Do faba bean (*Vicia faba* L.) accessions from environments with contrasting seasonal moisture availabilities differ in stomatal characteristics and related traits?

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Running title: **Stomatal characteristics in faba bean**

Abstract

Drought is a major constraint to faba bean (*Vicia faba* L.) production, and there are many mechanisms by which leaves can regulate water loss. Our primary objective was to test if the origin of the faba bean accessions, from drought-prone and non-drought-prone environments, was associated with differences in measurable aspects of stomatal morphology and physiology related to water use. Two sets, each consisting of 201 faba bean accessions, were chosen from environments with contrasting seasonal moisture profiles following the Focused Identification of Germplasm Strategy, and then screened under well watered conditions. From these, two subsets of 10 accessions each were chosen to test for differences in response to drought. Parameters related to stomatal function and water status were measured. The dry-adapted set had bigger stomata, higher leaf relative water content (LRWC) and cooler leaves under well watered conditions. Stomatal density and stomatal area per unit area of leaflet were negatively correlated with gas exchange parameters and positively correlated with intrinsic water use efficiency. Drought caused stomatal densities to increase in the dry set while stomatal length decreased in both sets. The moisture deficit was sufficient to decrease gas exchange in both sets to similar levels, but the dry-adapted set maintained warmer leaves and a higher LRWC that showed no significant correlations with leaf morphology or gas exchange, demonstrating more effective stomatal regulation. The results also support that collection site data from the environment where genetic resources are collected can be used as indicators of adaptive traits in an herbaceous annual species.

Keywords drought; focused identification of germplasm strategy; leaf temperature; stomatal conductance; stomatal morphology; *Vicia faba* L.; water use.

Abbreviations: A_{net}, photosynthetic rate. E, transpiration rate. FIGS, focused identification of germplasm strategy. g_s, stomatal conductance. LRWC, leaf relative water content. CTd, canopy temperature difference from air temperature. LTd, leaflet temperature difference from air temperature. WU, water used. WUE_b, biomass water use efficiency. WUE_i, intrinsic water use efficiency.

Introduction

Drought, defined as a temporary water deficit, is considered to be the environmental constraint most responsible for heavy production losses in crops (Boyer 1982). Faba bean (*Vicia faba* L.) is reputed to be relatively more sensitive to terminal drought stress, and possibly transient drought, than other temperate season grain legumes (Khan et al. 2007, 2010). The exchange of water and carbon dioxide between the plant and the atmosphere is a critical morpho-physiological process that affects drought response. Thus, stomatal characteristics such as density, size and responsiveness are considered key determinants of plant growth and water balance while under stress. Despite this, there is little information about how morphological characteristics of stomata relate to water loss, drought adaptation, and transpiration efficiency in plants, and in the studies that exist, results are often inconsistent (*e.g.* Wang and Clarke 1993a, b; Khazaei et al. 2010). The inconsistency of this relationship is attributable to the influence of characteristics other than stomata in transpiration and water loss. As a consequence, no clear relationship has been established between the morphological characteristics of stomata and water status in plants.

Nerkar et al. (1981) reported that stomatal conductance (g_s) was the most important trait determining water consumption in five faba bean accessions and that stomatal density contributed significantly to differences in the conductance. Among 11 faba bean accessions, those with higher stomatal density had lower yield and less resistance to water deficit, while lower stomatal density was associated with better adaption to drought (Ricciardi 1989). Khan et al. (2007) reported that drought-tolerant faba bean inbred lines had low g_s combined with high water use efficiency and warmer leaves, whereas sensitive lines in the set of nine showed the opposite combination. Appropriate stomatal activity might be helpful for improving drought resistance adaptation in faba beans by reducing water loss and increasing transpiration efficiency (Dawish and Fahmy 1997).

The available information concerning the relationship between stomatal morphology and adaptation to different environments has largely been obtained from woody perennials rather than annual crops, and showed opposite trends (Abrams 1994; Abrams et al. 1990). Restricted water supply generally causes an increase in stomatal density (Quarrie and Jones 1977; Yang and Wang 2001; Zhang et al. 2006; Sekiya and Yano 2008; Fraser et al. 2009), whereas sometimes there is no such change (Buttery et al. 1993; Aminian et al. 2011) and in one grass species, *Leymus chinensis* (Trin.) Tzvel., the stomatal density decreased but in a non-linear manner following water deficit stress (Xu and Zhou 2008).

Stomatal closure is one of the first responses to drought stress allowing the plant to avoid dehydration by reducing water loss and maintaining a favourable water status during stress conditions. It is well known that gas exchange (*i.e.*, g_s) is strongly reduced under drought stress in many legume species, including faba bean (Leport et al. 1998; Khan et al. 2007), but greater g_s under water deficit conditions is linked to a higher growth rate and biomass production. In this context, screening faba beans for higher g_s under water stressed conditions might be a useful means to select material for drought resistance breeding.

Leaf temperature was suggested as a rapid surrogate for measuring g_s in faba bean (Khan et al. 2010). Thus leaf physiological traits, such as relative water content and leaf temperature could be useful tools for drought resistance screening with faba beans. A wider spectrum of germplasm than the handful of accessions screened in earlier studies needs to be surveyed in order to validate this suggestion.

