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Research Article

The response of mesophyll conductance to short- and long-term environmental conditions in chickpea genotypes

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Abstract. Mesophyll conductance (g_m) has been shown to vary between genotypes of a number of species and with growth environments, including nitrogen availability, but understanding of g_m variability in legumes is limited. We might expect g_m in legumes to respond differently to limited nitrogen availability, due to their ability to fix atmospheric N_2. Using online stable carbon isotope discrimination method, we quantified genetic variability in g_m under ideal conditions, investigated g_m response to N source (N_2-fixation or inorganic N) and determined the effects of N source and water availability on the rapid response of g_m to photosynthetic photon flux density (PPFD) and radiation wavelength in three genotypes of chickpea (*Cicer arietinum*). Genotypes varied 2-fold in g_m under non-limiting environments. N-fed plants had higher g_m than N_2-fixing plants in one genotype, while g_m in the other two genotypes was unaffected. g_m response to PPFD was altered by N source in one of three genotypes, in which the g_m response to PPFD was statistically significant in N-fed plants but not in N_2-fixing plants. There was no clear effect of moderate water stress on the g_m response to PPFD and radiation wavelength. Genotypes of a single legume species differ in the sensitivity of g_m to both long- and short-term environmental conditions, precluding utility in crop breeding programmes.

Keywords: *Cicer arietinum*; mesophyll conductance; nitrogen-fixation; nitrogen nutrition; photosynthetic photon flux density.

Introduction

Mesophyll conductance to CO_2 (g_m), which regulates the diffusion of CO_2 from substomatal cavities to the sites of carboxylation, is now recognized as a significant and variable limitation to photosynthesis (Flexas *et al.* 2008, 2012). 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). g_m has been shown to be influenced by different growth environments including water availability, photosynthetic photon flux density (PPFD), temperature, CO_2 concentration and nitrogen nutrition (Warren *et al.* 2007; Flexas *et al.* 2008; Loreto *et al.* 2009; Bunce 2010; Douthe *et al.* 2011; Perez-Martin *et al.* 2014; Xiong *et al.* 2015; Olsovska *et al.* 2016). g_m variability within and among species and in response to growth conditions has been associated with leaf structure and anatomical properties, particularly the

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surface area of chloroplasts exposed to the intercellular spaces (S_c), cell wall and chloroplast thickness (Evans *et al.* 2009; Tosens *et al.* 2012; Tomás *et al.* 2013), but see (Hanba *et al.* 2002; Tomás *et al.* 2014; Shrestha 2017). g_m variability may also result from the changes in leaf enzymatic processes including membrane permeability through aquaporins, AQPs (Terashima and Ono 2002; Hanba *et al.* 2004; Flexas *et al.* 2006, 2008, 2012) and CO_2/bicarbonate equilibration though carbonic anhydrase, CA (Gillon and Yakir 2000; Perez-Martin *et al.* 2014; Momayyezi and Guy 2017). g_m has been suggested as an appropriate selection target to improve crop water-use efficiency (Flexas *et al.* 2013) while maintaining photosynthetic rate. An increase in g_m will increase chloroplastic CO_2 concentration, and so increase photosynthetic rates, with no simultaneous increase in transpiration (assuming g_m and g_s can be decoupled; Barbour *et al.* 2010).

Grain legumes have received less attention than cereals in studies of g_m regulation. Unlike other plants, legumes can derive some of their nitrogen from symbiotic nitrogen-fixation in their root nodules (Graham and Vance 2003; Foyer *et al.* 2016). Nitrogen acquisition by these methods has been shown to differ in metabolic and transport processes (Schubert 1995), and studies have reported a higher energetic cost of symbiotic nitrogen-fixation compared to that of soil mineral N uptake and assimilation (Pate *et al.* 1979; Chapin *et al.* 1987; Andrews *et al.* 2009). Nitrogen source has also been shown to affect stomatal conductance (g_{sw}; but not intercellular CO_2 concentration) and photorespiratory rates, with lower g_{sw} and higher photorespiratory flux in NO_3^--fed plants than in N_2-fixing plants (Frechilla *et al.* 1999). Busch *et al.* (2018) recently showed that NO_3^- assimilation via the photorespiratory pathway can increase the rate of CO_2 assimilation by fixing carbon as amino acids, highlighting the intrinsic link between C and N metabolism in leaves. N_2-fixing plants have also been reported to have higher leaf area per unit dry weight than NO_3^--fed plants (Frechilla *et al.* 1999). Previous studies have reported a significant correlation between leaf anatomy (e.g. leaf thickness, leaf mass per area) and g_m (Syvertsen *et al.* 1995; Hanba *et al.* 1999). It is likely that different source of N nutrition could influence g_m through modifications in leaf anatomy or N assimilation processes. However, there are no reports to date whether nitrogen source influences g_m.

Mesophyll conductance has also been found to respond to short-term changes in environmental conditions such as temperature and CO_2 concentration (Flexas *et al.* 2008; von Caemmerer and Evans 2015; Xiong *et al.* 2015); however, there are conflicting results between studies regarding the short-term response of g_m to light environment. Positive relationships between g_m and PPFD have been observed in some studies (Gorton *et al.* 2003; Flexas *et al.* 2007; Douthe *et al.* 2011, 2012; Xiong *et al.* 2015, 2018) but not in others (Tazoe *et al.* 2009; Yamori *et al.* 2010). Théroux-Rancourt and Gilbert (2017) demonstrated that g_m response to PPFD is controlled by anatomical structure across the leaf profile highlighting the 3D nature of g_m. Further, there has been speculation that rapid changes in g_m with PPFD are methodological artefacts (Tholen *et al.* 2012; Gu and Sun 2014). The two most commonly used methods for estimating g_m are (i) gas exchange in combination with ^{13}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). Studies examining the importance of growth environments (e.g. water and nitrogen limitation) on the sensitivity of g_m to light environment in different species and genotypes would be valuable to our understanding of g_m regulation. Xiong *et al.* (2015) found that the rapid responses of g_m to changes of CO_2 concentration, temperature and PPFD were affected by nitrogen supplements in rice, and Barbour and Kaiser (2016) reported genotypic variation in the g_m response to nitrogen and water availability in wheat.

The present study was undertaken to investigate g_m regulation under a range of growth and environmental conditions in chickpea (*Cicer arietinum*). Chickpea is the second most important grain legume crop in terms of area and production globally (FAOSTAT 2014). Chickpea genotypes have been shown to differ in leaf gas exchange under ideal growth conditions (Mafakheri *et al.* 2010), but g_m variability has not yet been quantified in chickpea. In the present study, we attempted to address three questions: (i) Do chickpea genotypes differ in mesophyll conductance? (ii) Does the source of N influence g_m in chickpea and are there genotypic differences in this effect? (iii) Are there genotypic differences in the growth environment effects on the g_m response to PDF and radiation wavelength? Three experiments were conducted to answer these questions. The first experiment characterized g_m variability in 20 chickpea genotypes under controlled conditions. In the second experiment, three chickpea genotypes were grown employing either N_2-fixation or inorganic nitrogen and measured under a range of PPFD. The third experiment examined the interactive effects of water availability and short-term changes in PPFD and radiation wavelength on g_m in three chickpea genotypes.

