**Original Article**

**Identifying physiological and genetic determinants of faba bean transpiration response to evaporative demand**

Hend Mandour1,2\*, Hamid Khazaei3, Frederick L. Stoddard4, Ian C. Dodd1\*

1 Lancaster Environment Centre, Lancaster University, LA1 4YQ, United Kingdom

2 Genetic Engineering and Biotechnology Research Institute, National Research Centre, Egypt

3 Production Systems, Natural Resources Institute Finland (LUKE), Latokartanonkaari 9, 00790 Helsinki, Finland

4 Department of Agricultural Sciences, Viikki Plant Science Centre and Helsinki Institute of Sustainability Science, PO Box 27 (Latokartanonkaari 5-7), FI-00014 University of Helsinki, Helsinki, Finland

**Running title:** Transpiration response to evaporative demand in faba bean

\* Correspondence: [hendmandour144@gmail.com](mailto:hendmandour144@gmail.com), [h.mandour@lancaster.ac.uk](mailto:h.mandour@lancaster.ac.uk)

* **Background and aims** Limiting maximum transpiration rate (TR) under high vapor pressure deficit (VPD) works as a water conservation strategy. While some breeding programs have incorporated this trait into some crops to boost yields in water-limited environments, its underlying physiological mechanisms and genetic regulation remain unknown for faba bean (*Vicia faba*). Thus we aimed to identify genetic variation in TR response to VPD in a faba bean recombinant inbred lines (RILs) population derived from two parental lines with contrasting water use (Mélodie/2 and ILB 938/2).
* **Methods** Plants were grown in well-watered soil in a climate-controlled glasshouse with diurnally fluctuating VPD and light conditions. Whole plant transpiration was measured in a gas exchange chamber that tightly regulated VPD around the shoot under constant light, while whole-plant hydraulic conductance and its components (root and stem hydraulic conductance) were calculated from dividing TR by water potential gradients measured with a pressure chamber.
* **Key results** Although TR of Mélodie/2 increased linearly with VPD, ILB 938/2 limited its TR after 2.0 kPa. Nevertheless, Mélodie/2 had a higher leaf water potential than ILB 938/2 at both low (1.0 kPa) and high (3.2 kPa) VPD. Almost 90% of the RILs limited their TR at high VPD with a break-point (BP) range of 1.5<BP<3.0 kPa and about 10% had a linear TR response to VPD. Thirteen genomic regions contributing to minimum and maximum transpiration, and whole-plant and root hydraulic conductance, were identified on chromosomes 1 and 3, while one locus associated with BP transpiration was identified on chromosome 5.
* **Conclusions** This study provides insight into the physiological and genetic control of transpiration in faba bean and opportunities for marker-assisted selection to improve its performance in water-limited environments.

**Keywords:** faba bean, abiotic stress, transpiration, vapor pressure deficit, hydraulic conductance genetic control

INTRODUCTION

Roughly one-third of the world’s arable land suffers from water shortage, which is expected to double by 2050 (Vicente-Serrano *et al.,* 2012). Therefore, it is essential to provide farmers with drought-adapted crop varieties to improve yields in water-limited (and well-watered) environments. Among the traits that can ameliorate the effects of water deficits on plant development and performance is limited transpiration rate (TR) under high vapor pressure deficit (VPD), which works as a water conservation strategy to delay the harmful effects of late-season water deficit. Moreover, simulation models that incorporated this trait into crops such as soybean (*Glycine max* [L.] Merr.) (Sinclair *et al.,* 2010), maize (*Zea mays* L.) (Messina *et al.,* 2015), and lentil (*Lens culinaris* Medik.) (Guiguitant *et al.,* 2017) revealed that limiting TR after 1 to 2 kPa (according to the species) resulted in major yield gains under late-season drought environments. Under late-season water deficit, genotypes that limit TR at elevated VPD can potentially use conserved soil water to sustain their physiological performance during grain filling, so they yield more than genotypes that are not expressing the trait ([Sinclair *et al.,* 2016](https://www.sciencedirect.com/science/article/pii/S0378429016306050#bib0220)). Nevertheless, if there is late-season rainfall, the conserved soil water may not be beneficial; thus genotypes with limited TR trait would yield similar or lower than genotypes that do not express the trait ([Vadez *et al.,* 2014](https://www.sciencedirect.com/science/article/pii/S0378429016306050#bib0250); [Sinclair *et al.,* 2016](https://www.sciencedirect.com/science/article/pii/S0378429016306050#bib0220)). Hence, the yield benefits of the limited-transpiration trait are likely to vary across growing seasons and locations. Thus, limiting TR at high VPD appears to be a promising selection trait, especially in drought-prone areas where crops rely on stored soil moisture.

Selection for limited TR at high VPD in field conditions is always challenged by the requirement of phenotyping the trait in a wide range of environmental conditions (Ghanem *et al.,* 2015). Thus, detecting the locations and the effects of genes that regulate limited TR at high VPD is urgently needed using environment-independent DNA markers, especially in drought-sensitive crop species such as faba bean (e.g. Khazaei *et al.,* 2014; Muktadir *et al.,* 2020). Genomics and transcriptomic approaches now being applied in faba bean open new opportunities for fine mapping and uncovering candidate genes (Khazaei *et al.,* 2021). The way to highly saturated and cost-effective second-generation genetic maps has been facilitated by developing DNA markers based on single nucleotide polymorphisms (SNPs) in faba bean (Webb *et al.,* 2016; Carrillo-Perdomo *et al.,* 2020; Khazaei *et al.,* 2021; Gela *et al.,* 2022). The SNP markers provide low genotyping cost per data point, high genomic polymorphism, locus specificity in terms of accuracy and reproducibility (e.g. Yan *et al.,* 2010; Chongtham *et al.,* 2022), simple documentation, codominance, a common occurrence amongst elite germplasm (e.g. Cottage *et al.,* 2012; Skovbjerg *et al.,* 2022), and potential for high-throughput analysis (Wang *et al.,* 2021). Recently, a high-density faba bean genotyping array (‘Vfaba\_v2’) containing 24,929 polymorphic high-resolution SNP markers located in 15,846 different genes has been developed (O'Sullivan *et al.,* 2019; Donal M. O'Sullivan, personal communication). Thus, SNP markers are considered powerful tools in genetic mapping, association studies, assessing genetic diversity, and positional cloning in faba bean. In the absence of a published faba bean reference genome, a number of transcriptomes have been reported for faba bean looking for drought-adaptation related genes (*see* Alghamdi *et al.,* 2018; Khan *et al.,* 2019; Wu *et al.,* 2020). The large genome of faba bean is currently being assembled, which will further advance faba bean genomics and breeding (Jayakodi *et al.,* 2022).

