]$  are used in the model, and at present the model assumes constant  $g_m$ . The present results are helpful in future simulation analysis for legume crops at various environments under climate change scenarios.

#### **8.1.4. Mechanistic basis for $g_m$ variability**

Environmentally driven changes in leaf anatomical traits including leaf thickness, surface area of mesophyll ( $S_{mes}$ ) and chloroplast exposed to the intercellular air spaces ( $S_c$ ) and the fraction of intercellular air space ( $f_{ias}$ ) were not the major factors determining  $g_m$  in faba bean (chapter 5). Genotypes differed in the effects of leaf anatomy on total  $g_m$  and its components;  $\Delta^{18}O-g_m$  and  $g_{cm}$ . Leaf thickness was weakly, but significantly, related to  $g_m$ ,  $\Delta^{18}O-g_m$  and  $g_{cm}$  for two genotypes but not for the three others. Similarly,  $S_{mes}$  and  $S_c$  were weakly, but significantly, related to  $g_m$ ,  $\Delta^{18}O-g_m$  and  $g_{cm}$  for one genotype but not for the four others.  $f_{ias}$  was not related to  $g_m$  for any genotype. Variation in leaf thickness,  $S_{mes}$  and  $S_c$  may affect the number of parallel pathways, effective pathlength and area for  $CO_2$  diffusion and thus contribute to  $g_m$ .  $g_m$  has been found to correlate with leaf anatomical traits in several studies (Evans *et al.*, 1994, Scafaro *et al.*, 2011, Terashima *et al.*, 2011, Tholen & Zhu, 2011, Peguero-Pina *et al.*, 2012, Tosens *et al.*,

2012a, Xiong *et al.*, 2015a, Peguero-Pina *et al.*, 2016, Xiong *et al.*, 2017), but not in other (Evans & Vellen, 1996, Hanba *et al.*, 2001, Hanba *et al.*, 2002, Hanba *et al.*, 2004, Miyazawa *et al.*, 2008, Tomás *et al.*, 2014). The relationships between anatomical traits and  $g_m$  vary between plant species (Fini *et al.*, 2016) and the results of the current study showed that the importance of leaf anatomy in  $g_m$  also varies between genotypes within a species. Cell wall thickness has also been shown as one of the important determinants of  $g_m$ , particularly in species with thick leaves (Tomás *et al.*, 2013). Thick cell walls would increase effective path length for CO<sub>2</sub> diffusion, thus reducing  $g_m$ .

As discussed earlier, chapter 4 looked at the variability in  $\Delta^{18}\text{O}-g_m$  and  $g_{cm}$  between genotypes of different legume species.  $\Delta^{18}\text{O}-g_m$  did not vary between genotypes across species, suggesting that the observed genetic variation in  $g_m$  may be related to variation in chloroplast membrane conductance. Considering the weak relationships between leaf anatomy and  $g_m$  in faba bean in the current study and among soybean genotypes reported by Tomeo and Rosenthal (2017), as well as the observed variation in  $g_m$  in response to short-term environmental changes, I speculate the variation in  $g_m$  may be related to leaf physiological or biochemical traits controlling the transport of carbon in the liquid phase from the intercellular air spaces to chloroplast stroma. Variation in  $g_m$  in *Castanea sativa*, was related to differences in leaf protein content (Lautery *et al.* 1997). Aquaporins (AQPs) are membrane proteins that facilitate membrane water transport (Maurel and Chrispeels, 2001). There is evidence that certain AQPs can also facilitate CO<sub>2</sub> transport across the plasma and chloroplast membranes (Terashima & Ono, 2002, Uehlein *et al.*, 2003, Kaldenhoff, 2012, Mori *et al.*, 2014, Maurel *et al.*, 2015, Uehlein *et al.*, 2017). Genetic variation in  $g_m$  and its response to environment could be due to variation in aquaporin expression and/or activity within plasma membranes and/or chloroplast membranes (Bernacchi *et al.*, 2002, Hanba *et al.*, 2004). Carbonic anhydrase (CA) catalyses the reversible interconversion of CO<sub>2</sub> with HCO<sub>3</sub><sup>-</sup>, and could contribute to  $g_m$  by maintaining a nearly constant CO<sub>2</sub> concentration throughout the stroma, allowing a more effective use of the available Rubisco (Tholen & Zhu, 2011). Gillon and Yakir (2000) suggested that the contribution of CA to  $g_m$  is species dependent, and that CA may become more important when  $g_m$  is low as in sclerophyll species. Moreover, CA has been recently shown to interact with aquaporin as part of a transport metabolon regulating stomatal closure in response to internal leaf CO<sub>2</sub> concentrations (Wang *et al.*, 2016).