Methods

Plant material and experimental arrangements

Experiment 1: screening for g_m under non-limiting environments. Twenty genotypes of chickpea were grown in a controlled-environment growth room at the University of Sydney, Centre for Carbon Water and Food (Camden, NSW, Australia). Seeds were sown in 7 L pots filled with commercial potting mix supplemented with slow release fertilizer (Osmocote Exact, Scotts, NSW, Australia). Plants were maintained at 25 °C/17 °C in a 16-h photoperiod, 75 % relative humidity with irradiance (PPFD) of ~600 μmol m^{-2} s^{-1} at the top of the canopy. All plants were well-watered and fertilized throughout the experiment. Genotypes were sourced from: NSW Department of Primary Industries (DPI: Amethyst, Genesis 079, Kyabra, Jimbour and Yorker); NSW DPI in conjunction with Pulse Breeding Australia (PBA Hattrick, PBA Monarch and PBA Slasher); the WA Department of Agriculture and Fisheries (DAF: Sonali); the QLD DAF (Tyson) and ICARDA (Flip079C). In addition, nine breeding lines (BL1–9) were included which were sourced from the germplasm store at the University of Sydney Narrabri Campus. Of the 20 genotypes, 17 were desi and 3 kabuli **[see Supporting Information—Table S1]**. Desi types have small, dark, angular seeds, whereas kabuli types have large, rounded, light-coloured seeds (Leport *et al.* 2006).

Experiment 2: nitrogen source × PPFD × genotype. The nitrogen experiment was carried out on 3 of the 20 chickpea genotypes from the screening experiment; Flip079C and PBA Slasher and Sonali. The genotypes were selected based on their phenological similarity (all three genotypes are early varieties; C. Blessing, the University of Sydney, pers. comm.) so that physiological measurements could be made at the same growth stage. Flip079C belongs to kabuli type while PBA Slasher and Sonali are desi type. PBA Slasher and Sonali are parental genotypes in mapping population (A. L. Pattison, the University of Sydney, pers. comm.). The study was conducted in a controlled growth room with environmental condition similar to Experiment 1, except PPFD was 200 μmol m^{-2} s^{-1} at plant height. Plants were grown in 7 L pots, filled with washed river sand (N-free media) and lined with ~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 treatments were (i) inoculated with a peat-based Nodule N Rhizobium without mineral N supply (N_2-fixing) and (ii) uninoculated and supplied with 2.5 mM NH_4NO_3 (N-fed).

The plants in both treatments were provided with quarter-strength modified Herridge N-free mineral nutrient solution (Herridge 1977): 250 μM $CaCl_2{\cdot}2H_2O$, 250 μM KCl, 125 μM KH_2PO_4, 125 μM K_2HPO_4, 500 μM $MgSO_4{\cdot}7H_2O$, 25 μM FeEDDHA and 25 μM Trace Elements (2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.11 mg L^{-1} $ZnCl_2$; 0.05 mg L^{-1} $CuCl_2{\cdot}2H_2O$; 0.025 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$). For the first 10 days after planting, 0.5 mM KNO_3 was included in the Herridge nutrient solution for both treatments to help the plants establish. All the pots were then flushed with pure water to wash away any nitrogen residues from the media. Thereafter, inoculated plants received the N-free Herridge solution while the uninoculated plants received 2.5 mM NH_4NO_3 in addition to the Herridge solution. The pots in each N treatment (three genotypes × three pots × two replicate plants per pot) 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.

Experiment 3: water availability × PPFD × radiation wavelength × genotype. We used 3 of the 20 chickpea genotypes from the screening experiment: Amethyst, PBA Slasher and Sonali for the water availability experiment. PBA Slasher and Sonali were identified as among the drought tolerant genotypes, whereas Amethyst (desi type) was drought susceptible based on the grain yield ranking and drought indices (Kaloki 2017). The highest yielding genotype under well-watered conditions was PBA Slasher followed by Sonali, whereas under water limited conditions, Sonali was the highest yielding genotype. Amethyst has the lowest g_m value (from Experiment 1). 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 room at the University of Sydney, Centre for Carbon, Water and Food (Camden, NSW, Australia). The growth room was set to 25 °C/17 °C day/night temperature, 75 % relative humidity, 700 μmol m^{-2} s^{-1} PPFD 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 (three genotypes × three pots × two plants per pot) 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. Midday leaf water potential (Ψ_{leaf}) of upper fully expanded leaves was measured to monitor water stress using a Scholander pressure chamber (115, Soil

Moisture Equipment, Santa Barbara, CA, USA) and following the precautions recommended by Turner (1988). Midday Ψ_{leaf} measurements were performed on lateral branches for each genotype.

At the temporary wilting point (at which the apical leaves wilted at midday but recovered overnight, which occurred 7 days after the start of the water stress treatment), average midday leaf water potentials for WW and WS plants were −0.6 and −1.2 MPa, respectively. 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 (7 days) by weighing each pot daily at 1 h after the start of the light period and adding water to replace that transpired and evaporated.

Simultaneous gas exchange and mesophyll conductance measurements

Experiment 1: screening for g_m under non-limiting environments. Gas exchange measurements and regulation of leaf environmental conditions were conducted using a Li-6400XT portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA). Five weeks after sowing, each of five leaves per genotype were enclosed in 12 cm^2 (2 × 6) clear-top chamber of the Li-6400XT fitted with a red-green-blue LED light source (Li-6400 18A) set to 1300 μmol m^{-2} s^{-1} (10 % blue and 90 % red). The uppermost fully expanded leaves of the primary branches were used for the measurements. 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. CO_2 concentration inside the chamber was fixed at 400 μmol mol^{-1}, leaf temperature was set at 25 °C, and relative humidity was maintained between 70 and 80 %. CO_2 concentration differences between the air entering and leaving the chamber were in the range of 31–105 to obtain the precise and accurate estimation of g_m, considering the precautions recommended by Pons *et al.* (2009) for online isotope method. Data points with CO_2 differentials <30 were excluded because of the associated error in the discrimination measurements. Kyabra genotype had one unrealistically high g_m value (>3 mol m^{-2} s^{-1} bar^{-1}), and thus this data point was removed from ANOVA analysis. All the measurements were made at 21 % O_2. Each leaf remained in the chamber for at least 30 min to allow time for the leaf to adjust to the chamber conditions before gas exchange and online discrimination measurements were made. Gas exchange was recorded at 1-min intervals.

Mesophyll conductance was estimated using the online carbon isotope discrimination method (Evans *et al.* 1986; Tazoe *et al.* 2009) for all the experiments. The Li-6400XT was coupled to a Tunable-Diode Laser Absorption Spectrometer (TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA), which measured the stable carbon and oxygen isotope compositions of CO_2 ($^{13}CO_2$, $C^{18}O^{16}O$), as described by Barbour *et al.* (2007). Leaf chamber inlet and outlet air streams were subsampled to the TDL. Mesophyll conductance was estimated from the difference between calculated carbon isotope discrimination assuming infinite g_m ($\Delta^{13}C_i$), and that measured by the coupled system ($\Delta^{13}C_{obs}$), as described in Jahan *et al.* (2014), including the ternary corrections as described by Farquhar and Cernusak (2012).