For plants to replace transpirational losses, the soil needs to continuously supply water to the roots, otherwise plant transpiration is restricted under unfavourable conditions such as high VPD. Plants can regulate transpiration at high VPD by matching stomatal and hydraulic conductance to maintain a constant water potential ([Attia *et al.,* 2015](https://www.frontiersin.org/articles/10.3389/fpls.2018.01572/full#B3)). By decreasing stomatal conductance (gs) to water vapor, plants minimize water loss and maintain cellular hydration as VPD increases. Despite many reports on transpiration response to VPD in several crop species, the mechanism(s) of stomatal closure under high VPD remains unclear (Damour *et al.,* 2010; Jalakas *et al.,* 2021). Maximum transpiration rate and maximum whole-plant hydraulic conductance are positively related (Tsuda and Tyree, 2000) suggesting that hydraulic conductance of different plant organs such as leaves (Sadok and Sinclair, 2010b) and roots (Sinclair *et al.,* 2014; Sivasakthi *et al.,* 2020) can constrain transpiration at high VPDs. However, there is considerable species variation in which organ is perceived to limit TR at high VPD. Limited TR under high VPD was associated with low leaf hydraulic conductance in soybean (Sadok and Sinclair, 2010b) and sorghum (*Sorghum bicolor* L.) (Choudhary *et al.,* 2013), while limited root hydraulic conductance was correlated with restricted TR at high VPD in chickpea (*Cicer arietinum* L.) (Sivasakthi *et al.,* 2020). In maize, both leaf and root hydraulic conductance limit TR at high VPD (Choudhary *et al.,* 2014). Thus, plant hydraulic conductance seems to play a vital role in regulating the stomatal response to changes in VPD (Sperry *et al.,* 2002).

Although restricting transpiration at high VPD can maintain crop yields in dry environments, relatively few studies have sought to determine the genetic basis of this trait (e.g. Schoppach *et al.,* 2016; Affortit *et al.,* 2022; Tamang *et al.,* 2022)despite the availability of high throughput phenotyping of transpiration response to VPD (Ryan *et al.,* 2016; Jauregui *et al.,* 2018; Kar *et al.*, 2020).Screening segregation populations is essential to understand the genetics of limited transpiration at high VPD, particularly in drought-susceptible crops such as faba bean. This research utilised an advanced faba bean recombinant inbred lines (RILs) population with the following objectives: 1) to identify genotypic variation in TR to VPD, 2) to examine whole-plant hydraulic conductance and its components as a possible regulatory mechanism for limited TR, and (3) to identify genomic regions associated with transpiration response to VPD.

MATERIALS AND METHODS

*Plant material*

The mapping population comprised 165 RILs derived from a cross between Mélodie/2 and ILB 938/2 at the F8 generation (Khazaei *et al.,* 2014). Mélodie/2 is an inbred line from INRA (Institut National de la Recherche Agronomique, France) with a relatively high yield and highly efficient use of water, where it maximizes soil moisture capture for transpiration, minimizes water loss by soil evaporation by rapid vegetative growth and reduces non-stomatal transpiration. ILB 938/2 is a selection from an accession originating from the Andean region of Colombia and Ecuador, maintained at ICARDA (International Centre for Agricultural Research in the Dry Areas), with high water use efficiency (WUE, ratio of [biomass](https://en.wikipedia.org/wiki/Biomass) produced to the rate of transpiration) and relatively low productivity (Khan *et al.,* 2007; Khazaei *et al.,* 2013; Khazaei *et al.,* 2018). The parent lines differed in their responses to water deficit. Mélodie/2 had a cooler canopy under well-watered conditions and a much greater increase in canopy temperature under water deficit conditions than ILB 938/2, while gs followed the opposite trend. Water deficit induced in potted plants under glasshouse conditions had a 3-fold greater effect on biomass production of Mélodie/2 than ILB 938/2, but biomass in Mélodie/2 under water deficit conditions was the same as ILB 938/2 under well-watered conditions (Khazaei *et al.,* 2014). Thus, ILB 938/2 can maintain higher water status under water deficit conditions as it has high WUE with a relatively low yield. In contrast, Mélodie/2 had better productivity under drought conditions than ILB 938/2, by maintaining water uptake via a well-developed root system (Khazaei *et al.,* 2014). Furthermore, the parental lines differed in a wide range of agronomic and morphological characteristics, confirming the wide genetic variation between them and their suitability for genetic mapping and genomic studies. This genetic and geographic divergence made them ideal for building a promising segregating population for successful genetic and trait mapping (Würschum, 2012).

