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Genetic variation in transpiration response to evaporative demand in faba bean

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Declaration

Except where references are made to other sources, I declare that the contents in this thesis are my own work and have not been previously submitted, in part or full, for the award of a higher degree elsewhere.

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Abstract

Limiting maximum transpiration rate (TR) under high vapour pressure deficit (VPD) is considered a water conservation strategy, with genotypes expressing this trait considered desirable in high VPD environments where water deficits commonly develop later in the growing season. While breeding companies have incorporated this trait into some crops, there is uncertainty on the best way to phenotype this trait, its underlying physiological mechanisms, and genetic regulation, which hitherto remain unknown for faba bean (*Vicia faba* L.). Thus, this thesis aimed to 1) understand whether variation in this trait was consistent at single leaf *versus* whole-plant levels. 2) identify whether restricting transpiration under high VPD was associated with low plant hydraulic conductance and/or tissue abscisic acid (ABA) levels. 3) identify genetic variation in TR response to VPD in 165 faba bean recombinant inbred lines (RILs) derived from two parental lines with contrasting water use and other physiological traits (Mélodie/2 & ILB 938/2).

Two British faba bean cultivars (Masterpiece and Robin Hood) were grown in well-watered soil in a semi-controlled glasshouse with diurnally fluctuating VPD and light conditions. In the same plants, whole-plant transpiration was measured gravimetrically under these conditions, single leaf transpiration was measured using an infra-red gas analyzer that regulated VPD around the leaf and whole-plant transpiration was measured in a gas exchange chamber that regulated VPD around the shoot. Transpiration response to VPD consistently varied between the cultivars across the three measurement approaches and fitted a segmented transpiration model with a break-point (BP) averaging 3.05 and 2.33 kPa for Masterpiece and Robin Hood, respectively then stabilized, decreased, or slightly increased at a diminished rate as VPD increased further. Statistical analysis of model variables (Slope 1, the BP, and Slope 2 values) revealed no significant differences according to the measurement approach, indicating that different instruments can be used according to their availability. The response was also consistent across different times of the year that varied in light conditions, temperature, and VPD.

In both cultivars, limited transpiration rates under high VPD coincided with decreased root hydraulic conductance and higher root ABA concentrations. The lower VPD break-point of Robin Hood was correlated with lower root hydraulic conductance and higher root ABA and

root xylem sap ABA concentrations than Masterpiece at the applied VPD. Thus, genotypic differences in transpirational responses to high VPD in faba bean were more closely associated with root hydraulic conductance and root ABA concentrations than stem hydraulic conductance, leaf ABA and xylem sap ABA concentrations.

Measuring whole-plant TR and hydraulic conductance response to VPD in the whole-plant gas exchange chamber revealed contrasting TR responses in the parents of the RILs, with TR of Mélodie increasing linearly with VPD whereas ILB938/2 limited its TR after 2.02 kPa. The higher leaf water potential of Mélodie/2 than ILB 938/2 at the two tested VPDs indicates better control in water status at the leaf level than ILB 938/2. Almost 90 % of the RILs limited their TR at high VPD with a BP range of 1.5<BP<3 kPa and about 10 % had a linear TR response to VPD. Genotypic variation in the BP may allow specific cultivars to be developed for differing water-deficit environments. QTL analysis identified thirteen QTLs contributing to minimum and maximum transpiration, whole-plant and root hydraulic conductances traits on faba bean chromosomes 1 and 3, while one locus associated with break-point transpiration was identified on chromosome 5. These QTLs harboured many abiotic stress-responsive genes, thus they can be used as potential targets for marker-assisted breeding to genetically improve faba bean performance under water-limited environments particularly.

Taken together, the limited TR response under high VPD in faba bean is controlled by restricted root hydraulic conductance and higher root ABA accumulation. Further research of cross-talk between different hormones and aquaporins activity seem essential to understand how plant transpiration and/or hydraulic conductance decline at elevated VPD.

List of commonly - used abbreviations

AQPs	Aquaporins
ABA	Abscisic acid
B.C.	Before Christ
CO_2	Carbon dioxide
cM	Centimorgan
DI water	Deionised water
EF	Evaporative flux
FAO	Food and Agriculture Organization
gs	Stomatal conductance
GC	Canopy conductance
Ha	Hectare
IPCC	Intergovernmental Panel on Climate Change
K_{plant}	Whole-plant hydraulic conductance
K_{root}	Root hydraulic conductance
K_{stem}	Stem hydraulic conductance
LA	Leaf area
LG	Linkage group
LOD	Logarithm of odds
M.t	Million ton
MAS	Marker-assisted selection
N	Nitrogen
PPFD	Photosynthetic photon flux density
QTL	Quantitative trait locus
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
TR	Transpiration rate
VPD	Vapour pressure deficit
WUE	Water use efficiency

Ψ	Water potential
Ψ_{leaf}	Leaf water potential
Ψ_{stem}	Stem water potential
Ψ_{soil}	Soil water potential

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1.1 Faba bean (*Vicia faba* L.)

1.1.1 Evolution of faba bean

Legume crops are critical for both human nutrition and agricultural productivity since they are a primary food source in many regions of the world and key rotational crops for improving soil nitrogen status (Lakitan et al., 1992). Faba bean (*Vicia faba* L.) is one of the first domesticated edible legumes dating back to the early Neolithic period (8,000 B.C.). It is an annual herbaceous species that belongs to the family Fabaceae (Lewis et al., 2005). The genus *Vicia* contains a large number of species with an estimated 16,000–19,000 species in approximately 750 genera distributed around the globe (Chakraverty et al., 2013) growing in temperate and arid areas, humid tropics, highlands, savannas, and there are even a few aquatic legumes (Wrigley et al., 2015). According to its seed size, faba bean is classified into four types: paucijuga (a primitive form possibly likely close to the wild progenitor; 0.3-0.4 g / seed), minor (tick bean, ellipsoidal seed; 0.4-0.6 g / seed), equina (field bean, horse bean, flattened seed, 0.6-1 g / seed) and major (broad bean, flattened seed, 1-3 g / seed) (Muratova, 193; Cubero, 1974). The major type emerged in the Mediterranean basin and China, the equina type mostly in the Middle East and North Africa while, the minor and paucijuga types are mostly in northern Europe, Ethiopia, Nepal, and Bangladesh (Duc, 1997).