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

where C_a, C_s and C_i are the ambient, leaf surface and intercellular CO_2 partial pressures, a_b and a_s are the fractionations during diffusion through the leaf boundary layer and the stomata, respectively, b is the fractionation associated with carboxylation, f is the fractionation associated with photorespiration, α_b is the fractionation factor for carboxylation (1 + b), $\alpha_{\acute{e}}$ is the fractionation factor for day respiration (1 + $\acute{e}$), α_f is the fractionation factor for photorespiration (1 + f). The assumed values for various fractionation factors during CO_2 diffusion within the leaf, used for calculating g_m are shown in Table 1. R_d is the rate of day respiration and Γ^* is the compensation point in the absence of R_d. Both R_d and Γ^* were predicted from leaf temperature using the approach described by Bernacchi *et al.* (2001). R_d is known to vary between genotypes of crop species (e.g. Jahan *et al.* 2014 found R_d varied between wheat cultivars), so in the absence of R_d measurements for the chickpea, we conducted a sensitivity analysis to determine the effect of errors in the R_d assumption. We assumed R_d was 1.5 μmol m^{-1} s^{-1} at 25 °C for all genotypes in all experiments. When R_d was varied between 1 and 2 mol m^{-2} s^{-1}, g_m changed by 0.01–0.02 mol m^{-2} s^{-1} (2 %) for measurements made with red or red-blue light and by 0.02–0.03 mol m^{-2} s^{-1} with blue light (3 %). These negligible errors were deemed unlikely to alter conclusions drawn from the measurements.

In Equation (1), t is the ternary correction factor (Farquhar and Cernusak 2012), and is given by:

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

where E is the transpiration rate (mmol m^{-2} s^{-1}), α_{ac} is the fractionation factor of 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

Table 1. Fractionation factors used in the calculation of g_m. *Fractionation associated with day respiration (*é*) was corrected for disequilibrium between growth CO_2 $\delta^{13}C$ (−14 ‰; measured by a stable isotope cavity ring down laser, G11101-i, Picarro, Santa Clara, CA, USA) and measurement CO_2 $\delta^{13}C$ (−31 ‰ for Experiment 1 and −4 ‰ for Experiments 2 and 3; measured by Tunable-Diode Laser Absorption Spectrometer; TDL, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA).

	Symbol	Value (‰)	Reference
Fractionation during leaf boundary layer diffusion	a_b	2.9	Evans *et al.* (1986)
Fractionation during stomata diffusion	a_s	4.4	Farquhar and Richards (1984)
Fractionation during CO_2 diffusion and dissolution	a_m	1.8	O'Leary (1984)
Fractionation during carboxylation	b	30	Guy *et al.* (1993)
Fractionation during day respiration*	e	−3	Tcherkez *et al.* (2010)
Fractionation during photorespiration	f	16.2	Evans and von Caemmerer (2013)

total conductance to CO_2 diffusion including the boundary layer and stomatal conductance.

Then, mesophyll resistance (r_m = $1/g_m$) is given by Farquhar and Cernusak (2012):

$$r_m = \frac{1-t}{1+t}\left(\Delta^{13}C_i - \Delta^{13}C_{obs}\right)\frac{C_a}{A\left(b - a_m - \frac{\alpha_b}{\alpha_{\acute{e}}}\acute{e}\frac{R_d}{A+R_d}\right)} \quad (3)$$

A is the CO_2 assimilation rate (μmol m^{-2} s^{-1}), a_m is the fractionation factor for liquid phase CO_2 diffusion and dissolution (‰).

$\Delta^{13}C_{obs}$ is calculated from the following equation (Evans *et al.* 1986):

$$\Delta^{13}C_{obs} = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)} \quad (4)$$

where

$$\xi = \frac{C_e}{C_e - C_o} \quad (5)$$

C_e and δ_e are concentrations and isotope compositions of CO_2 of dry air entering the leaf chamber and C_o and δ_o are concentrations and isotope compositions of CO_2 of dry air exiting the chamber, respectively. Carbon and oxygen isotope compositions of CO_2 were obtained from the TDL.

Two calibration cylinders were used to calibrate the TDL, spanning the range in concentrations of the isotopologues of the leaf chamber inlet and outlet air streams. Total CO_2 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. Carbon isotope ratios are presented relative to the Vienna Pee Dee belemnite standard, and oxygen isotope ratios of CO_2 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.).

Experiment 2: nitrogen source × PPFD × genotype. Leaf gas exchange and mesophyll conductance measurements were conducted 5 weeks after planting. The Li-6400XT was fitted with a custom-built leaf chamber of area 38 cm^2 (Loucos *et al.* 2017) and red-green-blue light source (Li-6400 18A) for this experiment. The boundary layer conductance for the chamber was estimated using the method described in Barbour *et al.* (2007). To examine leaf responses to rapidly changing PPFD, simultaneous leaf gas exchange and isotopic discrimination measurements were made in the order 1000, 800, 600, 400, 300 μmol m^{-2} s^{-1}, with the light colour was set to 10 % blue and 90 % red. The measurements were made for plants in both N treatments and leaves remained in the chamber for at least 15 min at each irradiance. Throughout the measurements, CO_2 concentration in the sample cell was maintained at 400 μmol mol^{-1}, flow rate at 500 μmol s^{-1} and leaf temperature at 25 °C. CO_2 concentration differences between the air entering and leaving the chamber were in the range of 40–90 (corresponding to the lowest and the highest PPFD, respectively). All the measurements were made at 21 % O_2.

Experiment 3: water availability × PPFD × radiation wavelength × genotype. Leaf gas exchange and mesophyll conductance measurements were performed as for Experiment 2, except that PPFD was set at (in order) 950, 700 and 400 μmol m^{-2} s^{-1}, under red radiation and then under blue radiation. The blue radiation had a peak emission at 457 nm, with a range from 424 to 524 nm, while the red radiation peak emission was centred at 636 nm, ranging from 584 to 661 nm. The leaves remained in the chamber for at least 15 min at each 'PPFD-wavelength' step. The measurements were made

for both the well-watered and water-stressed plants at 21 % O_2. CO_2 concentration differences between the air entering and leaving the chamber were in the range of 37–148 (for the lowest intensity of blue radiation to the highest intensity of red radiation, respectively). Leaf water potential (Ψ_{leaf}) was measured for all leaves immediately after gas exchange measurements.

Crop traits

In the nitrogen source experiment (Experiment 2), the youngest fully expanded leaf samples were collected after the gas exchange measurements and were oven-dried at 65 °C for 72 h. Samples were then ground to a fine powder and analysed for total N content (N%) and ^{15}N composition using isotope ratio mass spectrometry (Delta V, Thermo Fisher Scientific, Bremen, Germany). The plants were harvested, cleaned of sand and roots were washed. Roots and nodules were separated and oven-dried at 65 °C for 72 h for measurement of dry weight. The proportion of N derived from N-fixation (%Ndfa) for the N-fed plants was determined using the $\delta^{15}N$ Natural Abundance Method (Unkovich *et al.* 2008).