Natural agro-biodiversity stored in genebanks can be used to enhance the diversity of cultivated plants with trait variation that confer tolerance to biotic and abiotic stresses. There are often hundreds or thousands of times as many genebank accessions than can be screened, so choosing appropriate accessions is an important issue. The Focused Identification Germplasm Strategy (FIGS) uses environmental data associated with the collection sites of genebank accessions to make predictions about the selection pressures placed on the in-situ populations, and hence allows the identification of relatively small trait-specific, best-bet sets of genetic resource material that can be evaluated for adaptive traits, including those associated with drought tolerance (Mackay and Street 2004).

This study aimed to test whether faba bean germplasm from drought-prone and drought-free environments differed in measurable aspects of stomatal morphology and physiology under conditions of adequate water supply. The initial findings were then tested in a subset of material under both water deficit and water-sufficient conditions.

Materials and methods

Germplasm survey

Plant material

Two sets of faba bean (*Vicia faba* L.) accessions, 201 from environments with relatively high seasonal moisture regimes (here after referred to as the “wet set”) and 201 from dry regions with comparatively low seasonal moisture regimes (here after referred to as the “dry set”) were chosen according to the principles of FIGS (Mackay and Street 2004). The dry adapted set was constructed using accessions from the ICARDA (International Centre for Agricultural Research in the Dry Areas) genebank for which collection site geo-references were available. One accession was chosen at random from each collection site where the annual rainfall was between 300 and 550 mm/year. These sites were then grouped using the the SPSS hierarchical cluster analysis procedure (Version 17.0.1). The collection site agro-climatic descriptors used in the analyses were: long-term yearly precipitation, long-term yearly aridity index, long-term yearly minimum temperature, long-term yearly maximum temperature, temperature seasonality, precipitation seasonality, precipitation of wettest quarter and precipitation of coldest quarter (Worldclim dataset, http://www.worldclim.org/bioclim). The number of clusters that the procedure created was set at 20. The between groups linkage option was set as the clustering algorithm using squared euclidian distances as the distance measure. Accessions contained in 6 clusters were dropped because the average aridity index for the cluster was either above 0.6 or below 0.1 (indicating an irrigated site). The accessions in the remaining clusters were sorted on precipitation seasonality of their respective collection sites and anything with a score of 50 or lower was discarded. The remaining accessions were sorted on collection site long-term yearly precipitation and 200 accessions were chosen using the lowest scores. Selection ILB938/2 was added to the set as a benchmark for high water use efficiency (Abdelmula et al. 1999; Khan et al. 2007).

The wet set was constructed using one randomly selected accession from each site receiving more than 800 mm/year (long-term average), and then the 200 accessions from the sites with the highest yearly average aridity indices were chosen. Cultivar Aurora was added to the set as the benchmark for drought susceptibility (Amede et al. 1999; Khan et al. 2007).

Most of the wet set accessions originated from moist regions of China (79), Nepal (54), Bangladesh (24), Ethiopia (10) and Russia (7). The dry set originated mostly from the Middle East (Syria (75), Cyprus (32), Afghanistan (17), Morocco (10), Algeria (7), Turkey (7), and Tunisia (7)) and southern Europe (Spain (8) and Greece (7)). The ICARDA identifier numbers and details of origins are presented in supplementary Tables 1 and 2.

Experimental conditions

Experiments were conducted in the climate-controlled glasshouse of the Department of Agricultural Sciences, University of Helsinki, Finland using a randomized complete block design with 4 replicates in 2010. Soil moisture level was maintained at field capacity with automatic irrigation for all plants. Seeds of all accessions were inoculated with *Rhizobium* *leguminosarum* biovar. *viciae* (faba bean strain, Elomestari Oy, Tornio, Finland) before sowing. Three seeds were sown in 2 L plastic pots containing a mixture of sand and peat (White 420 W, Kekkilä Oy, Vantaa, Finland) (3:1 *v/v*) containing all essential nutrients. After 10 days the seedlings were thinned to one per pot. At three and five weeks after sowing, 70 ml of nitrogen-free fertilizer (equivalent to 20 kg of P and 24 kg of K per hectare) was added to each pot. Photoperiod was adjusted to 14 h light and 10 h dark, and the temperature was maintained at 21°C day/15°C night ±2°C. Photosynthetic photon flux density (PPFD) was approximately 300 µmol m^{-2} s^{-1} at the canopy level. Relative humidity was maintained at 60%. The temperature, humidity and light conditions were automatically recorded throughout the experiments.

Measurements

Stomatal morphology

Stomatal density, length and width, were measured on the middle part of the abaxial surface of the youngest, fully expanded leaflet of 8-week-old plants by the impression method (Wang and Clarke 1993a), whereby Xantopren® and its activator (Heraeus Kulzer GmbH, Germany) were used for taking impression from the leaflets, then bright nail polish impressions were made on the surface of the impression and used for microscopic observations (Leica® M-Series Stereo-microscopes, Ernst Leitz Wetzlar GMBH, Heerbrugg, Switzerland). The number of stomata was counted from ten different microscopic fields at 250x magnification and converted to the number per mm^2 of leaflet using a standard scale.

Stomatal length and width were measured on 10 stomata on the same leaf surface used for the stomatal density measurements from the impressions using an eyepiece micrometer at 500x magnification and converted to µm. Stomatal area was calculated as the product of stomatal length by width. Stomatal area per unit area of leaflet was calculated as the product of stomatal area and stomatal density.

Leaflet area

Leaflet area was measured using a LI-6200 leaf area meter (LI-COR Inc., Lincoln, NE, USA). The means of four leaflets per plant were used for statistical analysis.