1.1.2 Importance of Faba bean

Faba bean is the fourth most important legume crop worldwide after pea, chickpea, and lentil that can be utilized as green manure and for stock feed (López-Pedrouso et al., 2012; FAO, 2020). It is one of the cheapest protein sources for human consumption in the Middle East, Latin America and Africa, and in many developed countries for animal feed (mostly pigs and poultry). This makes it one of the best alternative sources for the plant-based protein industry for food, feed, and extractable protein. The protein content of the dry seed is about 30 % (Warsame et al., 2018) and is very rich in lysine making it one of the best alternatives for plant-based protein diets. Its high nutritional value and capacity to grow over a wide range of environmental conditions (Crépon et al., 2010; Preissel et al., 2015) make it one of the most effective solutions to malnutrition, particularly in the developing countries (Haciseferogullari et al., 2003).

Like most other legumes, it is symbiotic with nodule-forming bacteria with nitrogen (N)-fixing ability, which is critical for low-N environments and hence can provide major benefits to the cropping systems (Sprent, 2009; Liu et al., 2019). It has been identified as the most efficient N fixer among the cool-season legumes (Mekkei, 2014), including chickpea, field pea, and lentil (Bremer et al., 1988; Carranca et al., 1999; Turpin et al., 2002; Schwenke et al., 2015; Liu et al., 2019). It is also considered a valuable break crop in environmentally sustainable arable production systems across the world (Köpke and Nemecek, 2010) to boost soil fertility for sustainable production of cereal crops, mainly wheat. It can improve the economic value of the following crop by enhancing the yield and/or the protein content of the grain (López-Bellido et al., 1998). Crop rotations with faba bean improve soil fertility not only through biological N fixation, but also by solubilizing insoluble phosphorus (P) in the soil, improving the soil's physical environment, and boosting soil microbial activity and hence, a greater yield of the subsequent crop (Köpke and Nemecek, 2010; Rashid et al., 2016). However, the

greatest benefits of biologically fixed nitrogen are acquired when the following crop has a relatively long growth period allowing for the most efficient use of N mineralization to occur later in the growing season (Jensen et al., 2010). The amount of biologically fixed nitrogen from Faba bean in drylands has been reported to be between 50 and 200 kg N ha^{-1}, with an average of 90 and 120 kg N ha^{-1}, only for above-ground biomass (Schwenke et al.; 1998; Carranca et al., 1999; Kumar and Goh, 1999; Kessel and Hartley, 2000; Unkovich and Pate, 2000). The impact of faba bean on the nitrogen dynamics of the following crops has been well documented. For example, it increased the subsequent wheat and barley yields by 12 and 21 %, respectively, which was equivalent to applying about 120 kg N ha^{-1} of nitrogenous fertilizer (Wright, 1990). The residual N benefit to cotton yield from a previous faba bean crop was about 50 % higher in comparison to other non-legume rotations, which required 150-200 kg/ ha of nitrogenous fertilizer to achieve equivalent yields to those achieved following faba bean (Peoples et al., 2009). These outcomes contribute to better defining the role of faba bean in cropping systems as a fertility-building crop.

1.1.3 Cultivation of faba bean

Faba bean can be grown in all soil types, but it best grows in fine-textured soils (Jensen et al., 2010) with a pH ≥ 7.0 (Köpke and Nemecek, 2010). Any other environments could be suitable with some additional practices, e.g., in sandy soils, frequent irrigation will be essential, while liming is required when soil pH level is below 6.0 in acidic areas with relatively high precipitation. Seed germination is better in large-seeded than in small-seeded cultivars at 12.5 °C (Kang et al., 2008). Due to its shallow roots, the crop may suffer from drought stress

in quick-drying soils (Tekalign et al., 2016). On the other hand, it can tolerate cold soils better than most other legumes if its seed germination is sensitive to low soil temperature (Etemadi et al., 2019).

1.1.4 World Production of faba bean

According to FAO statistics in 2020, faba bean world production was 5.66 million tons from 2.67 million ha. The production is dominated by four countries (China, Ethiopia, the United Kingdom, and Australia) which contribute more than 65 % of the total world production (Fig. 1.1 A). Recently, the global cultivated area of faba bean has increased by 6 % in the last decade which lead to enhancing the world production and yield by 14 and 7.5 %, respectively (Fig.1.1 B). In the last 10 years, the cultivated area and productivity in the UK, for example have increased by 66 and 59 %, respectively (Fig.1.1 C). Despite this massive expansion of the UK cropping area, the UK yield declined by 3 % in the last decade (Fig.1.1 E). This is likely due to the reduction in the UK rainfall amounts by 22.5 % in the last decade (Statista, 2020, www.statista.com, Fig. 1.1 F). In contrast, the cultivated area and productivity in Egypt (the largest faba bean consumer) has declined by 34 % and 27 % respectively in the last decade (Fig.1.1 C&D), however, the yield increased by 11.6 % (Fig.1.1 E). The reduced productivity in Egypt can be attributed to the limited cropping area rather than any environmental stresses. As the Egyptian population within the Nile River delta (which is about 30 % of the national area with deserts occupying most of the country) has progressively increased, urbanisation along the banks of the Nile has decreased the productivity of all crops, not only faba bean.