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2\textit{-fixing legume}}{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2} \times \frac{100}{1} \quad (6)$$

where $\delta^{15}N$ of N_2-fixing legume represents the $\delta^{15}N$ value of the non-inoculated legume supplied with NH_4NO_3, and $\delta^{15}N$ of N_2 is the $\delta^{15}N$ value of the inoculated legume grown with atmospheric N_2 as the sole source of N. $\delta^{15}N$ of soil N (NH_4NO_3 fertilizer supplied to N-fed plants) was estimated using isotope ratio mass spectrometry.

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 $P < 0.05$.

Results

Do chickpea genotypes differ in mesophyll conductance?

The screening experiment results showed ~1.7-fold range in net photosynthetic rate (A) and stomatal conductance to water vapour (g_{sw}) among the 20 chickpea genotypes, while g_m ranged >2-fold from 0.29 to 0.88 mol m^{-2} s^{-1} bar^{-1} (BL9 and Jimbour, respectively; Fig. 1 and see **Supporting Information–TableS3**). Average leaf intrinsic water-use efficiency (A/g_{sw}) varied between 40

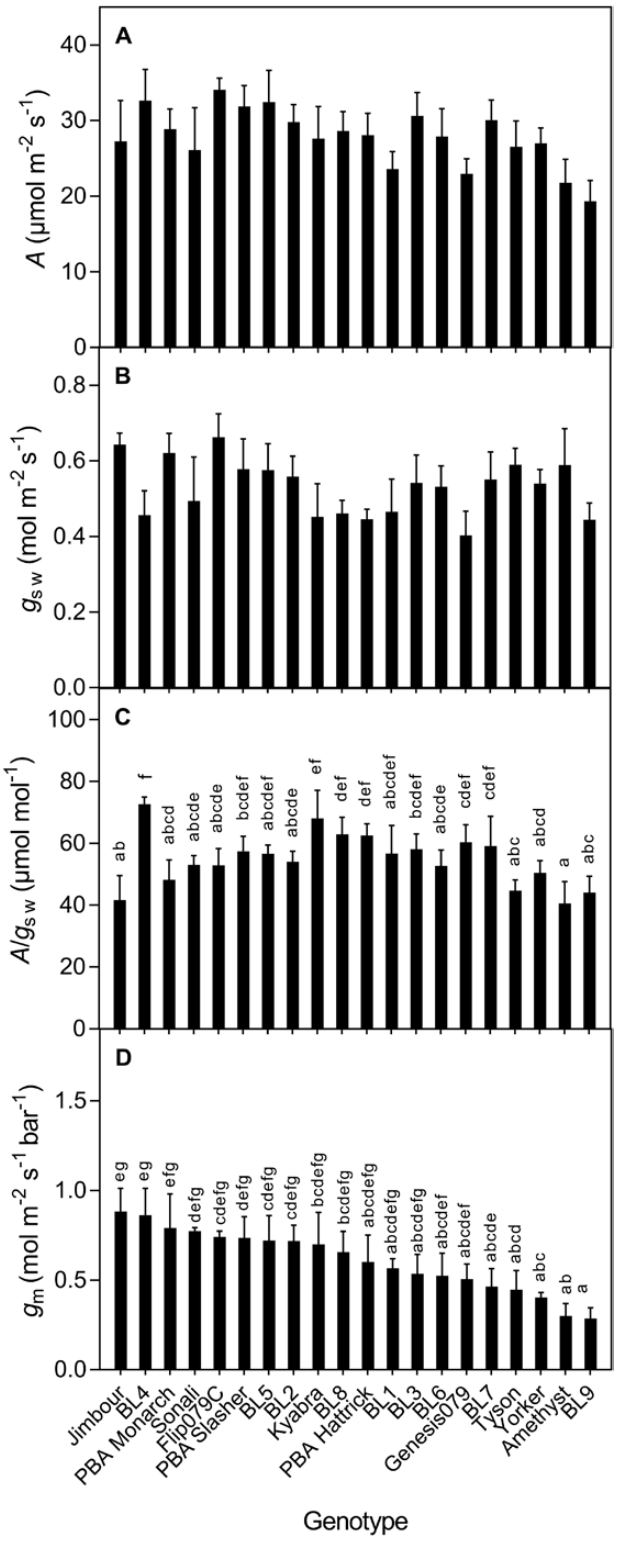

Figure 1. Photosynthetic rate (A; A), stomatal conductance to water vapour (g_{sw}; B), leaf-intrinsic water use efficiency (A/g_{sw}; C) and mesophyll conductance (g_m; D) of 20 chickpea genotypes grown and measured under non-limiting controlled environmental conditions. Mean and SE are shown (n = 3–5). Letters indicate significant differences ($P < 0.05$) between genotypes.

(BL9) and 73 μmol mol^{-1} (BL4), and was positively, but weakly, related to g_m (A/g_{sw} = 22.1 + 41.1g_m, R^2 = 0.25, P = 0.023, data not shown). Genotypic differences in A and g_{sw} were not statistically significant, but g_m and A/g_{sw} differed significantly between genotypes (P = 0.023 and P = 0.011, respectively; Fig. 1). In water availability and nitrogen source experiments, Sonali had significantly higher average g_m than the other genotypes (Amethyst, PBA Slasher and Flip079C) when grown and measured under ideal conditions.

Does the source of N influence g_m in chickpea and are there genotypic differences in this effect?

Three of the 20 chickpea genotypes (Flip079C, PBA Slasher and Sonali) were used to compare g_m of uninoculated, N-fed (2.5 mM NH_4NO_3) plants with that of inoculated, N_2-fixing plants. Some nodulation was observed in uninoculated, N-fed plants (Fig. 5). However, the nodule size and nodule number in N-fed plants was less than one-twentieth than that in N_2-fixing plants (P < 0.001, df = 14). Leaves of N_2-fixing plants were depleted in ^{15}N compared to N-fed leaves (P < 0.001; genotype averages: 1.8 ± 0.2 ‰ N-fed and −1.8 ± 0.09 ‰ for N_2-fixing leaves) indicating that different nitrogen sources were used. The $\delta^{15}N$ value of NH_4NO_3 fertilizer supplied to N-fed plants was 2.4 ‰. N-fed PBA Slasher and N-fed 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 ‰) was lower (P = 0.01) than that of the N fertilizer and so the proportion of N derived from N-fixation was higher, at 25 %.