Gas exchange traits

Gas exchange was measured on each plant at 6 weeks and 8 weeks after sowing, by using a LI-6400 portable photosynthesis system (LI-COR, Inc.) equipped with a 2×3 cm leaf chamber with a LED light source (6400-02B, 90% red and 10% blue). From a preliminary light source curve (following the manufacturer's instructions), it was determined that the appropriate photosynthesis photon flux density (PPFD) for faba bean was 1000 μmol m^{-2} s^{-1}. A CO_2-injecting cartridge was attached to the system to control reference CO_2 concentration at 400 μmol mol^{-1}, a value close to that during plant growth. The flow rate was 400 μmol s^{-1}. Temperature and relative humidity (RH) conditions were similar to those in the growth chamber. Chamber temperature and vapour pressure deficit (VPD) were held at 21˚C and 1.2±0.2 kPa, respectively, in all experiments. Mean cuvette temperature and VPD did not differ between experiments (≤ 2%). All the gas exchange measurements were done between 0900 and 1100 using the youngest, fully expanded leaflet that was also used for stomatal morphology and leaflet area measurements. Measurements were logged only when the stability criteria were met, according to the manufacturer's instructions. For logistical reasons, each replicate was measured on a separate day.

The measurements taken were photosynthetic rate (A_{net}), stomatal conductance (g_s), and transpiration rate (E). Intrinsic water use efficiency (WUE_i) was calculated as photosynthetic rate divided by stomatal conductance (A_{net}/g_s) (Centritto et al. 2009; Suriyagoda et al. 2010).

Temperature measurements

Leaflet temperature was determined using the fine-wire (0.127 mm diameter) chromel-constantan thermocouple within the LI-COR instrument. Canopy temperature was measured 6 weeks and 8 weeks after sowing using an infrared thermometer (IRT, FLUKE® thermometer gun 574, Everett, WA, USA) from the fully expanded leaves used for the other measurements. The protocol of using the IRT was followed carefully to avoid large variance error and non-repeatable results (Blum 2011). Air temperature was recorded at the time of measuring leaf temperatures, and the leaflet and canopy temperatures were subtracted from it to give the values of leaflet (LTd) and canopy (CTd) temperature difference, respectively.

Leaf relative water content

Five leaflets were used for determining leaf relative water content (LRWC%). First, fresh weight (FW) was determined. Turgid weight (TW) was measured after floating the sample on distilled water in Petri dishes in darkness at 4˚C for 24 h. Dry weight (DW) was taken after drying the samples for 48 h in a 60˚C oven. LRWC was calculated as LRWC (%) = ((FW–DW) / (TW–DW)) × 100 (Barrs and Weatherley 1962).

Transient drought response of 19 accessions

On the basis of principal component analysis on studied traits (Supplementary Figure 1) ten representative accessions from each set (wet and dry) were selected for exposure to transient moisture stress (marked with asterisks in supplementary Tables 1 and 2). One of the accessions from the dry set did not germinate adequately and was deleted from the experiment. Seeds of the selected accessions were inoculated with the appropriate Rhizobium as detailed above and sown in 2 L plastic pots (three seeds per pot) filled with 1.42 kg of mixture of sand and peat (3:1 *v/v*) that had a water holding capacity of 20% (*w/w*) in 2011. Pots were placed in a greenhouse in a completely randomized factorial design with four replicates. Each pot was brought to water holding capacity by adding 285 ml of water. Pots were weighed every 2 days and amounts of water equal to the loss in weight were added. Ten days after sowing, two seedlings were removed from each pot leaving the most vigorous one, and 60 g of perlite was added to the top of each pot to reduce soil evaporation. Unplanted pots were distributed among the planted pots, weighed and irrigated, in order to quantify evaporative water loss. In the well-watered treatment, the pots were irrigated as described above until harvesting at 13 weeks (when the main tiller of the plants turned yellow). Half of the plants were exposed to a gradual and uniform water deficit starting at 5 weeks by reducing water 2% (*w/w*) of available water per every two days to bring the moisture level down from the field capacity (20% *w/w*) to moisture stress (2–4% *w/w*). Pots were weighed and where water use exceeded 2%, irrigation was applied. Measurements were done 2 weeks after of the induction of moisture stress.

Water use and biomass water use efficiency

Shoots and roots were collected separately. Roots were carefully washed to eliminate the potting mix. Shoots and roots were dried at 70˚C for 2 days before weighing. The amount of water used was calculated as the difference between final and initial weight of the pot plus the total amount of water supplied to each pot, thus the total water used included both transpired and evaporated water, with the unplanted pots providing the correction for evaporation. Total dry matter per plant (shoot and root) was determined and biomass water use efficiency

(WUE_b) ($g.kg^{-1}$) was calculated as the ratio of dry matter produced to water used. Stomatal morphology, leaf gas exchange traits, leaf temperatures and leaf relative water content were determined using the same methods as in the germplasm survey.

Statistical analysis

The R statistical package (R Development Core Team, 2012) was used for all data analysis. Correlation analysis was performed to determine the relationship between the traits and regressions coefficients were calculated where appropriate. Statistical differences among treatments and interactions were determined using analysis of variance (ANOVA) after testing for normality. Comparisons of treatment means (well-watered *vs.* drought) as well as sets (wet *vs.* dry) in the transient drought response experiment were made using contrast analysis. Standard errors of means (S.E.M.) were calculated.