The coupling of CA and aquaporin could enhance  $g_m$  by creating a CO<sub>2</sub> concentration gradient adjacent to the chloroplast membranes.

The reduction of  $g_m$  under blue light, observed in chapter 6, could be related to chloroplast movement away from the blue light to avoid the photodamage to photosynthetic machinery in the avoidance response. The avoidance response would result in lower  $S_c$  under high blue light, as reported by Tholen *et al.* (2008) in *Arabidopsis thaliana*. However, Loreto *et al.* (2009) showed that the rapid reduction of  $g_m$  under blue light in *Nicotiana* and *Platanus* leaves was faster than any possible chloroplast movement and the response was still observed after the chloroplast movement inhibition. They suggested that the observed response of  $g_m$  to blue light might be the effect of blue light on photosynthesis through changes in photochemical efficiency. Alternatively, the  $g_m$  response to blue light might be related to changes in membrane permeability through aquaporin.

As well as changes in position, changes in chloroplast shape may also affect  $S_c$ . Holzinger *et al.* (2007a) observed an increase in chloroplast protrusions in mesophyll cells in *A. thaliana* in response to an increase in temperature. However, I did not find clear indication of chloroplast protrusions (which if present would have increased  $S_c$  and thus affected  $g_m$ ) at different temperatures in soybean genotypes and *A. thaliana* (chapter 7), and studies have found no significant temperature response of  $g_m$  in *A. thaliana* (Walker *et al.*, 2013, von Caemmerer & Evans, 2015). Therefore, the increase in chloroplast protrusions observed by Holzinger *et al.* (2007a) could not be related to  $g_m$  response to temperature. Moreover, chloroplast protrusions have not been observed in all plant species (Lütz, 2010), and Moser *et al.* (2015) demonstrated that in *Ranunculus glacialis* L., chloroplast protrusions were not a result of heat or light stress but were most abundant under moderate temperature and non-stress irradiation conditions.

$g_m$  is a complex leaf variable and is determined not by a single trait, but rather by several different and often covarying structural and biochemical elements, all of which are incompletely understood. von Caemmerer and Evans (2015) proposed that the differences in the temperature response of  $g_m$  between species may be due to variation in the activation energy for membrane permeability to CO<sub>2</sub> (suggesting the involvement of fast biochemical components like

aquaporins in the regulation of  $g_m$ ) and the effective pathlength for liquid phase diffusion (referring mostly to cell wall thickness, but also to cytosol thickness). They used a simplified two-component model outlined in Evans and von Caemmerer (2013) to describe the temperature response of  $g_m$ . Flexas and Diaz-Espejo (2015) published a commentary on von Caemmerer and Evans (2015) and highlighted three potential mechanisms for the rapid response of  $g_m$ : changes in cell wall properties (nature of chemical interactions between  $\text{CO}_2$  and cell wall components), regulation of membrane properties (aquaporins) and reshaping and redistribution of chloroplasts (changes in  $S_c$ ).  $g_m$  variability observed in the current study could also be due to variation/covariation of afore-mentioned factors plus other as yet unknown factors.

#### **8.1.5. Coordination between $g_m$ and $K_{\text{leaf}}$**

Chapter 5 examined the  $g_m$ - $K_{\text{leaf}}$  relationships in faba bean when variation in the traits was related to leaf anatomy through genotype and growth environment effects. I partitioned the total  $g_m$  into cell wall/plasma membrane conductance and chloroplast membrane conductance using  $\Delta^{18}\text{O}$  and  $\Delta^{13}\text{C}$  techniques, assuming  $\text{CO}_2$ - $\text{H}_2\text{O}$  equilibration at the chloroplast surface.  $K_{\text{leaf}}$  was not correlated to total  $g_m$  or to the components of  $g_m$ , and  $g_m$  and  $K_{\text{leaf}}$  did not share any of the studied anatomical determinants within each genotype of faba bean. These results were contrary to previous studies, which reported  $g_m$  and  $K_{\text{leaf}}$  correlation across species (Flexas *et al.*, 2013b), within a single genus mediated by leaf anatomy (Xiong *et al.*, 2017), and within a single species but only as drought progressed (Theroux-Rancourt *et al.*, 2014). A recent study in cotton (Loucos *et al.*, 2017) reported weak correlations between  $\Delta^{18}\text{O}$ - $g_m$  and  $K_{\text{leaf}}$  and between total  $g_m$  and  $K_{\text{leaf}}$  through differences in leaf anatomy. The lack of a significant relationship in the present study might be due to separation of the  $\text{CO}_2$  and water pathways or due to the independent regulation of  $\text{CO}_2$  and water transport in leaves, perhaps as a result of  $\text{CO}_2$ - and  $\text{H}_2\text{O}$ - specific aquaporin.