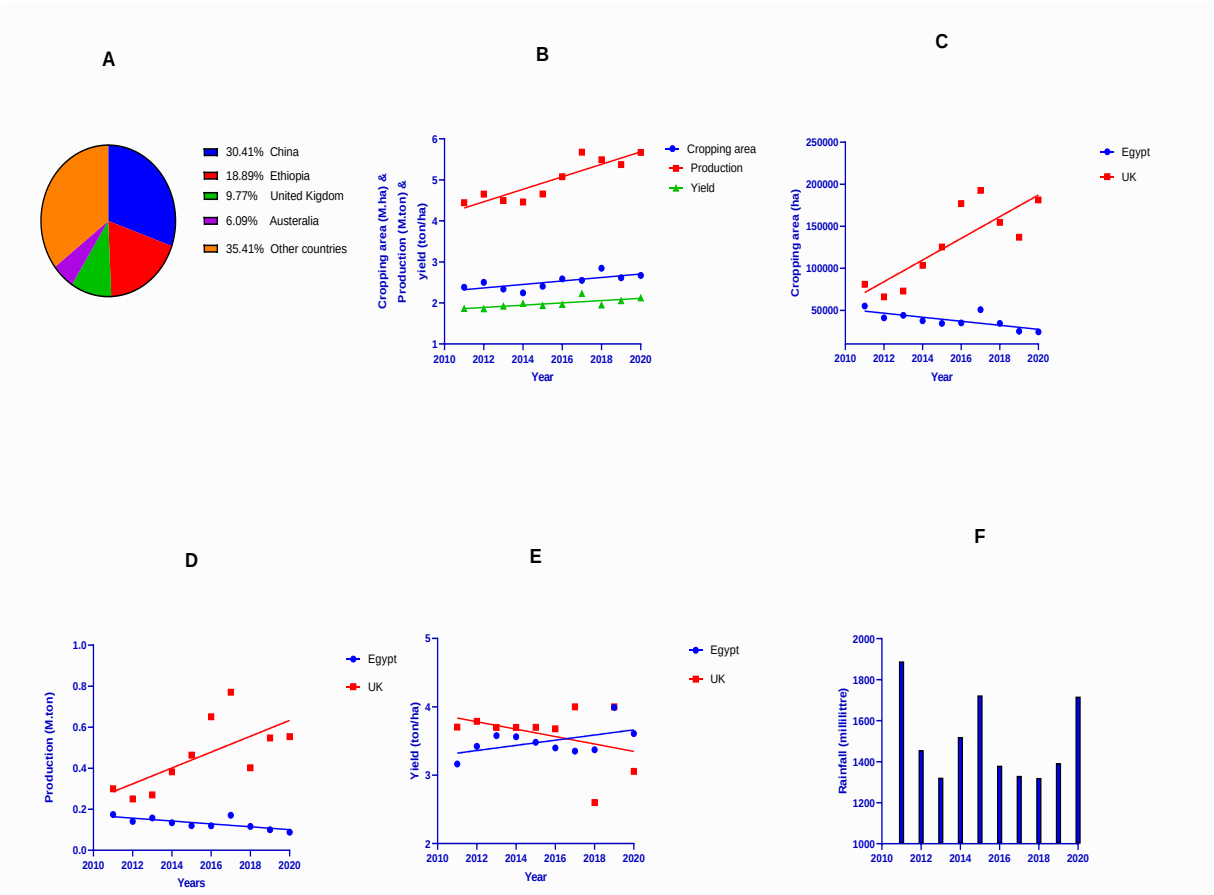


Figure 1.1 Faba bean top world producers in 2020 (A), changes in total world cropping area & production quantity & Yield (B), UK vs Egypt in cropping area (C), production (D) and yield (E) over the last decade, (FAO, 2020) and changes in UK rainfall amount across the last decade, (Statista, 2020).

1.2 Plant response to water deficit

Since global climate change has dramatically changed rainfall frequency over the last century, certain geographical areas are now experiencing longer drought seasons (Hirt and Shinozaki, 2003; Solomon et al., 2007). The decrease in precipitation as a result of infrequent rain events together with the predicted rise in atmospheric temperatures, will increase the intensity and frequency of drought incidents and hence, will negatively affect crop performance (Dai, 2013; IPCC, 2014; Spinoni et al., 2018). Another restricting factor is the reduction in water supplies in many parts of the world including North Africa. Indeed the new mega-dam being built by Ethiopia on the Nile River is threatening to spark a war over water rights in northeastern Africa, particularly Egypt (the estuary of the Nile).

Water deficit is one of the most prominent environmental stresses worldwide that substantially decreases crop yields (Ceccarelli et al., 2010; Farooq et al., 2017; Hong et al. 2020). It induces a range of morphological, physiological, and biochemical responses in plants (Ghahfarokhi et al., 2015; Le Gall et al., 2015) that ultimately decrease crop yields. The effect of drought stress on the plants depends on many factors, i.e. genotype, developmental stage, soil water depletion, and the time course of water shortage (Mahajan and Tuteja, 2005; Reddy et al., 2004). All these factors interact with each other to cause a significant drop in crop performance. Thus, understanding these complex responses may offer opportunities to improve crop management and genetics to enhance the drought tolerance of faba beans.

Various key physiological attributes include water use efficiency (ratio of crop yield to water use) (Amede et al., 1999), stomatal conductance (Bond et al., 1994), and transpiration response to vapour pressure deficit (Khan et al., 2010), that may be useful in improving plants for water-limited environments.

In general, plants cope with the lack of water via three basic strategies (Levitt 1980; Chaves et al*.,* 2003; Larcher 2003; Athar and Ashraf, 2009). The first approach aims to escape drought, thereby minimizing the effect of adverse drought conditions on a plant (Blum, 1988). This includes early vigour with accelerated plant development and a short life cycle with early flowering. The second approach is drought avoidance and includes minimizing tissue dehydration by maintaining high cellular water potential and/or maximizing water uptake by roots thus, maintaining high water status (Price et al., 2002). The third app roch is drought tolerance which represents the major strategy in plants and involves an adaptation of plant physiological functions to a limited water supply and a decreased plant cell water potential to reach a sustainable balance between root water uptake and shoot water loss (Tardieu and Tuberosa, 2010).

Water scarcity is common in Mediterranean environments and usually occurs in spring, which coincides with the grain filling stage in faba bean. Hence, increasing grain output does not necessitate crops with higher water use efficiency, but rather more effective use of water to maintain transpiration, especially in water-deficit environments (Blum, 2009; Lopes et al., 2011). So, it is important to identify drought-tolerant traits and incorporate them into high-yielding genotypes to sustain agricultural viability under current and upcoming climates with restricted amounts and /or frequency of precipitation.

Faba bean is characterized by its low tolerance to water deficit compared with other grain legumes (Amede et al., 2004; Khan et al., 2010; Khazaei et al., 2013 a & b), which affects its development, growth, and yield components (Ouji et al., 2017). Therefore, faba bean yields have remained lower and more variable than those of legumes, mainly because of the biological limitations of the traditional cultivars and the effect of environmental stresses

mainly drought (Gasim et al., 2013). So, improving drought tolerance in faba bean plants by either improving crop water use efficiency (WUE) or developing new improved drought-tolerant faba bean varieties is considered a promising approach for sustainable production in water-limited areas to overcome the constraints posed by the current and upcoming climate change.