Results

Morphological and physiological parameters measured under well watered conditions

The dry set had longer stomata (4%), greater stomatal area (4%), more stomatal area per unit of leaflet (3%) and 48% more leaflet area (Table 1) than the wet set. Stomatal width and density did not, however, differ significantly between the sets.

Measures of gas exchange and photosynthesis did not differ significantly between the sets except for photosynthetic rate and transpiration rate which bothwere 5% higher in the dry set. LTd and CTd values were more negative (by 0.55° and 0.79°, respectively) in the dry set than in the wet set, indicating that the dry set cooled its leaves further below ambient temperatures than the wet set (Table 2). Also, greater variation was observed within the wet-adapted set for all traits studied (Table 1 and 2).

Relationships among stomatal morpho-physiological traits

Stomatal density was negatively associated with photosynthetic rate (Fig. 1D), stomatal conductance (Fig. 1C) and transpiration rate (Fig. 1B) in both sets. Stomatal area was positively correlated with photosynthetic rate (Fig. 1H), stomatal conductance (Fig. 1G), transpiration rate (Fig. 1F) while intrinsic water use efficiency (Fig. 1E) showed the opposite trend.

Since stomatal density and size were highly correlated to each other (wet set: R^2=0.61, P<0.001, dry set: R^2=0.62, P<0.001), stomatal area per unit area of leaflet was calculated, and it showed a difference between sets in its relation to gas exchange behaviour.

The sets differed in the relationships between morpho-physiological traits in the following manner. Stomatal density was positively correlated with intrinsic water use efficiency in the wet set only (Fig. 1A). Stomatal area per unit area of leaflet showed a weak negative association with intrinsic water use efficiency in the dry set and a weak positive association in the wet set (Fig. 1I). A negative association was found between stomatal area per unit area of leaflet and transpiration rate (Fig. 1J), stomatal conductance (Fig. 1K) and photosynthetic rate (Fig. 1L) in the wet set only. In all cases except intrinsic water use efficiency, the R^2 values were higher in the wet set than in the dry set.

Relationships between leaf temperatures and morpho-physiological traits under well-watered conditions

LTd and CTd showed strong positive correlations with stomatal density and intrinsic water use efficiency, and strong negative correlations with stomatal area, stomatal conductance and transpiration rate in both sets. By contrast stomatal area per unit area of leaf was positively correlated with LTd and CTd in the wet set only (Table 3).

While a positive correlation was found between LTd and CTd in both sets (wet set: R^2=0.08, P<0.001, dry set: R^2=0.18, P<0.001), in all cases, the covariate was more strongly correlated with LTd than with CTd (Table 3).

Relationships between leaf relative water content and morpho-physiological traits under well-watered conditions

In the wet set, LRWC showed a positive correlation with stomatal density, stomatal area per unit of leaflet area, and LTd, and a negative correlation with stomatal area, leaflet area, gas exchange rate, stomatal conductance and transpiration rate. By contrast, in the dry set only leaflet area was correlated to LRWC (Table 4).

Response of morphological and physiological traits to water deficit

There was a significant fall in biomass produced by both subsets under drought conditions (Fig. 2). The distribution pattern about the fitted line differed between well watered and dry treatments. Under drought conditions, the standard error was smaller about the fitted line. However, the dry subset was more evenly distributed along the line while the wet subset tended to use more water and yield more dry matter.

Drought conditions affected both stomatal morphology and water relations in both subsets. While stomatal area per unit of leaflet remained the same under the drought treatment, leaflet area fell by 31% to 36% with an accompanying drop in stomatal length (3 to 10%) and stomatal area (6 to 11%) (Table 5). Stomatal width did not differ between the sets and treatments (data not shown).

Drought caused a reduction in LRWC in both subsets in spite of the sizable reduction in stomatal conductance and thus gas exchange. Stomatal conductance fell by 84% to 91% (Table 6), associated with a 21 – 23% fall in water use by both subsets and a corresponding 3-

fold increase in intrinsic water use efficiency with no significant differences between subsets (Table 7). Leaflet and canopy temperatures were appreciably warmer under drought conditions in both subsets (Table 6).

There was significant variation within the dry and wet subsets in water relations. Accession 72420 from Syria had by far the lowest water use, the highest intrinsic water use efficiency, LRWC and stomatal density as well as the lowest stomatal conductance under drought conditions. Finally, there was a positive relationship between water used and total dry matter for both subsets (well watered: R^2=0.22, P=0.012, drought: R^2=0.57, P<0.001, n=19) (Fig. 2).

The subsets differed in response to drought in the following ways. Stomatal density in the dry subset increased by 21% (Table 5), while there was no change in the wet subset. There was a greater reduction in LRWC in the wet subset (27%) than in the dry set (19%) (Table 6). While the drought treatment did not significantly affect biomass water use efficiency of either subset (control vs. treatment), under drought conditions the dry subset used less water and had warmer leaves than the wet subset.

Discussion

Difference between the two germplasm sets

The results supported the opening proposition that germplasm sets originating from environments with contrasting seasonal water availability will display morpho-physiological differences associated with water use. The dry set had greater leaflet area, larger stomata, higher transpiration rates and cooler leaves. When drought was imposed, the dry set displayed an increase in stomatal density, maintained a greater LRWC, used water more efficiently and had warmer leaves.