#### **8.1.6. $g_m$ as a potential target for legume crop improvement**

$g_m$  relationships with  $A$  and  $A/g_{\text{sw}}$  were studied across the experiments in this thesis.  $g_m$  was strongly correlated to leaf photosynthetic rate under a range of environments (growth and measurement) for different legume species, as reported for cereal and other crops (Flexas *et al.*, 2008, Barbour *et al.*, 2010, Jahan *et al.*, 2014), supporting the suggestion of enhancing  $A$  through

selection for increased  $g_m$ . However, the closeness of  $g_m$  relationships with leaf water use efficiency depended on species and environmental conditions. Under non-limiting environments (chapter 4),  $g_m$  was significantly, though not strongly, related to  $A/g_{sw}$  across field pea genotypes. Of the field pea genotypes studied, Para had among the highest  $A$ ,  $g_m$  and  $A/g_{sw}$  and among the lowest  $g_{sw}$ . I also found a positive correlation between  $g_m$  and  $A/g_{sw}$  for chickpea genotypes grown under well-watered or water-stressed conditions, and the correlation was stronger under water-limiting environments (chapter 6).  $g_m$  was not correlated to  $g_{sw}$  in field peas under ideal conditions or in chickpea under different water availability. The combination of low  $g_{sw}$  and high  $g_m$  would produce high leaf-intrinsic water-use efficiency while maintaining or increasing photosynthetic rates (Barbour *et al.*, 2010, Buckley & Warren, 2014, Flexas *et al.*, 2016). The presence of genetic variation in  $g_m$  and the positive relationships of  $g_m$  with  $A$  and with  $A/g_{sw}$  but not with  $g_{sw}$ , especially in field peas and in chickpeas under moderate water-stress conditions, demonstrates the potential of  $g_m$  in improving photosynthetic rates and leaf intrinsic water use efficiency within a crop improvement program. However, there was generally a large genotype  $\times$  environment interaction in chickpea, as discussed earlier and in chapter 6. This suggests that screening for  $g_m$  should be carried out under a range of environmental conditions.

$g_m$  was not related to  $A/g_{sw}$  in common bean, faba bean and garden peas grown and measured under non-limiting environments. Further, the study on faba bean genotypes grown and measured under differing irradiance and  $CO_2$  partial pressure did not find any relationship between  $g_m$  and  $A/g_{sw}$ , but a significant relationship  $g_m$  and  $g_{sw}$ . Correlation between  $g_m$  and  $g_{sw}$  might imply a trade-off between photosynthesis and WUE in faba bean under the growth conditions, as also reported by Tomeo and Rosenthal (2017) in soybean cultivars grown and measured under ideal conditions. Positive relationships between  $g_m$  and  $A$  in these species suggest considerable scope for improvement of  $A$  through increased  $g_m$  but concurrent improvement in  $A/g_{sw}$  would likely require simultaneous stabilizing selection of  $g_{sw}$ .

#### **8.1.7. Methodological issues/measurement sensitivity**

Variability in temperature responses of  $g_m$  between individual leaves within a genotype (chapter 7) reflects the sensitivities of the measurements/model parameters to environments and/or instrumentation noise in measurements. Reliability of the  $g_m$  estimates from the current

techniques depends on model assumptions and estimates of the values of the model parameters for the calculation of  $g_m$ . Despite some progress achieved in limiting uncertainties induced by model parameters (Tholen *et al.*, 2012, Gu & Sun, 2014), mesophyll conductance estimation has still been technically challenging. It would be necessary in future studies to conduct sensitivity analysis of the model parameters to a wide range of measurement and growth conditions. Experimental testing of the assumptions is core to the reliability of the  $g_m$  estimates, and further enhancement on the laser technology to limit the instrumentation noises would be of great benefit.