*Growing conditions*

A total of 165 RILs from cross Mélodie/2 × ILB 938/2 at F8 generation were used to study transpiration, leaf water potential, and hydraulic conductance under a range of VPDs ina whole-plant gas exchange chamber (Jauregui *et al.*, 2018) between 2019-2021. Seeds were chosen randomly and germinated at about 2.5 cm depth in rectangular 2 L pots (12.5×10.5×21 cm) containing a mixture of commercial John Innes No. 2 substrate (Westland Horticulture Ltd, UK) and silver sand (Royal Horticultural Society, UK) in a ratio of 3:1 (v/v). Depending on seed availability, each RIL was represented by three to four plants with seeds planted at different times of the year (Supplementary Table S1) in a semi-controlled glasshouse to ensure the replicates were randomly distributed across varying atmospheric conditions in the glasshouse at Lancaster Environment Centre, Lancaster University, UK. Supplementary lighting (high-pressure sodium lamps, Osram Plantastar 600W, Munich, Germany) maintained the photoperiod at 12 hours (08:00-20:00 h). The light intensity during the photoperiod was 551 ± 3 µmol m−2 s−1 PPFD (photosynthetic photon flux density) (mean ± SE, n=3600, comprising 12 h × 300 days) at the pot surface ∼ 2 m below the lamp. Air temperature and relative humidity in the centre of the glasshouse were recorded hourly with a Hortimax system (HortiMax Ektron III, hortisystems.co.uk). Day/night temperature ranges were 26.1 ± 0.06 °C and 19.7 ± 0.04 °C (mean ± SE, n=3600), respectively. Relative humidity day/night ranges were 31 ± 0.2% and 44 ± 0.3% (mean ± SE, n=3600), respectively across the entire period of experiment. These ranges generated a day/night VPD range of 2.32 ± 0.61 kPa to 1.28 ± 0.60 kPa (mean ± SE, n=3600), respectively. The plants were grown for approximately four weeks, daily irrigated to the upper limit of pot drained capacity, and fertilized weekly with 0.3% (w/v) Miracle-Gro All Purpose Plant Food (The Scotts Company Ltd, UK), supplying 20.5:3.5:3.5 (N:P:K). Homogeneous plants (leaf area = 299 ± 4 cm2, mean ± SE, n=560) were selected based on their developmental stage (7-8 fully expanded leaves) rather than chronological age. Although leaf area did not significantly differ among RILs, it differed over the year (Supplementary Table S1). For example, plants had 35% higher LA in May than those in August (Supplementary Fig. S1). However, neither genotype nor the genotype × month interaction was significant (Supplementary Table S1) which was expected since the basic criteria for choosing the plants was leaf number (7-8 leaves). Plants were assigned to measure transpiration response to VPD in the whole-plant gas exchange between December 2019 to January 2022. Each date assigned a number from 1 (January 1st) to 365 (December 31st) with no measurements occurring on 28th February.

*Measuring transpiration and hydraulic conductance responses to VPD*

Transpiration rate (TR) responses to elevated VPD in the whole-plant gas exchange system were measured on three plants per day, as described by Jauregui *et al.* (2018) from 09:00 h to 20:00 h under six VPD levels within the range of ∼ 1.0-3.5 kPa, with three plants measured per day. Previous experiments with two commercial faba bean cultivars revealed no time of the day (morning, afternoon, late-afternoon), or year (July vs. October), effect on the transpiration response to VPD (unpublished data).

Briefly, the plants were watered to maximum pot drained capacity and left to drain for about 15 min during which two leaves (the 3rd and the 4th ones from the base of the plant) were covered with aluminium foil to estimate stem water potential (Ψstem) under the lowest and the highest VPDs. The plants were then sealed into the chamber and left to acclimate to the chamber lights for about 30 minutes. The measurement started by increasing chamber relative humidity to its maximum of 70% ± 0.6 (mean ± SE, n=560) to generate the lowest VPD while the temperature is stable (25.9 ± 0.09, mean ± SE, n=560). After CO2 and H2O exchange had been steady for at least 5 min (steady-state), averaged values were logged every minute for 5 min. Then the chamber was opened, and the xylem pressure potential of the aluminium foil covered leaf (Ψstem) and one fully expanded transpiring leaf (Ψleaf) (15-20% of total leaf area) across both leaves was measured using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). After closing the chamber again, relative humidity inside the system was further decreased by introducing a mixture of dry and humidified air to the chamber. After the following relative humidity level was achieved, plant gas exchange was allowed to stabilize (typically 30-45 min) and CO2 and H2O values were logged again. Each plant was exposed to six sequentially decreasing humidity levels (70, 58, 43, 31, 18, and 11%), achieved by increasing the ratio of dry to humid air approximately corresponding to VPD values of 1.00, 1.41, 1.91, 2.32, 2.75 and 3.20 (± 0.02) kPa (mean ± SE, n= 560). Each genotype had at least one plant measured at each time of day. Thus each plant took 3-4 hours to quantify its transpiration response to VPD, as demonstrated in Mandour (2022).

Across the entire period of measurements (morning, afternoon, and late-afternoon), the main driving force for the VPD treatments was variation in the humidity levels established in the whole-plant gas exchange chamber as a result of differing air source humidity, since chamber temperature was stable resulting in VPD ranging from ∼ 1.0 to 3.2 kPa for all RILs. At the highest VPD, Ψleaf and Ψstem were determined again. The covered leaves were not included in leaf area calculations for transpiration measurements but were included in total leaf area calculations. After measuring the whole-plant gas exchange response to changing VPD, the plant was removed from the chamber to determine its leaf area using a leaf area meter (Model LI-3100C, LI-COR, Lincoln, NE, USA).. Data were then downloaded from the infra-red gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) comprising records of transpiration in mg H2O (which later was normalized to time in minutes and leaf area to get transpiration rate TR), VPD, and other physiological parameters. Whole-plant hydraulic conductance (Kplant) and its components, i.e., root hydraulic conductance (Kroot) and stem hydraulic conductance (Kstem) were measured only at the lowest and the highest VPD by dividing transpiration rate (TR) by the Ψgradient, as described by Tsuda and Tyree (2000), as follows:

Kplant-min = TRmin/ (Ψsoil–Ψleaf) at the lowest VPD

Kplant-max = TRmax/ (Ψsoil–Ψleaf) at the highest VPD

Kroot-min = TRmin/ (Ψsoil–Ψstem) at the lowest VPD

Kroot-max = TRmax/ (Ψsoil–Ψstem) at the highest VPD

Kstem-min = TRmin/ (Ψstem–Ψleaf) at the lowest VPD

Kstem-max = TRmax/ (Ψstem–Ψleaf) at the highest VPD

Since the plants were well watered, Ψsoil was considered to equal zero.