Ecotypic variation in leaf morphology and factors affecting water use have been observed in other species. For example, in the grass *Leymus chinensis* (Trin.) Tzvel., stomatal density and length were greater in ecotypes collected from drier sites along a transect in northeast China (Yang et al. 2007). Further, osmotic adjustment was shown to be greater in landraces of sorghum (*Sorghum* sp.) and pearl millet (*Pennisetum glaucum* (L.) R.Br.) from dry regions than in those from humid regions (Blum and Sullivan 1986). In wild emmer wheat (*Triticum turgidum* spp. *dicoccoides* (Körn.) Thell.), the most drought-tolerant populations were those from hot dry locations (Peleg et al. 2005). Our results and those cited above support the theoretical basis for FIGS as described by Mackay and Street (2004).

Plants adapt to dry conditions using a combination of strategies that differs between species (Passioura and Angus 2010). The faba bean is an annual plant and plant strategies to avoid drought can be different between annual and perennial species. Stomatal density did not differ between the wet and dry sets of faba bean, or between two populations of the tree *Eucalyptus globulus* Labill. from high and low rainfall conditions in south-western Australia (Franks et al. 2009), but the density was higher in populations of the perennial grass *Leymus chinensis* from drier regions (Yang et al. 2007). Similarly, as found here, no differences were found in photosynthetic parameters among three populations of the tree *Quercus ilex* L. from wet, intermediate and dry regions (StPaul et al. 2012), under well watered conditions. While faba bean accessions from drier sites did not have higher stomatal densities, their stomata were longer and, in the first experiment, covered a greater proportion of the leaf area, the latter being positively correlated with intrinsic water use efficiency. Furthermore, when water deficit was applied, the stomatal density of the dry set increased and water use efficiency improved.

Relationship between traits

When a plant is exposed to a water deficit, one of the first responses is the regulation of gas exchange through stomatal closure, so that the internal water status can be maintained. Thus intra-specific variation in a crop plant's ability to regulate water loss in an appropriate manner is of particular interest to plant breeders focused on drought adaptation. As demonstrated here, there is indeed a significant amount of variation in stomatal density and area as well as the associated measures of gas exchange (Fig. 1), which supports the findings of other studies on faba bean (Tanzarella et al. 1984; Grzesiak et al. 1997a).

To understand how plants regulate the loss of water, it is useful to examine the relationship between the parameters that affect gas exchange. In this context, the literature does not allow us to draw definitive conclusions about the nature of these interactions. Intuitively one would expect a positive correlation between measures of gas exchange and stomatal density, as has been demonstrated for both annuals and woody perennials (*e.g.* Galmés et al. 2007; Tanaka et al. 2008; Xu and Zhou 2008; Aminian et al. 2011); the logic being that more stomata will promote higher rates of gas exchange. However, in this study, stomatal density and stomatal area per unit area of leaflet were both negatively correlated with the gas exchange parameters, as has also been reported for cowpea (*Vigna unguiculata* (L.) Walp.) (Sekiya and Yano 2008), durum wheat (*Triticum durum* Desf.) (Merah et al. 2001) and rice (*Oryza sativa* L.) (Ohsumi et al. 2007). Further, as density increased, the size of the stomata decreased, which is a well-recorded relationship (*e.g.* Miskin and Rasmusson 1970; Hetherington and Woodward 2003; Khazaei et al. 2010). Since smaller stomata allowed less gas exchange (Fig. 1 F, G and H) the overall effect of having more and smaller stomata was a reduction in gas exchange.
Accessions that demonstrated this behaviour also had improved transpiration efficiencies as opposed to those which had lower densities and larger stomata. Indeed, in the current experiment, the drought hardy accession ILB938/2 had significantly higher stomatal density

and WUE_i than the highly susceptible cultivar Aurora. In contrast, Ricciardi (1989) found that faba bean accessions with higher stomatal density had less resistance to moisture stress, while those with lower stomatal densities performed better under stress conditions. The present study has several times more entries than previous ones, and was on transient rather than terminal drought, so differences in outcome are not surprising.

Response to drought

As has been widely reported (*e.g.* Quarrie and Jones 1977; Grzesiak et al. 1997b; Fraser et al. 2009), drought caused a reduction in biomass production with a corresponding drop in leaflet area for both subsets. While drought reduced the stomatal size in both sets, only the dry set showed a significant increase in stomatal density, of 17%. In contrast, both sets showed massive reductions in gas exchange parameters, owing to regulation of the stomatal aperture; g_s for example, fell by up to 90% in the dry adapted set.

While these results are consistent with those of Xu and Zhou (2008) on *Leymus chinensis*, and previous studies on faba bean (Spence et al. 1986), it must be noted that many other moisture stress experiments have not shown any significant effect of drought on stomatal morphology (*e.g.* Buttery et al. 1993, Aminian et al. 2011). This further indicates that stomatal response to stress is not straightforward and requires further research if stomatal characteristics are to be used as selection criteria in plant breeding efforts.

The ability of genotypes to maintain LRWC when moisture is limiting is a good indicator of drought tolerance. In this study the LRWC fell for both subsets when moisture stress was imposed, but it is notable that the material from dry environments on average maintained higher LRWC and WUE values than the wet set, indicating some ecotypic differentiation. Furthermore, LRWC was correlated to stomatal morphology and gas exchange parameters in the wet set but not in the dry set (Table 4). The negative correlation between stomatal conductance and LRWC in the wet set is intuitively expected; a higher rate of water passing through the stoma increases the likelihood that under water-limited situations the LRWC will fall. However, if the relationship in this study was as straightforward as this, then we would expect that stomatal conductance would be lower in the dry set than in the wet set, which was not the case. This lack of correlation between stomatal morphology and conductance and LRWC has also been demonstrated in common wheat (Wang and Clarke 1993b) and cotton, *Gossypium hirsutum* L. (Malik et al. 2006). This indicates that the internal water status of the dry set was influenced by factors other than just stomatal conductance under water limiting conditions.