N-fed plants had higher photosynthetic rates than N_2-fixing plants when measured at high PPFD across the three genotypes (Fig. 2). g_{sw} was higher for N_2-fixing plants than for N-fed plants but the differences were not significant at each PPFD (Fig. 2). Interestingly, there was a significant interactive effect of genotype by nitrogen source (P = 0.017) for g_m (Table 2; Fig. 2 and **see**

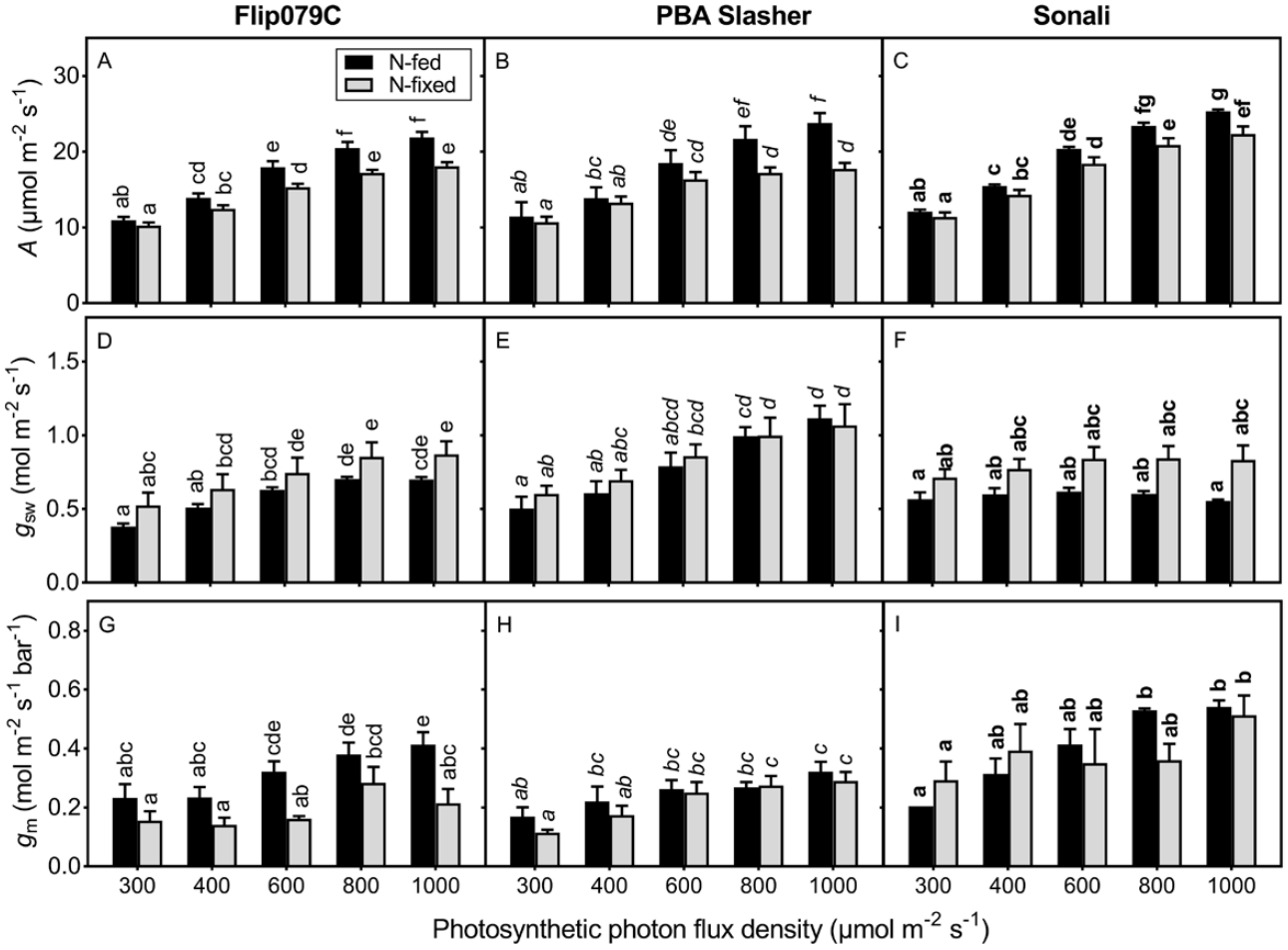

Figure 2. Photosynthetic rate (A; A, B, C), stomatal conductance to water vapour (g_{sw}; D, E, F) and mesophyll conductance (g_m; G, H, I) of three chickpea genotypes grown under two nitrogen source treatments and measured under different photon flux densities. Means and SE are shown (n = 5–6). Letters indicate significant differences (P < 0.05) between the treatments within each genotypes.

Table 2. Effects of PPFD, 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 PPFD = 4, nitrogen source = 1 and genotypes = 2.

		A	g_{sw}	g_m
PPFD	*F*	160.16	15.71	16.06
	P	<0.001	<0.001	<0.001
Nitrogen source	*F*	61.28	19.99	14.67
	P	<0.001	<0.001	<0.001
Genotypes	*F*	23.04	8.88	32.86
	P	<0.001	<0.001	<0.001
PPFD × nitrogen source	*F*	4.94	NS	NS
	P	0.001	NS	NS
PPFD × genotypes	*F*	NS	2.55	NS
	P	NS	0.014	NS
Nitrogen source × genotypes	*F*	NS	2.77	4.26
	P	NS	0.067	0.017
PPFD × nitrogen source × genotypes	*F*	NS	NS	NS
	P	NS	NS	NS

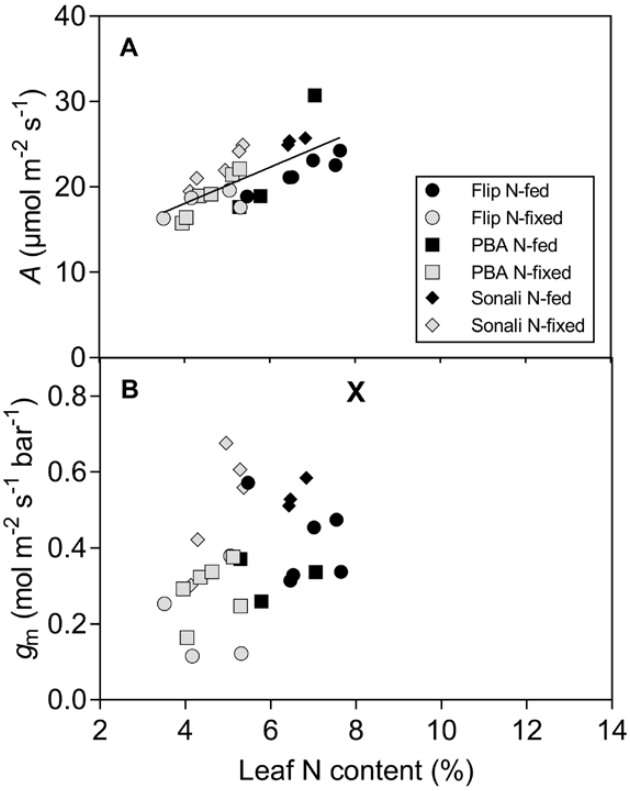

Figure 3. Relationships between leaf N content and photosynthetic rate (*A*; A) and mesophyll conductance to CO_2 (g_m; B), measured at 1000 μmol m^{-2} s^{-1} PPFD, for three chickpea genotypes grown under two nitrogen source treatments. The solid line in plot A indicates a significant linear regression ($P < 0.001$, $R^2 = 0.51$).

Supporting Information–TableS4). N_2-fixing Flip079C plants had lower g_m values than N-fed Flip079C plants and the difference was significant at higher PPFD. However, nitrogen source did not affect g_m in PBA Slasher and Sonali. The chloroplastic CO_2 concentration (C_c) was not affected by nitrogen source for any genotype.

Leaf N content (%N) was affected by the nitrogen source ($P < 0.001$) and was significantly lower for N_2-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 ($P < 0.0001$, $R^2 = 0.51$) (Fig. 3). However, we did not find any relationship between g_m and %N (Fig. 3).