*Identifying genomic regions associated with transpiration response to VPD*

*Genotyping*

Details on genotypic data and linkage map construction of the Mélodie/2 × ILB 938/2 population at F8 generation are explained in Gela *et al.* (2022). Briefly, DNA was isolated from three-day-old germinated embryo axes for 165 RILs as well as the parental lines using the CTAB (cetyl trimethyl ammonium bromide) method, as described previously (Björnsdotter *et al.,* 2021). Genotypic data for this population was generated from the Axiom 'Vfaba\_v2' 60K array (O'Sullivan *et al.,* 2019).

*Linkage map construction*

The linkage map was developed by Gela *et al.* (2022). The 35,363 SNP markers were filtered based on polymorphism between parents, segregation distortion using a chi-square (χ2) test, and missing data. The linkage map was built using both ASMap software (Taylor and Butler, 2017) and MapDisto v.2.1.8 (Heffelfinger *et al.,* 2017) with a logarithm of odds (LOD) score of 3.0 and a cut-off recombination value of 0.35. The Kosambi function was used to calculate the map distance in centiMorgans (cM) (Kosambi, 1943). The map included 4,089 markers, distributed in six linkage groups corresponding to the six chromosomes of faba bean, and spanned 1,229.5 cM (Gela *et al.,* 2022).

*QTL mapping of transpiration response to VPD and candidate gene identification*

Composite interval mapping (CIM) was used to detect putative QTL locations of TRmin, TRmax, TRBP, Kplant-min, Kplant-max, Kroot-min, and Kroot-max by Windows QTL Cartographer v. 2.5 (Wang *et al.,* 2012). The cofactors were determined using the forward and backward method in the standard CIM model with a probability of 0.1 and window size of 5 cM. QTL significance thresholds were determined by 1,000 permutations at a significance level of *P*=0.05. Phenotyping data of 142 RILs with available genotyping data was used for QTL analysis. To determine candidate genes, the sequences of SNP markers that appeared within the QTL intervals were searched using BLASTn (Goodstein *et al.,* 2012) in Phytozome v.13 on the reference genome for *Medicago truncatula*. It was notable that no QTLs were identified for the slopes of the TR versus VPD relationships, and the actual BP values.

*Statistical analysis*

Analysis of TR response to VPD utilised the segmented linear regression model of GraphPad GraphPad Prism 9.3.1 (GraphPad Software Inc., San Diego, CA, 2007), which provides a break-point (BP) value (when the slopes of the fitted regression differ significantly), values of the slopes and their standard errors as well as the regression coefficient. A simple linear regression was applied when the slopes did not significantly differ (Devi *et al.,* 2010; Shekoofa *et al.,* 2020). Significant genotypic differences (*P*<0.05) in regression parameters (slopes and BPs), TR, Ψleaf, and hydraulic conductance for the entire population were discriminated using student’s t-test. ANCOVA (for main effects of genotypes, VPD, and their interaction) between the parental lines in their TR, Ψleaf, and Kplant response to VPD and for the effects of planting month on the genotypic differences in leaf area was carried out with SPSS 27.0 for Windows statistical software package (SPSS, Inc., Cary, NC). Differences between means were considered statistically significant for values of *P*<0.05.

RESULTS

*Physiological responses of the parental lines*

The parental genotypes differed in their TR response to VPD, as indicated by a significant genotype × VPD interaction (*P*=0.036, Table 1). While TR of Mélodie/2 increased linearly over the range of VPDs tested (Fig. 1A), ILB 938/2 was well characterized by the two-segmental analysis (Fig. 1B). Its TR increased linearly with increasing VPD to reach 30.45 ± 2.35 mg H2O m−2 min−1 at 2.12 ± 0.04 kPa, which represented a break point, BP. Thereafter, TR was relatively stable despite increases in VPD, reaching 32.53 ± 2.25 mg H2O m−2 min−1 at 3.20 ± 0.07 kPa. Although the minimum TR of ILB 938/2 was 17% higher than that of Mélodie/2 at the lowest VPD, they did not significantly differ in their maximum TR (Fig. 1C). Thus, ILB 938/2 restricted its transpiration at high VPD.

Leaf water potential (Ψleaf) and stem water potential (Ψstem) responded similarly to VPD in the two genotypes (no significant genotype × VPD interactions – Table 1), with high VPD decreasing Ψleaf by 0.15 MPa (averaged across genotypes). However, Ψleaf of Mélodie/2 was significantly higher than ILB 938/2 at the lowest (by 14%) and the highest VPD (by 9%), respectively (Fig. 2A). Stem water potential (Ψstem) exceeded Ψleaf by ∼ 25%, with high VPD decreasing Ψstem by 0.12 MPa (averaged across genotypes) at the two tested VPDs and decreased by 30 and 26% at the highest VPD in Mélodie/2 and ILB 938/2, respectively(Fig. 2B). Whole-plant hydraulic conductance (Kplant) did not differ between genotypes but was increased similarly by 12% (averaged across genotypes) at the highest VPD, as indicated by no significant genotype × VPD interaction (Table 1; Fig. 2B). Thus lower Ψleaf and Ψstem of ILB 938/2 (irrespective of VPD) could not be attributed to impaired Kplant.