1.3 Selection for improving drought tolerance

Plants have several adaptive strategies to cope with the lack of water (Maroco et al., 1997; Borrell et al., 2006; Araus et al., 2008). However, most of these traits relate to plant survival under drought and have little or no economic contribution to annual crop production. When severe drought incidents threaten plant survival, there is insufficient water for plant growth and yield formation, and consequently, yields remain extremely low. Any putative drought tolerance trait must benefit yields and have genetic variability within the target crop species (Sadok and Sinclair, 2011). In this regard, various investigators have worked on enhancing the efficiency of selection for drought-tolerant genotypes based on yield and specific physiological traits. Most of these efforts have been challenged by the lack of accurate screening techniques and the complex inheritance of yield traits that are highly affected by the environment (Cooper and Hammer, 1996; Mitra, 2001). Hence, improving drought tolerance response by selecting for physiological characters requires a comprehensive understanding of the nature of any putative trait, its responsiveness to the environment, and most importantly, its contribution to enhancing crop yield (Sheshshayee et al., 2003; Sinclair, 2011; Sinclair et al., 2016). The desirable traits to improve crop performance under water deficit conditions are generally associated with limiting the use of water early in the growing

season and hence conserving water for use later in the most critical flowering and grain filling periods, to maintain high productivity (Richards and Passioura, 1989; Sinclair et al., 2005; Zaman-Allah et al., 2011 a & b).

1.3.1 Transpiration response to evaporative demand

Up to 90 % of water absorbed by the plant from the soil is lost by transpiration (Pei et al., 1998), through minute pores in the leaf epidermis called stomata. To compensate for these transpirational losses, the plant should be well-watered to ensure that the soil can supply sufficient water to the roots, otherwise plant transpiration will be limited. Changes in transpiration rates (TR) are reportedly independent of plant leaf area and only marginally associated with phenology, which is the timing of plant growth and development (Schoppach et al., 2017). TR is driven by changes in evaporative demand (leaf to air vapour pressure deficit, VPD), which is a combined function of air temperature and relative humidity (Farquhar and Sharkey, 1982; Belko et al., 2012) and is calculated as the difference between the saturated vapour pressure and the actual vapour pressure. Since the saturated vapour pressure is sensitive to temperature, it is difficult to separate the effect of temperature on VPD and of temperature directly on plants (Sinclair et al., 2007). The predicted rise in atmospheric temperature and drought events, driven by climate change, will consequently result in severe VPD incidents. Since soil water deficit is often accompanied by drier air, it is difficult to separate the effects of both stressors on transpiration (Novick et al., 2016). One strategy to conserve water is for plants to lower their leaf gas exchange during periods of high VPD so that TR is decreased to match water flux into the leaf. In general, TR and VPD follow a diurnal pattern, being lowest from night to sunrise and increasing to maximum around

midday (Bunce, 1981; Hirasawa and Hsiao, 1999; Shekoofa et al., 2014). However, TR cannot exceed plant hydraulic conductance nor the rate of soil water uptake (Jackson et al., 2000). TR response to VPD could either be linear with genotypic differences in the slope and intercept of the transpiration response or segmented with a break-point (BP) - the VPD value after which transpiration slightly increases, stabilizes or even decreases- and genotypic differences in the BP value and/or the slopes before and after the break-point (Bunce, 1981; Turner et al., 1984; Vadez et al., 2013) generally reflect the water transport capacity in the soil-plant system.

A limited-transpiration trait has been confirmed as a water conservation strategy under well-watered conditions in several crop species including legumes such as soybean (*Glycine max* L.) (Fletcher et al., 2007; Sadok and Sinclair 2009 a & b), chickpea (*Cicer arietinum* L.) (Zaman-Allah et al., 2011 a & b), cowpea (*Vigna unguiculata* L.) (Belko et al., 2012) and peanut (*Arachis hypogaea* L.) (Devi et al., 2010). Their BP varied from 1.1 to 2.92 kPa in both controlled environments and field conditions. Also, within a species, BP values vary by about 0.6 – 1 kPa, indicating the existence of genotypic difference in this response (Table 1.1). The "slow-wilting" soybean genotype (PI 416937) (Sloane et al., 1990) had a constant TR after 2.0 kPa in contrast to the other studied cultivars that showed little or no further change in TR across the applied VPD range (Fletcher et al., 2007). The slow-wilting phenotype of soybean genotype PI 416937 in the field may be a consequence of limited water use resulting in soil water conservation. Moreover, phenotyping of commercial and recombinant inbred line (RIL) populations that had PI416937 in their pedigree resulted in a large genetic variation in transpiration response to VPD (Sadok and Sinclair, 2009 a & b). Such genetic variation suggests that much larger variability could be expected in bigger populations due to the high

genetic diversity as well as complex inheritance of this trait, which suggests more than one controlling mechanism.

Limited TR at high VPD has been theoretically and experimentally correlated with increased yield under terminal drought (Gholipoor et al., 2010; Cooper et al., 2014; Lobell et al., 2014; Vadez et al., 2014). It has the dual effect of (1) improving crop WUE by limiting gas exchange (Sinclair et al., 1984), and (2) conserving soil water early in the growing season for use at late in the season under water deficit, thus delaying the harmful effects of water deficit on plant tissues (Sinclair et al., 2005; Yang et al., 2012; Seversike et al., 2013; Shekoofa et al., 2014) and consequently enhancing yield.

On the other hand, limited transpiration could negatively affect CO_2 assimilation rate due to the close relationship between water vapour and CO_2 exchange by leaves and crop canopies (Tanner and Sinclair, 1983). In a simulation analysis of the limited transpiration trait in soybean (*Glycine max* (L.) Merr.) based on 50 years of weather data across the USA, yield increased with the limited transpiration trait in most regions in more than 85 % of the study period (~ 43 years) (Sinclair et al., 2010). Yield gains occurred in most locations at percentile rankings of both 25 % (dry years) and 75 % (wet years), with the greatest yield increases noted in the drier years. A similar analysis was conducted in Africa for the same crop. Genotypes with limited TR trait had higher yields in more than 70 % of the growing seasons in many locations, however, yield benefits were small in the higher rainfall regions (Sinclair et al., 2014). In another simulation study in Tunisia, wheat genotypes with limited TR trait at high VPD resulted in 40-80 % yield gain in the dry centre (food insecure) and south of Tunisia, with relatively marginal yield penalties in the less arid north. This outcome was due to both water savings for flowering and grain filling periods and increasing transpiration efficiency (TE) (the ratio of biomass produced per unit of water transpired by a crop) while allowing maximal gas

exchange at favourable times of the day (i.e., VPD < 1 kPa). Yield losses occurred when limiting TR unnecessarily restricted carbon assimilation when enough soil moisture was available to trade transpirational water loss for CO_2 uptake (Sadok et al., 2019). Similar findings were obtained in sorghum (Sinclair et al., 2005), maize (Messina et al., 2015), and lentil (Guiguitant et al., 2017). Therefore, yield benefits from restricting transpiration at high VPDs vary with geography, environment type, and/or the nature of the soil, especially its water holding capacity and infiltration rate. Thus, aside from the need to investigate the agronomic implications of genotypic variations in transpiration response to VPD, additional work is needed to figure out the underlying physiological mechanisms and the genetic controls of traits that potentially reduce plant water consumption and improve productivity (Vadez et al., 2014).