Are there genotypic differences in the growth environment effects on the g_m response to PPFD and wavelength?

g_m response to PPFD was assessed in N-fed and N_2-fixing plants of three genotypes (Flip079C, PBA Slasher and Sonali). Table 2 shows the result of the ANOVA. Our results showed genotypic differences in the effect of N source on the g_m sensitivity to PPFD **[see Supporting Information—Table S2]**. The linear relationships between g_m and PPFD (regression fitted to the individual data) were significant for N-fed plants of each genotype (Flip079C: $P < 0.001$; PBA Slasher: $P = 0.004$; Sonali: $P < 0.001$), while in N_2-fixing plants, the linear relationship between g_m and PPFD was significant for PBA Slasher ($P < 0.001$) and Flip079C ($P = 0.038$) but not for Sonali ($P > 0.05$).

Three of the 20 genotypes (Amethyst, PBA Slasher and Sonali) were examined for the effect of water availability on the short-term response of g_m to PPFD and wavelength (Table 3 and **see Supporting Information–TableS5**). Water stress lowered leaf water potential, Ψ_{leaf} ($P < 0.001$). The average midday Ψ_{leaf} for WW and WS plants were −0.66 and −1.32 MPa, respectively, i.e. the WS plants were moderately stressed, but we did not find genotypic differences in Ψ_{leaf}. g_m decreased linearly with decreasing PPFD but the g_m reduction was not significant for the water-stressed PBA Slasher, water-stressed Sonali measured under blue radiation and well-watered Sonali under red radiation ($P > 0.05$; Fig. 4) **[see Supporting Information—Table S2]**.

Switching from red radiation to blue radiation while maintaining constant PPFD reduced *A* and g_m but not g_{sw} in both WW and WS plants of the three genotypes (Table 3; Fig. 4). There was also a significant interactive effect of genotype by water stress by radiation wavelength for g_m ($P = 0.008$; Table 3; Fig. 4). Water stress

reduced g_m only in Sonali when measured under red radiation. g_m was unaffected by water availability under blue radiation in Sonali and under any radiation wavelength in Amethyst and PBA Slasher.

Discussion

Mesophyll conductance varies between genotypes

g_m has been recognized as a significant and variable limitation to photosynthesis in a range of species, but there is limited information on g_m variability in legumes including chickpea. The 20 genotypes screened here showed a significant difference in g_m values. 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), a few other crop species (Lauteri *et al.* 1997; Galmés *et al.* 2011; Tomás *et al.* 2014) and recently among soybean edamame genotypes (Tomeo and Rosenthal 2017), faba and field pea genotypes (Shrestha 2017). We did not observe any clear differences in g_m values between the two types of chickpea (desi or kabuli) under non-limiting growth conditions. Barbour *et al.* (2016) reported the first hints of genetic control of g_m in bread wheat. Genotypic variation in g_m values in chickpea in our study might be due to the leaf anatomical or biochemical differences (not evaluated in the current study) between the genotypes.

Table 3. Effects of PPFD, radiation wavelength, 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 PPFD = 2, wavelength = 1, water stress = 1 and genotypes = 2.

		A	g_{sw}	g_m
PPFD	*F*	205.78	NS	41.43
	P	<0.001	NS	<0.001
Wavelength	*F*	365.35	NS	157.79
	P	<0.001	NS	<0.001
Water stress	*F*	120.97	250.92	5.96
	P	<0.001	<0.001	0.016
Genotypes	*F*	10.7	20.32	3.18
	P	<0.001	<0.001	0.044
PPFD × wavelength	*F*	6.19	NS	NS
	P	0.003	NS	NS
PPFD × water stress	*F*	8.64	NS	NS
	P	<0.001	NS	NS
Wavelength × water stress	*F*	20.02	NS	2.61
	P	<0.001	NS	0.10
PPFD × genotypes	*F*	NS	NS	NS
	P	NS	NS	NS
Wavelength × genotypes	*F*	NS	NS	NS
	P	NS	NS	NS
Water stress × genotypes	*F*	21.57	3.62	22.72
	P	<0.001	0.029	<0.001
Wavelength × water stress	*F*	2.31	NS	4.92
× genotypes	*P*	0.1	NS	0.008

When N-fixation is the sole source of plant N, g_m is reduced in one genotype but not in two others

The current study showed that chickpea genotypes differed in their g_m response to nitrogen source. The genotype Flip079C had higher g_m when fertilized with nitrogen than when nitrogen was fixed by *Rhizobium inocula*; however, nitrogen source did not affect g_m in PBA Slasher and Sonali. Conversely, genotypes responded similarly to nitrogen source in terms of photosynthetic rate and leaf N content. Leaf N content was significantly lower for N_2-fixing than for N-fed plants, as reported by Lodeiro *et al.* (2000) in common beans. We found a significant positive correlation between *A* and leaf N content, 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 see Adams *et al.* 2016). A higher photorespiratory flux in NO_3^--fed plants than in N_2-fixing plants was reported by Frechilla *et al.* (1999) and Busch *et al.* (2018) showed that NO_3^- assimilation via the photorespiratory pathway can increase the rate of CO_2 assimilation. However, the results of our study suggest that inorganic N source allowed higher assimilation through higher leaf N content.

There are no published studies on variability of g_m between N_2-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.* 2015). The mechanism of g_m regulation under different nitrogen sources is unclear. g_m response to nitrogen availability has been shown to be strongly correlated to S_c (Xiong *et al.* 2015) and chloroplast size (Li *et al.* 2012). Leaf ultrastructural 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. Regarding the biochemical component of g_m, Warren (2004) suggested that a correlation between nutrient supply and abundance or activity of CA and/or AQPs seems unlikely since CA and AQPs have a very low N cost. On the other hand, several studies have shown that AQP gene