Stem hydraulic conductance (Kstem) and root hydraulic conductance (Kroot) responded similarly to VPD in the 2 genotypes (no significant genotype × VPD interactions, Table 1), even if Kstem was 4 and 4.5-fold higher than Kroot in Mélodie/2 and ILB 938/2 respectively at the two tested VPDs (Fig. 3A and B). Whereas both genotypes had similar Kstem at the lowest VPD, Kstem of Mélodie/2 was 8% higher than that of ILB 938/2 at the highest VPD (Fig. 3A). In contrast, Kroot did not significantly differ between the genotypes at any VPD (Fig. 3B). While Kstem of Mélodie/2 increased by 30% as VPD increased, ILB 938/2 increased its Kstem by 18% at the highest VPD (Fig. 3A). Overall, the component hydraulic conductances (Kstem and Kroot) did not show genotypic differences (as with Kplant) but increased at the highest VPD.

*Genotypic variation in TR response to VPD in the RILs*

As the parental lines differed, there was considerable variability in TR response to VPD amongst the progeny lines. Segmented regression analysis identified a significant BP in more than 90% of the population (150 RILs) ranging from 1.5<BP<3.0 kPa, while only 15 RILs had a linear TR model (Fig. 4A). The RILs with a segmented TR response were divided into three sub-groups based on the BP value as follows: 1) 1.5<BP<2.0 (61 RILs), 2) 2.0<BP< 2.5 (65 RILs), and BP>2.5 (24 RILs). At the lowest VPD, the groups of RILs (linear and segmented TR) differed slightly in their TR, with TR of the groups 1.5<BP<2.0 and 2.0<BP<2.5 exceeding that of the linear group and BP>2.5 groups by 5% averaging 25.0 ± 0.73 mg H2O m−2 min−1. The highest VPD increased TR by 42-51%, with the group having a BP between 2.0 and 2.5 kPa having the highest TR of 39.72 ± 1.45 mg H2O m−2 min−1, the group showing a linear response to TR having the lowest TR of 34.36 ± 1.24 mg H2O m−2 min−1 and the other two groups showing an intermediate response. Thus, the different groups varied in their maximum TR (Fig. 4B).

In the group where TR was linearly related to VPD (R2 averaged 0.95 ± 0.01), the slope averaged 4.86 ± 0.35 mg H2O m−2 min−1 kPa−1,similar to the parental line Mélodie/2 (4.92 ± 0.94 mg H2O m−2 min−1 kPa−1)(Fig. 5A). For the 61 RILs with a low BP (1.5<BP<2.0), Slope 1 (below the BP) was substantially (28-47%) greater than in the other groups. For this group, slope 2 (above the BP) was 0.5-2.5-fold greater than in the other groups (Fig. 5B-D; Table 2). In genotypes that restricted their transpiration at lower VPDs (lower BP), their transpiration was less constrained by increasing VPDs.

Similar to the parental lines, leaf water potential (Ψleaf) did not differ between the four groups at the two tested VPDs, averaging -0.567 ± 0.012 MPa at the lowest VPD and -0.756 ± 0.016 MPa at the highest VPD (Fig. 6A). Whole-plant hydraulic conductance (Kplant) differed significantly across the four groups, with greater values in the 2.0<BP<2.5 kPa group i.e 47.85 ± 0.93 mg H2O m−2 min−1 kPa−1 and 54.19 ± 1.15 mg H2O m−2 min−1 kPa−1 at the lowest and the highest VPD, respectively. These values were 6-12% higher than Kplant in the other groups and 10-24% higher than the parental lines (Fig. 6B). Tripling the VPD increased Kplant by 9-14% across the four groups with the highest increase in the 2.0<BP<2.5 group and the lowest in the linear group, causing significant differences between the four groups at the two tested VPDs.

Across the entire population, Kstem and Kroot differed significantly between the groups at the two tested VPDs with about 3-fold lower Kroot values than Kstem (Fig. 7A and B). The highest VPD increased Kstem and Kroot by 8-15% across the four groups, with the greatest increase in the 2.0<BP<2.5 group and the lowest within the linear one (Fig. 7A and B). At the lowest VPD, Kstem of the 1.5<BP<2.0 and 2.0<BP<2.5 groups exceeded the other groups by 8% (averaged across the groups) while at the highest VPD, Kstem significantly differed between all four groups with the highest values recorded for the 2.0<BP<2.5 group (Fig. 7A). Similar differences in Kroot were detected at the highest VPD but were absent at the lowest VPD (Fig. 7B). Taken together, the 2.0<BP<2.5 group sustained higher transpiration rates at high VPD, associated with its higher Kplant and its components.

*QTL analysis and candidate gene identification*

Thirteen QTLs were identified in total: three for the TRmin on chromosomes 1 and 3, one for TRmax on chromosome 3, one for TRBP on chromosome 5, two for Kplant-min on chromosome 1, two for Kplant-max maximum hydraulic conductance on chromosomes 1 and 3, two for Kroot-min on chromosome 1, and two for Kroot-max on chromosomes 1 and 3 (Table 3; Fig. 8; Supplementary Fig. S2). The QTLs qTRmin1.1, qTRmin1.2, and qTRmin3.1 each accounted for over 9% of phenotypic variance explained (PVE). The qTRmin1.1 and qTRmin3.1 showed a negative additive effect, suggesting that the positive allele came from Mélodie/2, whereas qTRmin1.2 showed a positive additive effect, indicating that its positive allele came from ILB 938/2. The qKplant-min1.1, qKplant-min1.2, qKplant-max1.1, and qKplant-max3.1 accounted for 12, 13, 10 and 10% of PVE, respectively. All of these QTLs showed positive additive effects, so alleles for higher values were likely associated with ILB 938/2. QTLs qKroot-min1.1, qKroot-min1.2, qKroot-max1.1, and qKroot-max3.1, explained 11, 9, 10, and 11% of the PVE, respectively. The positive additive effect suggests that its positive allele came from ILB 938/2. Similarly, qTRBP showed about 11% of variation with a positive additive effect. QTLs governing TRmin, Kplant-min, Kplant-max, Kroot-min, and Kroot-max were co-located on the same region on chromosome 1. Likewise, QTLs for TRmin and TRmax and QTLs for Kplant-max and Kroot-max were co-located on chromosome 3 (Table 3; Fig. 8). Several candidate genes were also identified in the corresponding regions that may play a role in transpiration efficiency and plant water-relations related traits (Supplementary Table S2).