Although reducing water use under drought stress may be a useful adaptation in some situations, maintaining open stomata, and thus a relatively lower canopy temperature under drought conditions indicates a relatively better capacity for taking water from deeper down the soil profile and consequently result in better water status in plants (Blum 2009).

Leaf and canopy temperatures

Since stomata close due to a water deficit, the transpirational cooling effect is impeded, causing increased leaf temperatures, so leaf temperature can be an indicator of overall plant water status. In this study, leaflet and canopy temperature differences were correlated with WUE_i and stomatal conductance (Table 3), which has also been reported elsewhere for smaller sets of faba bean (Khan et al. 2007, 2010) and common wheat (Amani et al. 1996; Fischer et al. 1998).

Amani et al. (1996) demonstrated that leaf temperature depression was linearly related to stomatal conductance in spring wheat. Hence leaf temperature can serve as an effective and economic surrogate measurement for stomatal conductance. In a further refinement of this, thermal imaging has been suggested as a screening tool for large numbers of accessions to detect variation in stomatal conductance as water deficits are imposed (Munns et al. 2010). The present study indicates that faba bean leaflet and canopy temperature differences from ambient can be used as surrogates for stomatal density and area as well as stomatal conductance and intrinsic water use efficiency, under well-watered conditions.

However the case is not so clear when drought is imposed. In the model detailed above, reduced stomatal conductance should lead to warmer leaves. In this study, when drought was imposed, the dry set warmed and maintained higher LRWC, both of which indicate reduced stomatal conductance, but the difference in stomatal conductance between the sets was not significant (P = 0.060).

Conclusions

Faba beans originating from environments with contrasting moisture profiles displayed ecotypic differentiation in terms of morphology and the regulation of water use. This study therefore supports the model proposed by Mackay and Street (2004), that genetic resource collections can be more efficiently utilized by using collection site environmental data to predict adaptive traits. For many years, some plant breeders and crop physiologists have asserted that reduced stomatal densities and size were key objectives to enhance drought adaptation. For faba bean at least, the present study does not support this approach. Rather, it indicates that stomatal function, and other as yet unidentified processes such as root morphology and function, cuticular wax content and osmotic adjustment (Khan et al. 2010), play a more important role than stomatal density and size in gas exchange, since a) stomatal area and density were negatively correlated to each other, so reducing one is the same as increasing the other, b) stomatal densities actually increased for both sets under drought conditions while gas exchange fell dramatically, and c) subsets had the same stomatal area per unit of leaflet, densities and gas exchange measurements under control and drought conditions but the set from the dry environment maintained higher LRWC and used water more efficiently. This study confirms that leaflet and canopy temperature measures are correlated with morphological measurements as well as gas exchange parameters and LRWC. Hence, it supports those studies that suggest temperature can be used as a surrogate for the more expensive leaf morpho-physiological measurements. However, the temperature measurements need to be interpreted with caution. In this study, while LTd and CTd were correlated with stomatal density, area and gas exchange traits, the relationships between these traits were not able to give a clear indication of the mechanisms that caused the different LRWC realized for the two sets under drought stress. Nevertheless, the temperature measurements were able to point towards the more favourable LRWC.

Supplementary data

Supplementary Table 1 List of wet set germplasm used in the study, including ICARDA accession number, country and province of origin and geographic coordinates.

Supplementary Table 2 List of dry set germplasm used in the study, including ICARDA accession number, country and province of origin and geographic coordinates.

Supplementary Figure 1 Results of principal component analysis of studied traits of the wet (A) and dry (B) sets of faba bean. Ten accessions for transient drought response experiment were chosen from those within the circle.

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Table 1 The mean and significance of different source of variation for stomatal morphology measurements and leaflet area for faba bean sets collected from dry environments and relatively wet environments measured under well-watered conditions.

	Density (No. mm^{-2})	Length (μm)	Width (μm)	Area ($μm^2$)	Area per unit of leaflet (10^3 $μm^2.mm^{-2}$)	Leaflet area (cm^2)
Means						
Wet set	**49.6**	**53.2**	**30.4**	**1622**	**77.9**	**11.4**
S.E.M.	0.87	0.28	0.114	13.0	0.88	0.41
Dry set	**48.3**	**55.4**	**30.3**	**1685**	**80.6**	**16.9**
S.E.M.	0.44	0.19	0.065	5.4	0.48	0.37
Source of variation						
Wet	***	***	***	***	***	***
Dry	***	***	***	***	***	***
Between sets	ns	***	ns	***	**	***

S.E.M., standard error of means.

ns, non significant; ** and *** indicate significance at P <0.01 and P <0.001, respectively.

Table 2 The mean and significance of different source of variation for leaf physiological measurements for faba bean sets collected from dry environments and relatively wet environments measured under well-watered conditions.