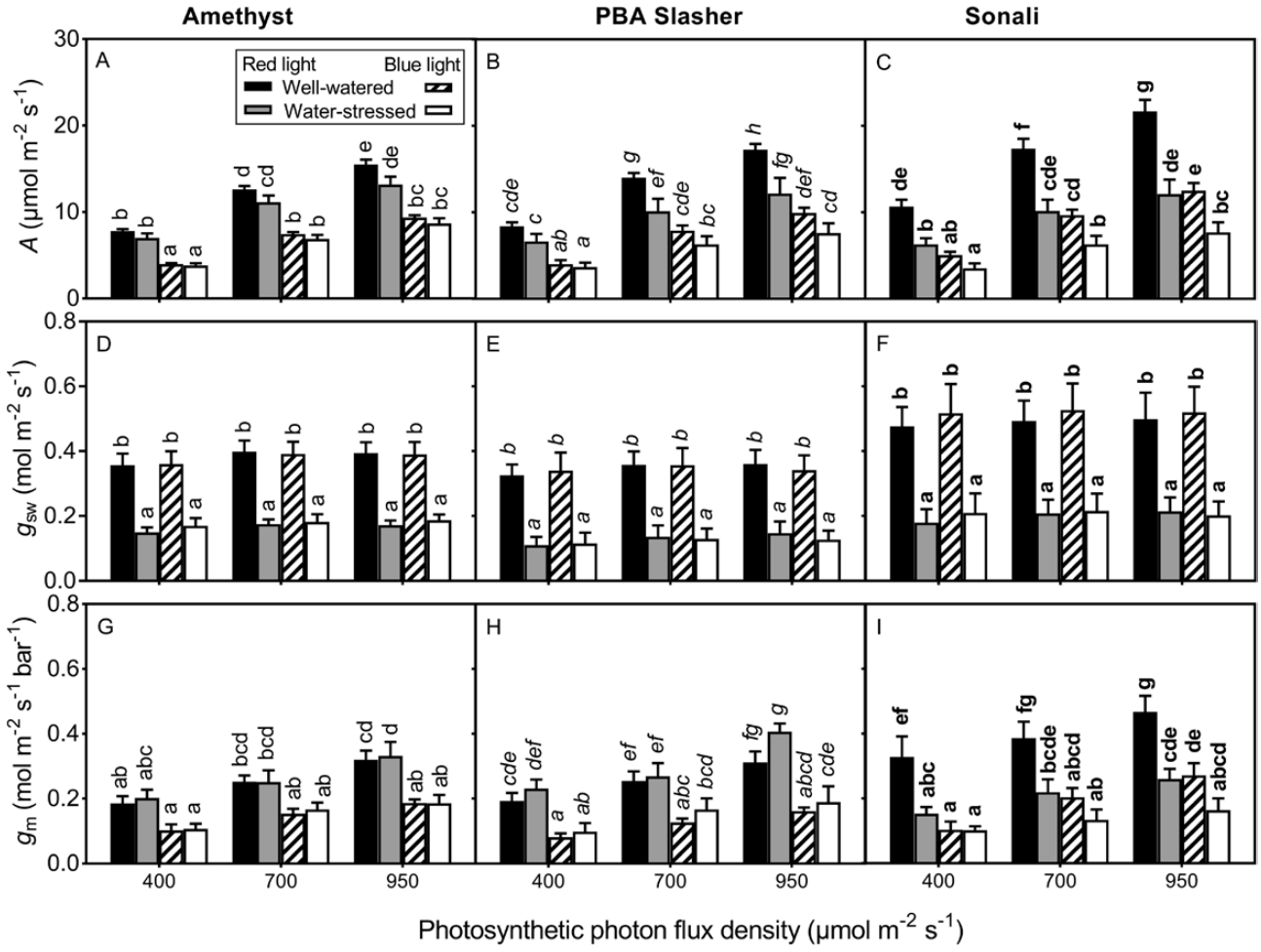

Figure 4. Photosynthetic rate (*A*; A, B, C), stomatal conductance to water vapour (g_{sw}; D, E, F) and mesophyll conductance (g_m; G, H, I) of three chickpea genotypes grown under well-watered or water-stressed conditions and measured under varying photon flux density and radiation wavelength. Means and SE are shown (n = 5–6). Letters indicate significant differences ($P < 0.05$) between the treatments within each genotypes.

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. Whether g_m is limited by nitrogen investment in one or more enzymes or membrane proteins remains to be investigated. In the current study, we did not find any relationship between leaf N content and g_m, consistent with previous studies reporting weak N–g_m relationships (Warren 2004; Barbour and Kaiser 2016). Higher g_m in N-fed Flip079C could simply reflect the relationship between *A* and g_m ($P < 0.001$, $R^2 = 0.64$, data not shown). Further, the chloroplastic CO_2 concentration (C_c) was not affected by nitrogen source, suggesting that mesophyll limitation may not be responsible for the lower photosynthetic rate in N_2-fixing plants.

It is not clear how nitrogen source could affect g_m in some genotypes but not in others. Flip079C is a kabuli chickpea and PBA Slasher and Sonali belong to the desi group. Studies have shown that the two types differ in morphology, nutrition and response to abiotic stresses (Porta-Puglia *et al.* 2000; Walley *et al.* 2005; Leport *et al.* 2006; Purushothaman *et al.* 2014; Imran *et al.* 2015). The gene pools for desi and kabuli types have been separate for many years (Gowda *et al.* 1987; Porta-Puglia *et al.* 2000) and genes associated with g_m may differ between the two types. It would be interesting to elucidate whether the genotypic difference observed here is related to the types of chickpea. The proportion of N derived from N-fixation (%Ndfa) was higher for N-fed Flip079C than for N-fed PBA Slasher and Sonali. N_2-fixing plants had reduced root biomass compared to N-fed plants in PBA Slasher and Sonali, but nitrogen source had no effect on the root biomass of Flip079C (Fig. 5). von Caemmerer and Evans (2015) observed that the temperature response of g_m differed greatly between

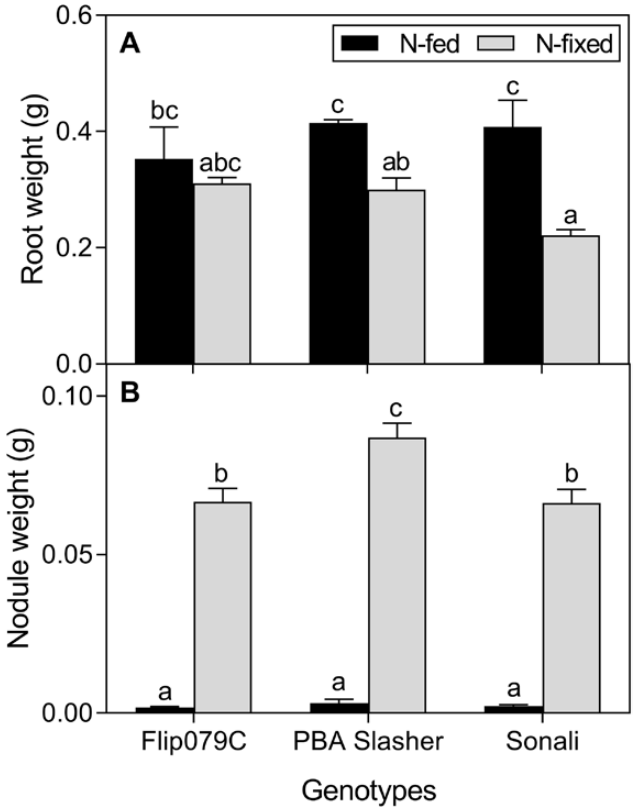

Figure 5. Root (A) and nodule weight (B) of three chickpea genotypes grown under two nitrogen source treatments. Means and SE are shown (n = 5–6). Letters indicate significant differences ($P < 0.05$) between the treatments.

species, and proposed that variation in the g_m response may be due to variation in the activation energy for membrane permeability to CO_2 (AQPs) and the effective path length for liquid phase diffusion (cell wall thickness). Future studies should investigate genotypic differences in leaf anatomy, enzymatic processes and the role of photorespiration in carbon and nitrogen assimilation under different sources of N nutrition (Busch *et al.* 2018).

Despite a lack of clear understanding of the underlying mechanisms of g_m regulation under different nitrogen sources, the observed genotypic variation in g_m sensitivity is interesting in the context of the recognized importance of legume-based farming systems and thus warrants further research.