DISCUSSION

This study identified genomic regions governing the transpiration rate and hydraulic conductance response to evaporative demand in a bi-parental mapping population of faba bean. Although both parental lines (Mélodie/2 and ILB 938/2) were considered drought-adapted (Abdelmula *et al.,* 1999; Khan *et al.,* 2007; Khazaei *et al.,* 2014), they significantly differed in their TR response to VPD, where TR of Mélodie/2 increased linearly as VPD increased while ILB 938/2 restricted its TR once the VPD exceeded 2.1 kPa. Nevertheless, both genotypes achieved the same maximum transpiration rate. However, this transpirational restriction of ILB 938/2 did not prevent it from having a lower Ψleaf than Mélodie/2 irrespective of VPD (Fig. 2A), and Ψleaf of both genotypes declined similarly as VPD increased (Table 1). Similarly, whole plant hydraulic conductance (Kplant) did not differ between genotypes irrespective of VPD (Fig. 2C), disproving our hypothesis that limited Kplant restricted transpiration. Although stem hydraulic conductance of ILB 938/2 was less than Mélodie/2 at high VPD (Fig. 3A), this difference was insufficient to significantly affect Kplant, and both genotypes achieved a similar maximum transpiration rate (Fig. 1C). Limited agreement between variation in hydraulic conductance and transpiration responses to VPD suggests that alternative (non-hydraulic) mechanisms may restrict transpiration at high VPD, with foliar ABA accumulation possible (Kholová *et al.,* 2010; McAdam *et al.,* 2016). Indeed, ABA-deficient mutants fail to show stomatal closure at high VPD (McAdam *et al.,* 2015; McAdam *et al.,* 2016). ~~accumulation at high VPD (Veselov~~ *~~et al.,~~* ~~2018).~~

Since transpiration of the parental lines differed in response to VPD, it was likely that RILs derived from these lines would also show differences. Only 15 RILs (10%) were represented by a single linear regression over the entire range of VPD that matched Mélodie/2, and 65 RILs (40%) exhibited segment TR response with a 2.0<BP<2.5 that matched ILB 938/2. Interestingly, half of the population revealed a segmented TR response with a BP lower (1.5<BP<2.0 kPa, 61 RILs) and higher (BP>2.5 kPa, 24 RILs) than the BP of ILB 938/2. The observed stability or even the slightly higher TR after the BP is beneficial for improving crop performance under mild abiotic stress as an alternative to stomatal closure under severe stress conditions (Collins *et al.,* [2008](https://link.springer.com/article/10.1007/s00425-010-1286-7#ref-CR5)). The low BP of 61 RILs suggests that whole plant hydraulic conductance may be restricted more than either parent. Since the TR response to VPD in almost half of the RILs differed from the parents, this trait has a complex inheritance consistent with a previous study in soybean (Sadok and Sinclair, 2009). None of the soybean genotypes that had a segmented TR genotype in their pedigree expressed the segmented TR trait, indicating that the trait(s) responsible for this response is either recessive or depends on a combination of alleles.

Better understanding the genetic basis of this variability requires QTL analysis of TR response to VPD. Theoretically, genotypes with a low BP are likely better suited for a dry environment than those with a high BP, as they restrict transpirational depletion of soil moisture reserves. However, genotypes with a low BP may not necessarily be the most water-conserving if their transpiration increases more rapidly at VPDs below the BP (higher Slope 1 values). Paradoxically, those genotypes with a moderate BP (2.0-2.5 kPa) sustained a higher maximum transpiration rate than those with higher and lower BPs and the linear group (Fig. 4B), indicating the VPD of the BP (or its occurrence) did not coincide with the lowest maximum transpiration rate. Instead, genotypes that did not restrict transpiration as VPD increased actually had the lowest maximum transpiration rate (Weatherley, 1982; Else *et al.,* 1995; Steudle and Peterson, 1998) consistent with the response of faba bean (Fig. 4B). These findings suggest considerable diversity in the relationships between transpiration responses to evaporative demand and the existence of any hydraulic restrictions.

In the mapping population used in this study, ψleaf decreased by 30-35% at the highest VPD (Fig. 6A), although independently of whether the genotypes restricted transpiration at high VPD. Possibly the stomata directly sense high VPD independently of the bulk leaf water potential and close before the leaf experiences water shortage. In control terms, this may be regarded as a feed-forward response of the stomata to high evaporative demand ([Bunce, 1997](https://www.frontiersin.org/articles/10.3389/fpls.2018.01572/full#B7);Dewar, [2002](https://onlinelibrary.wiley.com/doi/full/10.1111/pce.12137#pce12137-bib-0008); [Buckley, 2005](https://www.frontiersin.org/articles/10.3389/fpls.2018.01572/full#B6)).

By measuring components of hydraulic conductance *in planta*, root hydraulic conductance was identified as the most limiting to whole-plant hydraulic conductance across the entire population, indicating that roots restrict the flow of water to the guard cells and hence limit TR at high VPD. Similarly, Sivasakthi *et al.* (2020) postulated that limited root hydraulic conductance restricted chickpea TR at high VPD (although leaf/stem hydraulic conductance were not measured). Root hydraulic conductance affects the point at which plants reach their maximum TR or begin to reduce TR in response to elevated VPD, allowing plants to maintain higher gs and preventing a decline in TR in response to high VPD (Sadok and Sinclair, 2010b). In contrast, limited leaf hydraulic conductance restricted transpiration at high VPD in soybean (Sinclair *et al.,* 2008; [Sadok and Sinclair, 2010a](https://www.frontiersin.org/articles/10.3389/fpls.2021.779834/full#B42), [2012](https://www.frontiersin.org/articles/10.3389/fpls.2021.779834/full#B44); [Devi *et al.,* 2016](https://www.frontiersin.org/articles/10.3389/fpls.2021.779834/full#B22)) and peanut (*Arachis hypogaea* L.) (Devi *et al.,* 2012), suggesting variation in the site of hydraulic limitations in these species.