Table 1.1 List of break-point (BP) ranges in some legume species.

Species	Break-point (kPa)	Reference
Soybean	1.1-1.88	Sadok and Sinclair 2009
Peanut	1.98-2.56	Devi et al., 2010
Cowpea	1.81-2.92	Belko et al., 2012

1.4 Plant signalling under high VPD/water deficit

For about 200 years, biologists have been interested in the stomatal response to changes in environmental conditions (Darwin, 1898). Soil water depletion limits leaf gas exchange by decreasing stomatal aperture (Dodd, 2003; Schachtman and Goodger, 2008). Changes in atmospheric relative humidity or more precisely VPD trigger the movement of guard cells.

Stomatal regulation of leaf water balance has been suggested to be controlled by passive hydraulic processes and/or active metabolic processes (McAdam and Brodribb, 2014).

1.4.1 Hydraulic signalling

To protect plants from excessive water loss, natural selection has limited water flows within roots (Tharanya et al., 2018) and leaves (Sack et al., 2004) as part of the whole-plant hydraulic system, thereby decreasing transpiration at a higher VPDs (Fletcher et al., 2007). Changes in root and leaf tissues in response to water stress can have significant consequences on plant hydraulics. The underlying physiological mechanisms responsible for such responses to atmospheric stimuli are still largely unexplained. Hydraulic signals or passive stomatal regulations refer to the changes in water potential gradients throughout different tissues that could regulate turgor and water status and consequently induce stomatal closure (Brodribb and Holbrook, 2003; Franks, 2013). In this model, the conductance of water to and into the leaves is considered to be a critical variable in determining transpiration rate. When VPD increases, a relatively low hydraulic conductance would restrict water flux into the leaves thereby necessitating partial stomatal closure (Bunce, 2006). A few studies have evaluated how the hydraulic conductance of individual organs contributes to overall whole plant conductance (Pratt et al., 2010). Leaves are a major bottleneck in the whole-plant water transport pathway that accounts for 30-80 % of the whole-plant hydraulic resistance depending on the species (Sack et al., 2003 & 2005). In dicotyledons, leaf hydraulic limitations form about 50 % of the hydraulic resistance of the aerial plant parts, which is about 30 % of the whole-plant resistance (Fig. A-1 A), (Sack et al., 2003; Sack and Holbrook, 2006).

While most of the resistance to water flow through plants is due to the stomatal closure, the root system can still represent a significant barrier (Steudle, 1994; Steudle and Peterson, 1998) and can contribute up to approx. 50 % of the overall hydraulic resistance of the plant (Martre et al., 2001). Thus, roots establish a critical link in the soil-plant–air continuum, this link should be maintained in the most adverse environmental conditions (Jackson et al., 2000; Steudle, 2001). The contribution of faba bean organs to whole-plant hydraulic resistance is yet to be addressed.

Whole-plant hydraulic conductance (K_{plant}) and organ conductance components regulate transpiration, carbon gain and growth rate (Stiller et al., 2003; Tyree, 2003; Brodribb, 2009). The high association between transpiration and hydraulic conductance (Tsuda and Tyree, 2000) supports the hypothesis that hydraulic conductance can regulate stomatal aperture in response to VPD (Domec et al., 2009). Indeed, many existing studies reported that limited transpiration is attributed to restrictions in hydraulic conductance in different plant organs i.e., leaves (Sack et al., 2003 & 2005; Brodribb et al., 2005; Sadok and Sinclair, 2010 b), shoots (Yang and Tyree, 1993), stems (Nardini and Salleo, 2000; Martorell et al., 2014) or roots (Rodríguez-Gamir et al., 2011; Perrone et al., 2012). In soybean and sorghum, limited TR under high VPD is likely due to limitation in leaf hydraulic conductance between the xylem and the guard cells (Sinclair et al., 2008; Choudhary et al., 2013). While in wheat (Schoppach et al., 2014) and pearl millet (Tharanya et al., 2018), limited TR at high VPD results from limited root hydraulic conductance. In pearl millet, roots were characterized by low amounts of water channel proteins (aquaporins) that mediate water transport, while in wheat, root hydraulic limitation was explained by smaller meta-xylem vessels, thinner endodermis and a smaller population of mercury-sensitive aquaporins in the roots. In maize, limitation in both leaf and

root hydraulic conductance restricted TR at high VPD (Choudhary et al., 2014). Thus, the hydraulic architecture of plants seems to play a vital role in stomatal response to changes in plant hydration (Sperry et al., 2002) even though stomata respond to multiple plant signals triggered by a variety of environmental stresses (Hartung et al., 2002; Bunce 2006; Domec et al., 2009). A better understanding of the coordination between hydraulic architecture and stomatal responses to fluctuations in VPD will provide insight into the diurnal and seasonal growth patterns of plants. These assumptions require a mechanism that can alter hydraulic conductance which might involve abscisic acid (ABA) production (Hose et al., 2000; Thompson et al., 2007; Parent et al., 2009), differences in xylem anatomy, and/or changes in water channels (aquaporins) that regulate water flow in response to unfavourable conditions (Steudle and Henzler, 1995; Tyree et al., 1999).

1.4.2 Chemical signalling

Chemical signalling or active stomatal regulation is presumably driven by low bulk leaf water potential that triggers abscisic acid (ABA) synthesis (Xie et al., 2006; Bauer et al., 2013; Merilo et al., 2013; McAdam and Brodribb 2015; McAdam and Brodrib, 2016). ABA regulates various physiological processes throughout the plant life cycle including stomatal closure in response to water shortage and the expression of multiple stress-responsive genes.