	A_{net} (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	WUE_i (μmol mol^{-1})	E (mmol m^{-2} s^{-1})	LTd (°C)	CTd (°C)	LRWC (%)
Means							
Wet set	**7.8**	**0.329**	**25.6**	**3.26**	**–0.17**	**–0.80**	**82.3**
S.E.M.	0.14	0.008	0.55	0.06	0.003	0.009	0.28
Dry set	**8.2**	**0.320**	**26.4**	**3.42**	**–0.72**	**–1.59**	**86.3**
S.E.M.	0.08	0.005	0.30	0.03	0.001	0.003	0.17
Source of variation							
Wet	***	***	***	***	***	***	***
Dry	***	***	**	***	**	***	***
Between sets	*	ns	ns	*	***	***	***

A_{net}, photosynthetic rate; g_s, stomatal conductance; WUE_i, intrinsic water use efficiency; E, transpiration rate; CTd, canopy temperature difference from air temperature; LTd, leaflet temperature difference from air temperature; LRWC, leaf relative water content; S.E.M., standard error of means.

ns, non significant; *, ** and *** indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table 3 Pair-wise simple Pearson correlation coefficients between leaf and canopy temperature with some morpho-physiological traits (n=201) measured under well-watered conditions.

		Stomatal density	Stomatal area	Stomatal area per unit of leaflet	g_s	WUE_i	E
LTd	wet set	0.41***	–0.40***	0.30***	–0.60***	0.51***	–0.59***
	dry set	0.22***	–0.38***	–0.06ns	–0.85***	0.75***	–0.82***
CTd	wet set	0.28***	–0.33***	0.16*	–0.34***	0.28***	–0.35***
	dry set	0.23***	–0.24***	0.06ns	–0.43***	0.39***	–0.44***

g_s; stomatal conductance; WUE_i; intrinsic water use efficiency; E, transpiration rate; CTd, canopy temperature difference from air temperature; LTd, leaflet temperature difference from air temperature.

ns, non significant; * and *** indicate significance at *P* <0.05 and *P* <0.001, respectively.

Table 4 Correlations between leaf relative water content (LRWC) and some morpho-physiological traits (n=201) measured under well watered conditions.

Morpho-physiological traits	Correlation with LRWC	
	Wet set	Dry set
Stomatal density	0.456^{***}	0.019^{ns}
Stomatal area	$–0.257^{***}$	0.104^{ns}
Stomatal area per unit area of leaflet	0.486^{***}	0.128^{ns}
Leaflet area	$–0.363^{***}$	0.180^{*}
A_{net}	$–0.331^{***}$	0.088^{ns}
g_s	$–0.328^{***}$	0.007^{ns}
WUE_i	0.134^{ns}	0.020^{ns}
E	$–0.344^{***}$	0.012^{ns}
LTd	0.256^{***}	0.046^{ns}
CTd	0.127^{ns}	0.108^{ns}

A_{net}, photosynthetic rate; g_s, stomatal conductance; WUE_i, intrinsic water use efficiency; E, transpiration rate; CTd, canopy temperature difference from air temperature; LTd, leaflet temperature difference from air temperature.

ns, non significant; * and *** indicate significance at *P* <0.05 and *P* <0.001, respectively.

Table 5 The mean and significance of different source of variation for stomatal morphology measurements and leaflet area of faba bean subsets collected from dry environments (n = 9) and relatively wet environments (n = 10) measured under well-watered conditions and under an experimentally imposed drought.

	Density (No. mm^{-2})			Length (µm)			Area (µm^2)			Area per unit of leaflet (10^3 µm^2.mm^{-2})			Leaflet area (cm^2)		
Means	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*
Wet set	**46.6**	**50.0**	*0.064*	**53.6**	**51.9**	*0.018*	**1566**	**1468**	*0.009*	**72.3**	**72.6**	*0.856*	**12.1**	**8.3**	*<0.001*
S.E.M.	1.2	1.2		0.51	0.52		**25**	**22**		1.4	1.6		0.84	0.74	
Dry set	**43.1**	**52.43**	*<0.001*	**55.4**	**50.0**	*<0.001*	**1633**	**1446**	*<0.001*	**69.8**	**74.8**	*0.105*	**14.0**	**9.0**	*<0.001*
S.E.M.	1.2	1.8		0.57	0.64		23	35		1.6	2.4		0.91	0.79	
P	*0.054*	*0.267*		*0.035*	*0.623*		*0.067*	*0.586*		*0.264*	*0.460*		*0.629*	*0.213*	
Source of variation															
set			ns			ns			ns			ns			ns
Accession			**			***			*			***			***
Stress			***			***			***			ns			***
Set × Stress			*			*			ns			ns			*
Accession × Stress			ns			ns			ns			ns			ns

S.E.M., standard error of means.

ns, non significant; *, ** and *** indicate significance at P <0.05, P <0.01 and P <0.001, respectively.

Table 6 The mean and significance of different source of variation for leaf physiological measurements of faba bean subsets collected from dry environments (n = 9) and relatively wet environments (n = 10) measured under well watered conditions and under an experimentally imposed drought.