## 8.2. Future work

- Simultaneous measurements of  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  in this study provided better understanding of the relative contributions of the  $g_m$  components before and after  $\text{CO}_2\text{-H}_2\text{O}$  equilibration, and thus have the potential to identifying targets to genetically manipulate  $g_m$ . Therefore this topic deserves priority in future studies, ideally, together with the assessment of the location and activity of CA to identify the location of the equilibration, and thus correctly interpret  $\Delta^{18}\text{O}-g_m$  estimates.
- Concurrent  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  estimates under differing growth environments showed how the different components of  $g_m$  (cell wall/plasma membrane and chloroplast membrane conductance) were regulated. More studies on how  $\Delta^{18}\text{O}-g_m$  and  $g_{cm}$  vary in different species and environmental conditions would increase our mechanistic understanding of  $g_m$  response to environments and thus would increase our ability to improve plants under stressful conditions.
- The temperature responses of  $\Delta^{18}\text{O}-g_m$  and  $\Delta^{13}\text{C}-g_m$  may reveal the location of  $\text{CO}_2\text{-H}_2\text{O}$  equilibration, as highlighted by Barbour *et al.* (2016b). There was a variability in the temperature responses of  $\Delta^{13}\text{C}-g_m$  between soybean and common bean in this study and between different species in von Caemmerer and Evans (2015). It would be interesting to study the temperature responses of  $\Delta^{18}\text{O}-g_m$  in these species. There is an opposing effect of temperature on diffusivity and solubility of  $\text{CO}_2$ , and therefore the temperature response of  $\text{CO}_2$  diffusion through liquid is negligible (Evans & von Caemmerer, 2013). Temperature

affects the properties of the lipids and proteins that comprise the membrane and thus directly affects membrane permeability in the short-term, although there is limited experimental data available for the temperature response of membrane permeability to CO<sub>2</sub>.

- The commonly involved leaf anatomical traits for setting  $g_m$  (leaf thickness,  $S_{mes}$ ,  $S_c$ ) were not the major determinants of  $g_m$  variability in faba bean in this study. Substantial evidence has been accumulated implying causal links between changes in aquaporins and  $g_m$ , but was outside the scope of this thesis. More studies are needed which include procedures that modify aquaporin expression like knockout or antisense techniques to provide precise information about the role of aquaporin in membrane permeability and thus in  $g_m$  regulation. There could be a complex integrated roles of different structural and metabolic controls in  $g_m$  regulation, thus future studies should also look at how different traits interact with each other to enhance the possibility of completing our fragmentary picture of mesophyll conductance.
- Genotypic variation in the sensitivity of  $g_m$  to nitrogen source (NH<sub>4</sub>NO<sub>3</sub>-fed/non-inoculated versus N-fixing by Rhizobium inoculate) observed in this study may be of particular interest for future studies, given the involvement of nitrogen in leaf structure, physiology as well as in building or maintaining proteins like aquaporin. Investigating the link between the influence of N source/N assimilation on respiratory or photorespiratory processes and on mesophyll conductance could be helpful. Future studies could also consider integrated roles of aquaporins in carbon and nitrogen assimilation (Maurel *et al.*, 2008).
- Despite the lack of correlation between  $g_m$  and  $K_{leaf}$  in faba bean observed in this study, I believe that more detailed studies on  $g_m$ - $K_{leaf}$  relationships across different environmental conditions and species are needed in order to arrive at definite conclusion of the proposed coordination. I suggest that a better approach for precisely assessing the relationships between  $g_m$  and  $K_{leaf}$  would be to partition  $g_m$  into different components using  $\Delta^{18}O$  and  $\Delta^{13}C$  techniques (as described in this study and Loucos *et al.* (2017)) and partition  $K_{leaf}$  into xylem and extra-xylary components. Studies on protein-mediated  $g_m$ - $K_{leaf}$  coordination would also be of great benefit. Studies to map the water movement pathways outside the xylem and the

location of the phase change between liquid and vapour will further elucidate the relationships between leaf internal conductances to CO<sub>2</sub> and H<sub>2</sub>O.

- The experiments in this study were conducted in controlled environment. The key results of this study, particularly the genetic variation in the  $g_m$  response to water stress and the positive correlation between  $g_m$  with leaf water use efficiency, should be tested under field conditions including water-limiting environments, and at the crop stages that are most sensitive to drought.
- The contribution of increased  $A$  and  $A/g_{sw}$  through increased  $g_m$  to improvement in crop yield and WUE needs to be assessed under realistic field conditions. The potential scope for yield improvement by enhancing leaf photosynthesis in C<sub>3</sub> crops has been demonstrated by the effects of CO<sub>2</sub>-enrichment on yield and biomass (Kimball *et al.*, 2002, Ainsworth & Long, 2005). Barbour *et al.* (2010) stated that selecting for high  $g_m$  to improve  $A/g_{sw}$  will also improve crop WUE (so long as allocation of carbon to the harvested plant organ is not reduced). The recent detection of the quantitative trait locus (QTL) associated with  $g_m$  in bread wheat hints at the genetic basis for  $g_m$  (Barbour *et al.*, 2016). Identifying QTL associated with  $g_m$  in legumes and including this genomic information in the breeding program would provide additional improvements in legumes crop breeding.

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