Over the last 3 decades, it has been hypothesised that stomatal closure at high VPD is driven by increased flux of ABA to the guard cells (Tardieu and Davies 1993; Speirs et al., 2013). ABA is synthesised throughout the plant in response to decreased cell turgor but, it is still controversial whether it is primarily synthesized in the roots or aerial parts. One school of thought suggested that decreased root turgor enhances ABA synthesis with export to the

shoots via the transpiration stream, where it accumulates in the leaf apoplast and hence closes the stomata (Davies and Zhang; 1991; Tardieu and Davies 1993; Dodd, 2005; Wilkinson et al., 2012; Puertolas et al., 2013). As an alternative to the root-sourced model, ABA is synthesized in the aerial parts of the plant (e.g., leaves and stems) and then is transported to the roots to increase hydraulic conductance (Manzi et al., 2015; McAdam et al., 2016 a). Nevertheless, both hypotheses have been confounded by the idea that stomatal closure at high VPD occurs as plants actively regulate gene expression to change ABA levels. That is, plants activate *de novo* ABA biosynthesis in response to increased VPD (Bauerle et al., 2004; Bauer et al., 2013 a & b; McAdam and Brodribb 2015). This theory is supported by multiple lines of evidence as follows (i) the stomatal closure in response to VPD is compromised in ABA biosynthetic mutants (Xie et al., 2006; Merilo et al., 2013), (and ii) the increase in leaf ABA concentration as VPD increases occur over the time scale of minutes (McAdam and Brodribb 2015) to hours (Bauerle et al., 2004), and (iii) at elevated VPD, there is an increase in the expression of the gene encoding a 9-cis-epoxycarotenoid dioxygenase (NCED), the key protein required for catalysing the rate-limiting carotenoid cleavage step of ABA biosynthesis (Bauer et al., 2013 a; Pantin et al., 2013). However, changes in gene expression have only been tested in *Arabidopsis thaliana* plants during exposure to elevated VPD. The hypothesis that *de novo* ABA biosynthesis regulates ABA levels during VPD transitions has also been confounded with the idea that rapid functional increases in ABA levels seen in angiosperms (McAdam and Brodribb, 2015) could occur by hydrolysis of the conjugated form of ABA, ABA-glucose ester (ABA-GE), to ABA at high VPD. The conversion of ABA-GE to ABA by a single step through β-glucosidases has long been hypothesized as a means of dynamically increasing ABA levels in both the leaf and xylem sap in response to increased stress (Dietz et al., 2000). Molecular and physiological characterization of the genes encoding β-glucosidases, and their respective

mutants, suggests an important role for the hydrolysis of ABA-GE to ABA in plant response to severe water stress (Lee et al., 2006; Xu et al., 2012).

The role of ABA has also been confounded by the idea that so far, there is no evidence that leaf water deficits trigger an active metabolic process at the onset of the limited TR response, with neither leaf water potential (Ψ_{leaf}) nor water content (LWC) significantly decreased at the threshold of limited TR (VPD = $\sim$ 2.1 kPa). (Sinclair et al., 2017). The insensitivity to any decreases in leaf water status is consistent with the observation of McAdam and Brodrib, (2016) that excised pea (*Pisum sativum* L.) leaves had to be exposed to 1.0 MPa of pressure to induce a sufficient loss in turgor to increase ABA concentration. Since Ψ_{leaf} does not decrease as VPD increases for genotypes expressing limited TR, it is possible that a localized decrease in water status in the leaf causes the stomatal response to increased VPD. Meidner, (1975) proposed that the epidermis, including the stomatal guard cells, was the site of significant water evaporation within the leaf, and hence loss in cell water status. Since mesophyll cells evaporate little water, the bulk leaf water status might not change much with increased VPD. As the epidermis, particularly the guard cells, evaporates most of the water, these cells are the final destination of most of the water flow in leaves. As a result, guard cells may be particularly prone to turgor loss and stomatal closure at high VPD. Nevertheless, stomatal closure is not always the only response regulating water loss e.g., in wheat, the stomata of young plants do not necessarily close in response to air warming and high water potential can be maintained by an increase in root hydraulic conductance (Vysotskaya et al., 2004 a & b) indicating that it is not only stomatal closure that may regulate plant water loss. In another study, different stomatal responses to air temperature were observed among the studied wheat cultivars, where some closed their stomata at elevated VPD while others did

not (Kudoyarova et al., 2007). Thus, it appears there are two options for regulating water relations under these circumstances and it is important to understand the mechanisms involved (Fig. A-2).

1.5 Quantitative trait loci (QTL) for drought stress tolerance in Faba bean

Quantitative traits are amongst the most agriculturally important traits, including yield, quality factors and many forms of disease resistance that are controlled by many genes. Detecting the major quantitive trait loci of these traits (QTLs) is one of the most essential aspects of marker-assisted selection (MAS) application in plant breeding. In QTL mapping, it is widely assumed that there is a small number of major detectable genes in relatively small samples. Combining phenotypic data and allelic marker segregation along a genome allows the detection of QTLs, which increases knowledge of their inheritance and gene action. Faba bean is a diploid with six large chromosomes (2n = 12), which makes it an appropriate tool for cytogenetic studies. Faba bean genome size (~13,000 Mbp) is 2.6, 3.2, 17.6, and 26-fold larger than those of pea, lentil, chickpea, and *Medicago truncatula*, respectively (Sato et al., 2010) with more than 95 % of repetitive DNA and is considered to be one of the largest of any diploid crops (Bennett and Smith, 1976; Johnston et al., 1999). Therefore, the use of molecular markers and the development of suitable F2 and advanced inbred lines have allowed significant progress in mapping to enhance breeding strategies in this species.

Most of the functional genomics studies in faba bean are academic and not directly related to crop improvement. It was used as a model plant to study stomatal movement (Hanstein and Felli, 2002; Chen et al., 2004; Gao et al., 2005). A fia (faba bean impaired in ABA-induced stomatal closure) mutant was identified in which the stomatal movement was disturbed (Iwai

et al., 2003). Yield, its components, and flowering time (associated with drought avoidance) were fine mapped in faba bean (Cruz-Izquierdo et al., 2012).

Molecular approaches in faba bean breeding have been mostly limited to biotic stresses and anti-nutritional compounds (reviewed in Torres et al., 2012). Recently, some significant efforts have been made in identifying QTLs for abiotic stresses such as frost tolerance (Arbaoui et al., 2008; Sallam and Martsch, 2015; Sallam et al., 2016), traits related to drought adaptation (Ali et al., 2016; Khazaei et al., 2014 a), and yield (Ávila et al., 2017; Cruz-Izquierdo et al., 2012), fortified by comparative genomics with *Medicago truncatula* and other legumes (Ellwood et al., 2008; Kaur et al., 2012; Torres et al., 2012; Khazaei et al., 2014 b). The genome of *M. truncatula* has been an ideal comparative model in many genetic and genomic studies of legumes, where limited genetic resources in legume crops have categorized them as 'genomic orphans' (Varshney et al., 2009). Recently, linkage groups detected in faba bean (Ruiz-Rodriguez et al., 2014) have been directed to all faba bean chromosomes, which provides the source for integration of genetic maps that will facilitate good QTL mapping and gene identification in this species. Moreover, sequencing of the whole soybean genome (Schmutz et al., 2010) is a very useful tool in providing further insights into the genetics and biology of legumes.