The high genetic variation between the RILs in transpiration response to VPD and other related traits suggests that the limited transpiration trait at high VPD could be used in crop breeding programs. QTL analysis identified 13 QTLs associated with the minimum, maximum and BP transpiration and minimum and maximum whole-plant and root hydraulic conductance traits were identified on chromosomes 1, 3, and 5. Some genomic regions governing frost tolerance and winter hardiness have also been detected on faba bean chromosomes 1 and 3 neighbouring QTLs detected in this study (reviewed in Khazaei et al., 2021). Using the same mapping population at the F5 generation, Khazaei *et al.* (2014) reported QTLs for stomatal morphology and function mainly on chromosome 2. Most of the identified candidate genes in this study were previously reported to regulate plant response to several abiotic stresses (Supplementary Table S2). Of these, for instance, the property of qTRmin1.1 flanking markers were associated to alkaline ceramidase-related genes and 1-aminocyclopropane-1-carboxylate oxidase 3-related Greagenes (Zheng *et al.,* 2018; Kim *et al.,* 1998; Houben and Van de Poel, 2019). Greater ethylene biosynthesis was associated with stomatal opening of tomato grown at high (90%) relative humidity (Arve and Torre, 2015). The qTRmax1.1 and qTRmax1.3 were related to oligopeptide transporters (Zhu, 2016; Qi *et al.,* 2018; Gong *et al.,* 2020) and Basic-Leucine Zipper (BZIP) transcription factor family protein (Yu *et al.,* 2020) that are known to play crucial roles in plant responses to several abiotic stresses including water deficit and reactive oxygen species (ROS). Interestingly, the region on chromosome 1 where seven QTLs were co-located (qTRmin1.2, qKplant-min1.1, qKplant-min1.2, qKplant-max1.1, qKroot-max1.1, qKroot-min1.1, and qKroot-min1.2) accommodated a cytochrome c oxidase that when deficient in *Arabidopsis thaliana* lowered sensitivity to abscisic acid (Garcia *et al.,* 2016). In wheat (*Triticum aestivum* L.), six QTLs associated with transpiration response to VPD were identified, of which one major QTL included genes involved in root hydraulic conductance and ABA signalling (Schoppach *et al.,* 2016). Similarly, in soybean, limited transpiration was associated with two QTLs that harbour several candidate genes, including one involved in abiotic stress tolerance (Sarkar *et al.,* 2022). In chickpea, transpiration efficiency (ratio of biomass per unit of transpired water) was associated with the ‘*QTL-hotspo*t’ region that harboured four genes associated with drought adaptation (Barmukh *et al.,* 2022). These findings confirm the role of genetic populations in detecting genomic regions and candidate genes for water conservation traits under complex genetic control to discriminate the regulation of limited TR at elevated VPD.

*Conclusions*

More than 90% of the faba bean RILs used in this study restricted TR at high VPD, with considerable variation in the BP at which this occurred. Although genotypes with BPs that restrict TR at high VPD are regarded as suitable for water-deficit environments (Gholipoor *et al.*, 2010), in this faba bean population the RILs with a linear TR response to VPD actually had the lowest TR at high VPD. This suggests complex regulation of these transpiration and hydraulic responses to VPD with this study. This study provides the first reports on the genetic control of transpiration response to VPD in faba bean. These identified QTLs can be used as potential targets for further genetic studies, and after validation in appropriate germplasm, the linked DNA markers can enable the use of marker-assisted selection to help breed water-conserving faba bean genotypes.

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AUTHOR CONTRIBUTIONS

HM and ICD conceived the research and designed the experiments; HM performed the experiments; HM and HK analyzed the data; HK and FLS contributed reagents/materials/analysis tools; HM wrote the first draft of manuscript with contributions from all the authors.

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**Table 1.** *P*-values from ANCOVA describing the difference between Mélodie/2 and ILB 938/2 in transpiration rate (TR), leaf water potential (Ψleaf), stem water potential (Ψstem), whole-plant hydraulic conductance (Kplant), root hydraulic conductance (Kroot), and stem hydraulic conductance (Kstem) responses to VPD. Significant values italicised.

|  |  |  |  |
| --- | --- | --- | --- |
| **Trait** | **Genotype** | **VPD** | **Genotype × VPD** |
| TR | 0.15 | *<0.001* | *0.036* |
| Ψleaf | *0.03* | *<0.001* | 0.94 |
| Ψstem | *0.002* | *<0.001* | 0.41 |
| Kplant | 0.90 | *0.047* | 0.96 |
| Kroot | 0.76 | 0.10 | 0.79 |
| Kstem | 0.81 | 0.06 | 0.62 |

**Table 2.** Differences between the parental lines and the four groups of RILs in their slope 1, break point (BP), and slope 2 of transpiration response to VPD. Data are the mean ± SE of the individual parental lines, or the number of RILs indicates in parentheses.

| **Parental line/Group** | **Slope 1** | **BP** | **Slope 2** |
| --- | --- | --- | --- |
|
| Mélodie/2 | 4.92 ± 0.94d | NA | NA |
| ILB 938/2 | 6.81 ± 1.44c | 2.15 ± 0.09b | 1.82 ± 0.89b |
| Linear | 4.86 ± 0.35d | NA | NA |
| 1.5<BP<2.0 | 11.93 ± 0.54a | 1.75 ± 0.02c | 3.52 ± 0.16a |
| 2.0<BP<2.5 | 9.36 ± 0.36b | 2.24 ± 0.02b | 2.93 ± 0.18a |

Different letters indicating significant (*P*<0.05) differences according to the t-test. NA, not applicable.