The g_m response to PPFD and radiation wavelength varies between genotypes and with water and N availability

In the present study, g_m significantly differed only between the highest and the lowest PPFD with an average change of ≈40 % between 950 and 400 µmol m^{-2} s^{-1} in the water availability experiment (Experiment 3), and an average change of ≈48 % between 1000 and 300 µmol m^{-2} s^{-1} in the nitrogen source experiment (Experiment 2). The sensitivity of the PPFD response in our study was different from that observed by Douthe *et al.* (2011, 2012) in *Eucalyptus* species. They found a positive relationship between g_m and PPFD at low intensities (i.e. when PPFD was lowered from 600 or 500 to 200 µmol m^{-2} s^{-1}) but no change in g_m at higher intensities. The dissimilarity in results may be related to species-specific differences or to differences in growth environments.

g_m response to PPFD was altered by nitrogen source in only one of three genotypes, Sonali, in which the g_m response to PPFD was statistically significant in N-fed plants but not in N_2-fixing plants. However, the response of A to PPFD was significant for both N-fed and N_2-fixing plants in all three genotypes. Xiong *et al.* (2015) reported that the g_m response to PPFD differed with N supplement in rice, with g_m increasing with PPFD in high N leaves while remaining unaffected in low N leaves, suggesting an important role of N in rapid response of g_m. We are unable to explain, on the basis of the present results, the cause of the observed genotypic variability in the N source effect on g_m–PPFD relationships. The mechanism of g_m response to short-term changes in PPFD is not yet clear. Rapid responses of g_m to environmental factors have been attributed to CA and AQPs. Transcript abundance of two AQP isoforms has been shown to substantially up-regulated by radiation within minutes in *Juglans regia* (Cochard *et al.* 2007; Baaziz *et al.* 2012). Day respiration has been shown to be influenced by the source of nitrogen (NH_4^+ or NO_3^-) supplied to plants (Guo *et al.* 2005). The link between PPFD and day respiration (Noguchi 2005) and nitrogen source might have played some role in the N source effect on the apparent g_m–PPFD relationship, through the influence of respiratory fractionation on g_m estimates (Barbour *et al.* 2017).

The present study showed no general trend in the effects of water availability on the g_m–PPFD relationships. However, the response of g_m to PPFD was not significant for the water-stressed PBA Slasher and water-stressed Sonali when they were measured under blue radiation. All genotypes responded similarly to radiation wavelength under both WW and WS conditions. The reduction in A and g_m when leaves were exposed to blue radiation compared to red radiation of the same intensity was similar to reductions reported in previous studies in *Nicotiana tabacum*, *Platanus orientalis* (Loreto *et al.* 2009), *Populus* × *canadensis* and *Quercus ilex* (Pallozzi *et al.* 2013). g_m was measured using chlorophyll fluorescence-based methods in these two studies and Loreto *et al.* (2009) demonstrated that the g_m response to blue light is real, although approximately half of the observed effect of blue radiation on g_m might be attributable to experimental artefacts.

Nevertheless, the fact that two methods that rely on substantially different assumptions produce similar results supports the hypothesis that the response of g_m to radiation wavelength is real. Further, differential response of g_m and g_{sw} to radiation wavelength in our study suggest uncoupling of the two conductance in the studied genotypes and environmental conditions, as also observed under blue radiation by Loreto *et al.* (2009) and under water stress conditions by Bunce (2009) but in contrast to the usually coregulation observed in wider multispecies data sets (Flexas *et al.* 2013). Nevertheless, the interpretation of the result should be made cautiously as the light exposure was not long enough (leaves remained in the chamber for 15 min) to ensure complete stomatal response. Gago *et al.* (2016) linked leaf gas exchange with leaf primary metabolism and reported that some sugars (mostly related to cell wall composition and structure; such as arabinose, xylose and galactose) had a significant effect on g_m but not A or g_{sw}. However, cell wall properties are less likely to exert influence on g_m in short-term environmental changes.

The observation that g_m is lower under blue radiation than red radiation could be related to chloroplast movement away from blue radiation, the avoidance response, to avoid photodamage to the photosynthetic machinery (Kagawa and Wada 2002; Suetsugu and Wada 2007). The avoidance response would reduce S_c under high blue radiation, 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 radiation in *Nicotiana* and *Platanus* leaves was faster than any possible chloroplast movements and the response was still observed after chloroplast movement inhibition. They suggested that the reduction of photosynthesis due to photochemical limitation under blue light might have, to some extent, affected g_m. In our study, the radiation wavelength significantly affected the calculated C_c, implying some extent of g_m limitation to photosynthesis under blue radiation. The response of g_m to blue radiation may have been caused by unknown factors affecting AQP-facilitated CO_2 diffusion in the mesophyll (Kaldenhoff 2012).

Overall, these experiments demonstrate the considerable variability in measured g_m responses to both long-term and short-term changes in environmental conditions. Some of this variability is likely to result from measurement artefacts, because g_m is always the residual variation in measurements that include instrument noise. Part of the observed variability probably also results from the complex nature of the trait. That is, whether a response to a given environmental stimulus is present or not probably depends on the relative importance of the component resistance and if a given resistor is sensitive to a given stimulus.

Conclusions

The present study showed that g_m varies between chickpea genotypes under ideal conditions and in response to growth conditions. This is the first study to examine the response of g_m to N_2-fixing versus N-fed (uninoculated) legumes. Genotypes differed in the sensitivity of g_m to nitrogen source. Flip079C had higher g_m when fertilized with NH_4NO_3 than when nitrogen was fixed by *Rhizobium* inoculates. The g_m sensitivity to blue radiation was similar between the genotypes and growth environments. There was no clear indication of water availability effects on responses of g_m to PPFD. Genotypes differed in the effects of nitrogen source on the rapid response of g_m to PPFD. Little research has been done in the area of g_m regulation under different N sources, and future work should extend to examine a wide range of legumes and environments, and explore the underlying mechanisms of the results of this study in greater detail. The large g_m variability observed in our experiments indicates that it may be premature to recommend increased g_m as a target for improved productivity or water-use efficiency.

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Contributions by the Authors

A.S. and M.M.B conceived the study and designed the experiments. A.S. and E.L.L. carried out the experiments and analyzed the data. A.S. wrote the manuscript with input from all authors. M.M.B and T.N.B provided critical feedback and contributed to the interpretation of the results and to the final manuscript.

Conflict of Interest

None declared.

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Supporting Information

The following additional information is available in the online version of this article—

Table S1. List of chickpea genotypes and their types used in Experiment 1.

Table S2. Effects of photosynthetic photon flux density (PPFD) on mesophyll conductance to CO_2 (g_m) across genotypes and treatments including radiation wavelength, water availability and nitrogen source in Experiments 2 and 3.

Table S3. Leaf gas exchange, online carbon isotope discrimination and mesophyll conductance values of 20 chickpea genotypes grown and measured under non-limiting controlled environmental conditions.

Table S4. Leaf gas exchange, online carbon isotope discrimination and mesophyll conductance values of the three chickpea genotypes grown under two nitrogen source treatments and measured under different photon flux densities.

Table S5. Leaf gas exchange, online carbon isotope discrimination and mesophyll conductance values of the three chickpea genotypes grown under well-watered or water-stressed conditions and measured under varying photon flux density and radiation wavelength.

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