To date, the published faba bean studies have mostly involved bi-parental populations, derived from crosses between two inbred lines such as F2, backcrosses and recombinant inbred lines (RILs) that have been employed for genetic map construction and trait mapping. The relatively large set of interconnected bi-parental populations that segregate for diverse important traits in this species will help advance faba bean breeding. These types of populations are easy to construct and represent a powerful tool for QTL detection due to the relatively large segregations for diverse important traits in the bi-parental populations.

Moreover, their optimal allele frequency and low rate of linkage disequilibrium decay within chromosomes mean that only a few hundred RILs/markers are needed to map a QTL (Scott et al., 2020). Despite the advantages of bi-parental populations, their mapping precision is low due to the low total amount of genetic recombination, as only two alleles are present at any locus, and to the low amount of genetic diversity that can be created by only two founders. These factors may limit the number of QTLs captured. To overcome these limitations, multi-parental populations would be helpful. Indeed, a multi-parent population derived from 11 European winter faba bean founders was created and employed to identify genomic regions controlling frost adaptation (Sallam and Martsch, 2015). Furthermore, Khazaei et al., (2018 b) developed a multi-parent population from four founders (ILB 938/2, Disco/2, IG 114476, and IG 132238) for preliminary characterization of important morphological and biochemical traits. A MAGIC (multi-parent advanced-generation intercross) population comprising over 2000 F4 individuals is currently under development at ICARDA (International Center for Agricultural Research in Dry Areas), combining eight diverse parents with sources for several resistance to multiple abiotic and biotic stresses e.g., drought, heat, ascochyta blight, chocolate spot, rust and broomrape (Maalouf et al., 2019).

Khazaei et al., (2014) developed a bi-parental population from a cross between Mélodie/2 and ILB938/2 to study leaf morpho-physiological traits related to drought stress response e.g. stomatal characteristics and water status. The population was phenotyped for several morpho-physiological traits and the map revealed a high degree of synteny with the *M. truncatula* genome. Most of the detected QTLs for the morphology and function of stomata were in a single region of faba bean chromosome 2 syntenic that harbours receptor-like protein kinase. These results reveal that genomic data from model plant species can be easily translated to faba bean. Thus, to facilitate characterization of the genetic diversity within

germplasm collections as well as understanding their potential, it is essential to develop a reference genome, gene functional analyses, and genotype-phenotype association, together with the development of high-throughput genotyping platforms.

1.6 Aims of the study

This research aimed at identifying possible genetic variation in transpiration response to evaporative demand in faba bean along with determining the underlying physiological mechanisms regulating the response. Faba bean was chosen as a model species due to its dual importance for human consumption and animal feed. Moreover, to the best of my knowledge, there are no studies in faba bean relating the phenotyping characteristics of plant transpiration under high VPD to physiological performance and biochemical changes under controlled and semi-controlled conditions in the same lines.

Initially, two British faba bean cultivars, Masterpiece and Robin Hood were used to study TR to VPD using different phenotyping approaches (Chapter 2) to determine the most appropriate technique to facilitate later screening of a recombinant inbred population derived from faba bean (Khan et al., 2007 & 2010; Khazaei et al., 2013 b). Since the two cultivars consistently differed in their TR to VPD over the various phenotyping approaches, it was of interest to determine what regulates this variation. The role of tissue hydraulic conductance and tissue ABA were compared at low and high VPD (Chapter 3). The conclusions of Chapters 2 and 3 were adopted by determining genetic variation in TR to VPD in 165 faba RILs using a whole-plant gas exchange chamber and measuring hydraulic conductance as a potential controlling mechanism for the variation, in addition to identifying QTLs responsible for the response in the faba bean chromosomes (Chapter 4).

The aims proposed for this thesis were:

1. To develop a high throughput system for genetic studies of transpiration response to VPD.
2. To determine the role of hydraulic conductance and ABA as regulatory mechanisms for the limited transpiration trait under high VPD.
3. To identify genotypic variation in transpiration response to evaporative demand in faba bean RILs as well as the QTLs of the trait along the faba bean chromosome.

Chapter 2: Identifying genetic variation in transpiration response to evaporative demand in faba bean (*Vicia faba* L.) using different techniques

2.1 Introduction

Faba bean (*Vicia faba* L.) is one of the most important cool-season grain legumes because of its high nutritional value, which makes it one of the best solutions to combating malnutrition in developing countries in Africa and parts of Asia and Latin America (Haciseferogullari et al., 2003). It is considered a valuable break crop in environmentally sustainable arable production systems across the world (Köpke and Nemecek, 2010). Nevertheless, faba bean yields have remained lower and more variable than other legumes such as soybean, due to the effects of biotic and/or abiotic stresses (Gasim et al., 2013), and its sensitivity to water shortage (Khan et al., 2007 & 2010; Khazaei et al*.,* 2013 b) which affects its growth, development and yield components (Ouji et al., 2017).

Plants can minimize water losses via stomatal closure and the importance of this for plant adaptation to water-limited environments has been thoroughly discussed in Chapter 1. Plant TR is driven by changes in VPD, with high atmospheric VPD causing leaf dehydration if rates of water loss exceed those of water uptake. Thus, one strategy for plants to maintain their water status is to restrict their TR during periods of high VPD to match water flux into the leaves, thus saving soil water for the most critical periods e.g flowering and grain filling (Sinclair et al., 2005, Shekoofa et al., 2014) resulting in yield gains (Sinclair et al., 2010). Indeed, plant breeders have incorporated the restricted transpiration trait into some species such as maize, via Pioneer's AQUAmax® hybrids (Cooper et al., 2014). Extending this plant breeding strategy to other crop species (such as faba bean) requires the identification of

genetic variation in transpiration response to VPD, underpinned by rapid, precise, and reproducible measurements.