	A_{net} (μmol m^{-2} s^{-1})			g_s (mol m^{-2} s^{-1})			LTd (°C)			CTd (°C)			LRWC (%)		
Means	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*
Wet set	**5.03**	**1.47**	*<0.001*	**0.108**	**0.017**	*<0.001*	**–0.6**	**–0.2**	*0.002*	**–0.9**	**–0.4**	*<0.001*	**80.7**	**58.9**	*<0.001*
S.E.M.	0.19	0.21		0.011	0.002		0.007	0.007		0.013	0.016		1.1	1.7	
Dry set	**4.51**	**1.86**		**0.100**	**0.009**	*<0.001*	**–1.1**	**0.1**	*<0.001*	**–1.8**	**0.4**	*<0.001*	**81.2**	**65.0**	*<0.001*
S.E.M.	0.12	0.22	*<0.001*	0.005	0.001		0.005	0.003		0.011	0.024		1.5	2.2	
P	*0.035*	*0.211*		*0.568*	*0.060*		*<0.001*	*0.004*		*0.159*	*0.014*		*0.771*	*0.043*	
Source of variation															
Set			ns			ns			**			*			*
Accession			ns			*			ns			**			ns
Stress			***			***			***			***			***
Set × Accession			**			ns			***			***			ns
Accession × Stress			ns			ns			ns			ns			ns

A_{net}, photosynthetic rate; g_s, stomatal conductance; CTd, canopy temperature difference from air temperature; LTd, leaflet temperature difference from air temperature; LRWC, leaf relative water content; S.E.M., standard error of means.

ns, non significant; *, ** and *** indicate significance at P <0.05, P <0.01 and P <0.001, respectively.

Table 7 The mean and significance of different source of variation for water use parameters for faba bean subsets collected from dry environments (n = 9) and relatively wet environments (n = 10) measured under well-watered conditions and under an experimentally imposed drought.

	WU (kg.plant^{-1})			WUE$_b$ (g.kg^{-1})			WUE$_i$ (µmol mol^{-1})		
Means	Control	Drought	*P*	Control	Drought	*P*	Control	Drought	*P*
Wet set	**3.83**	**2.93**	*<0.001*	**4.69**	**5.05**	*0.189*	**54**	**149**	*<0.001*
S.E.M	0.069	0.060		0.19	0.18		3.6	8.2	
Dry set	**3.50**	**2.76**	*<0.001*	**4.55**	**4.37**	*0.590*	**55**	**158**	*<0.001*
S.E.M.	0.083	0.087		0.26	0.18		2.3	14.6	
P	*0.007*	*0.125*		*0.650*	*0.016*		*0.298*	*0.201*	
Source of variation									
Set			***			ns			ns
Accession			**			***			ns
Stress			***			ns			***
Set × Accession			ns			**			ns
Accession × Stress			ns			ns			ns

WU, water used; WUE$_b$, biomas water use efficiency; WUE$_i$, intrinsic water use efficiency; S.E.M., standard error of means.

ns, non significant; ** and *** indicate significance at $P \leq 0.01$ and $P \leq 0.001$, respectively.

Figure legends:

Figure 1 Correlations of stomatal density, area and area per unit area of leaflet with A_{net}, g_s, E and WUE_i among wet set (solid circles, solid line) and dry set (open circles, dotted lines) accessions (*n* = 201). Solid and open stars corresponded to cvs. Aurora and ILB938/2, respectively. Error bars for all graphs show mean ± 1 S.E.M.

(A) wet set: R^2=0.08, *P*<0.001, *y*=0.17*x*+17.03, dry set: R^2=0.01, *P*=0.159, *y*=0.067*x*+23.11

(B) wet set: R^2=0.44, *P*<0.001, *y*=–0.05*x*+5.82, dry set: R^2=0.07, *P*<0.001, *y*=–0.03*x*+ 4.30

(C) wet set: R^2=0.38, *P*<0.001, *y*=–0.01*x*+0.62, dry set: R^2=0.06, *P*<0.001, *y*=–0.01*x*+0.45

(D) wet set: R^2=0.52, *P*<0.001, *y*=–0.12*x*+13.69, dry set: R^2=0.14, *P*<0.001, *y*=–0.07*x*+11.45

(E) wet set: R^2=0.09, *P*<0.001, *y*=–0.01*x*+46.71, dry set: R^2=0.11, *P*<0.001, *y*=–0.01*x*+ 45.88

(F) wet set: R^2=0.48, *P*<0.001, *y*=0.003*x*–1.95, dry set: R^2=0.23, *P*<0.001, *y*=0.002*x*–0.23

(G) wet set: R^2=0.45, *P*<0.001, *y*=4.33*x*–0.37, dry set: R^2=0.20, *P*<0.001, *y*=2.69*x*– 0.13

(H) wet set: R^2=0.55, *P*<0.001, *y*=0.01*x*–5.55, dry set: R^2=0.26, *P*<0.001, *y*=0.005*x*+0.24

(I) wet set: R^2=0.03, *P*=0.010, *y*=1.14*x*+16.76, dry set: R^2=0.04, *P*=0.008, *y*=–1.16*x*+35.74

(J) wet set: R^2=0.22, *P*<0.001, *y*=–3.35*x*+6.07, dry set: R^2=0.004, *P*=0.350, *y*=0.005+2.78

(K) wet set: R^2=0.18, *P*<0.001, *y*=–4.11*x*+0.65, dry set: R^2=0.009, *P*=0.171, *y*=0.001+0.24

(L) wet set: R^2=0.26, *P*<0.001, *y*=–8.46*x*+14.39, dry set: R^2=0.005, *P*=0.314, *y*=–0.01+9.11

Figure 2 Correlations between water used and biomass under well-watered (open symbols) and drought (solid symbols) conditions (*n* = 19). Triangles and circles correspond to the dry and wet sets, respectively. Stars show Accession 72420. Error bars show ± 1 S.E.M. Well-watered: R^2=0.22, *P*=0.012, *y*=0.05*x*+2.68, Drought: R^2=0.57, *P*<0.001, *y*=0.07*x*+1.84.