**Table 3.** QTL information for minimum and maximum transpiration rate (TRmin and TRmax), whole-plant (Kplan-min and Kplant-max) and root hydraulic conductance (Kroot-min and Kroot-max), and break-point transpiration (TRBP) traits in Mélodie/2 × ILB 938/2 RIL population at F8.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| QTL | Chromosome | Peak (cM) | QTL interval | LOD | R2 (%)a | Addb |
| qTRmin1.1 | 1 | 67.01 | 65.0 - 69.0 | 3.59 | 9.13 | -1.02 |
| qTRmin1.2 | 1 | 222.01 | 217.0 - 219.0 | 3.41 | 9.40 | 1.18 |
| qKplant-min1.1 | 1 | 217.01 | 216.0 - 218.0 | 4.64 | 11.58 | 2.34 |
| qKplant-min1.2 | 1 | 222.01 | 221.0 - 226.0 | 5.07 | 12.54 | 2.40 |
| qKplant-max1.1 | 1 | 222.01 | 221.0 - 224.90 | 4.73 | 11.80 | 2.90 |
| qKroot-max1.1 | 1 | 222.01 | 220.30 - 224.80 | 3.57 | 9.90 | 3.50 |
| qKroot-min1.1 | 1 | 215.0 | 214.0 - 217.0 | 4.51 | 11.10 | 3.11 |
| qKroot-min1.2 | 1 | 222.01 | 221.0 - 225.30 | 3.51 | 8.70 | 2.80 |
| qTRmin3.1 | 3 | 5.01 | 1.90 - 9.50 | 3.35 | 9.36 | -1.12 |
| qTRmax3.1 | 3 | 5.01 | 0.40 - 8.60 | 4.02 | 10.80 | -2.03 |
| qKplant-max3.1 | 3 | 77.01 | 75.70 - 78.0 | 4.05 | 10.60 | 2.79 |
| qKroot-max3.1 | 3 | 77.01 | 75.8 - 79.0 | 4.04 | 10.88 | 3.73 |
| qTRBP5.1 | 5 | 66.0 | 63.0 - 68.0 | 4.01 | 10.80 | 1.55 |

aR2 - Percentage of phenotypic variance explained by QTL, bAdditive genetic effect.

**Figure captions:**

**Figure 1.** TR response to VPD of Mélodie/2 (A) and ILB 938/2 (B) in the whole-plant gas exchange chamber and the difference between the two genotypes in their minimum (1st column each genotype) and maximum (2nd column each genotype) TR (C). Each point and column represents 5 minutes of transpiration rate after 15 minutes of steady-state. Symbols are the mean of four plants per genotype. Error bars were omitted from A and B for clarity, while different letters in C indicate significant (*P*<0.001) differences according to the t-test. Linear (A) and broken-stick (B) regression lines (*P*<0.01) were fitted in Prism. Mean ± SE of regression variables i.e., slope 1 and R2 values of Mélodie/2 and BP, slopes, and R2 values of ILB 938/2 are shown on the top of panels A and B.

**Figure 2.** Changes in Ψleaf (A), Ψstem (B) and Kplant (C) in Mélodie/2 (blue columns) and ILB 938/2 (red columns) from the lowest (1 kPa) to the highest (3.2 kPa) VPD. Bars show the mean ± SE of four plants of each parental line, with different letters above the bars indicating significant (*P*<0.05) differences according to the t-test.

**Figure 3.** Changes in Kstem (A) and Kroot (B) in Mélodie/2 (blue columns) and ILB 938/2 (red columns) from the lowest to the highest VPD. Bars show the mean ± SE of four plants of each parental line, with different letters above the bars indicating significant (*P*<0.05) differences according to the t-test.

**Figure 4.** Frequency distribution (%) of TR response models to VPD of 165 RILs derived from Mélodie/2 and ILB 938/2 (A) and the difference between the groups in their minimum TR (1st column in each group) and maximum (2nd column in each group) TR (B). Bars show mean ± SE of TR of the genotypes in each group (n=585), with different letters above the bars indicating significant (*P*<0.05) differences according to the t-test.

**Figure 5.** TR response to VPD of the linear TR group (A), and the three segmented TR models i.e 1.5<BP<2 kPa (B), 2<BP<2.5 kPa (C) and BP>2.5 kPa (D). Data are presented as mean TR of genotypes in each group. Linear (A) and broken-stick (B, C, and D) regression lines (*P*<0.01) were fitted in Prism. Mean ± SE of regression variables i.e., BP, slopes, R2 and *P*-value are represented on the top of each panel. Symbols are the mean of four plants per genotype, , comprising a, b, c, d plants in panels A, B, C, and D respectively with error bars omitted for clarity.

**Figure 6.** Differences between the four TR models of 165 RILs in their Ψleaf (A) and Kplant (B) at the lowest (1st column in each group) and the highest (2nd column in each group) VPD levels. Data are presented as mean ± SE of 3-4 plants of each RIL comprising a, b, c, d plants for the linear, 1.5<BP<2, 2<BP<2.5, and BP<2.5 groups respectively, with different letters above the bars indicating significant (*P*<0.05) differences according to the t-test.

**Figure 7.** Differences between the four TR models of 165 RILs in their Kstem (A) and Kroot (B) at the lowest (1st column in each group) and the highest (2nd column in each group) VPD levels. Data are presented as mean ± SE of 3-4 plants of each RIL comprising a, b, c, d plants for the linear, 1.5<BP<2, 2<BP<2.5, and BP<2.5 groups respectively, with different letters above the bars indicating significant (*P*<0.05) differences according to t-test.

**Figure 8.** Location of QTLs on chromosomes 1, 3 and 5 using 142 RILs derived from cross Mélodie/2 × ILB 938/2. The QTL interval regions are shown with a green bar. QTLs are represented by boxes extended by lines representing the LOD-1 and LOD-2 confidence intervals. Only portions of the linkage map include the QTL positions are displayed. Full length chromosomes are presented at Gela *et al.* (2022).