Like most physiological characters, measuring TR response to VPD is always challenging with the requirement of phenotyping the traits under a wide range of environmental conditions (Ghanem et al., 2015). In this regard, there are several direct and indirect phenotypic approaches for limited TR at high VPD depending on the available resources and the developmental stage of the plant. An indirect simple field approach depends on observations of plant response after exposure to water shortage, where sustaining high water status via delayed leaf wilting would be likely due to limiting TR and conserving soil water (Sinclair et al., 2017). Although this approach is fast, easy, and can screen a large number of genotypes at the same time, it cannot be definitive as delayed wilting can result from several factors other than limited TR e.g., high leaf water potential. Another phenotyping approach involves remotely measuring temperature images of a field location where the higher temperature for well-watered plots under high VPDs indicates a partial stomatal closure because of limited TR (Sinclair et al., 2017). This also could not offer robust data as it requires unique environmental conditions, in addition, temperature variations can result from several possibilities. Alternatively, stomatal conductance (gs) measurements of field-grown plants during the diurnal variations in VPD are more representative (Gilbert et al., 2011; Shekoofa et al., 2014 & 2015). On the other hand, the field approaches may be restricted by the diurnal changes in the atmospheric conditions and the number of plants that can be measured per day. To overcome the limitations of the direct measurements, an alternative approach has been developed in which whole-plant transpiration can easily be measured in potted plants, as the difference in the pot weight between successive gravimetric measurements, in semi-controlled glasshouses (Belko et al., 2012; Ryan et al., 2016; Schoppach et al., 2016) or

unregulated fields (Belko et al., 2012; Vadez et al., 2014 & 2015; Kar et al., 2020) under naturally fluctuating VPD.

Another way to phenotype TR to VPD is to measure leaf transpiration with an infra-red gas analyzer (IRGA) that offers some control of VPD around the leaf (McAdam and Brodribb, 2015), but there is some uncertainty about whether such measurements can be readily scaled to the entire plant. Transpiration inside the infra-red gas analyzer cuvette reflects the controls imposed on that environment (i.e. chosen temperature, light source, flow rate, and leaf area) used for measurement. Also, the spatial variation in the light environment of different leaves and naturally occurring microclimates across the plant affect its interaction with the environment, thus single leaf measurements may not adequately describe the whole-plant response (Niinemets, 2013; Medrano et al., 2015). The most intense phenotyping approach involves direct measurements of water loss in response to fluctuated VPD in controlled chambers. This approach requires tight regulation of both temperature and relative humidity around the shoot in the chamber that measures transpiration with an IRGA, allowing VPD to be changed at variable (Fletcher et al., 2007) or almost stable (Jauregui et al., 2018) air temperature.

Statement of Research Objectives

Since previous investigators have utilized these approaches that vary in experimental throughput, plant levels (i.e. whole plant *versus* single leaf), and environmental regulation, it was not clear which was most suitable to determine possible genetic variation in faba bean transpiration responses to VPD. It was hypothesized that consistent genotypic variation in transpiration response to VPD could be detected at both individual leaf and whole-plant levels, independently of whether relative humidity and temperature fluctuated concurrently

(gravimetric measurements in the greenhouse) around the whole plant or whether relative humidity was tightly regulated in a leaf cuvette or whole-plant chamber. By comparing plants grown at different times of the year and measured with different techniques at different times of the day, we sought to determine the most appropriate technique to facilitate later screening of a recombinant inbred line population.

2.2 Materials and Methods

2.2.1 Plant culture

Vicia faba cvs. Masterpiece (MP) and Robin Hood (RH) (as commonly grown UK cultivars for which seed was readily available) were used to study transpiration response to VPD gravimetrically, and using infra-red gas analysis of individual leaves or using the whole-plant gas exchange chamber. In May 2018, seeds of the two faba bean cultivars (Moles Seeds, Colchester, UK) were germinated at about 2.5 cm depth in rectangular 2 L pots (12.5 top × 10.5 base × 21 cm height, two seeds/pot) containing a mixture of commercial John Innes No. 2 substrate (Westland Horticulture Ltd, UK) and silver sand (Royal Horticultural Society, UK) with a ratio of 3:1 (v/v) in a glasshouse at Lancaster Environment Centre, Lancaster University. 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 after sunset at the top of the canopy ~ 2 m below the lamp was 602 ± 15 µmol m−2 s−1 PPFD (mean ± SE, n= 336, comprising 12h x 28 days), where 1 W/m2 (supplied by high-pressure sodium lamps) = 3.56 µmol m^{-2} s^{-1} PPFD according to Apogee Light Unit Convertor (www.apksfull.com/light-unit-converter). Actual air temperature and relative humidity in the centre of the glasshouse were recorded with a Hortimax system (HortiMax

Ektron III, hortisystems.co.uk). Day/night temperature ranges were 29.3 ± 0.12°C, and 18.9 ± 0.12°C (mean ± SE, n= 336), respectively. Day/ night relative humidity ranges were 35 ± 0.32 % and 60 ± 2.35 % (mean ± SE, n= 336), respectively. After the first true leaf emerged, the plants were thinned to a single plant per pot. The plants were grown for 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 NPK. Homogenous plants with 6-7 fully expanded leaves (leaf area= 495 ± 10 and 423 ± 8 cm^2 for cv. Robin Hood (RH 1-3) and cv. Masterpiece (MP 1-3), respectively, mean ± SE, n= 3) were assigned to measure transpiration response to VPD using the single balance system, infra-red gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) and whole-plant gas exchange chamber in June 2018.

2.2.2 Experimental design

To test whether the type of measurement affected transpiration response to VPD, Experiment 1 measured transpiration response to VPD of three plants for each genotype, i.e RH 1-3 and MP 1-3, over eight consecutive days with the three systems. Transpiration was measured gravimetrically on a single balance (Ohaus Adventurer Pro AV8101, Ohaus Corporation, USA) over six consecutive days, one plant per day and alternating between genotypes. On the sixth day, single leaf transpiration to VPD was measured with the LI-6400XT for three of the plants previously measured with the balance. On the seventh day, the whole-plant gas exchange measurements were included, with the same plants measured over another two consecutive days, three plants per day. (Table B-1).

2.2.3 Gravimetrically measuring whole-plant transpiration response to VPD

Plant transpiration response to variation in ambient VPD was measured under glasshouse conditions. Pots were watered to drained capacity at 08:00 h and allowed to drain for 30 minutes. Thereafter, the pot was covered with aluminium foil to avoid evaporative losses from the soil and placed on a digital balance (Ohaus Adventurer Pro AV8101, Ohaus Corporation, USA) that was connected to a laptop running software (launch SPAD data collection v2.03.exe.) which recorded pot weight every minute for 24 h, with 15-minutes averages aligned with other environmental measurements (Fig. B-1 A). A data logger (OM-LE-USB-2, Omega, UK) was positioned close to the plant to regularly record (every 15 minutes) air temperature and relative humidity throughout the measurement period. After 24 hours on the balance to collect data, the plant was harvested and its leaf area was measured using a leaf area meter (Model LI-3100C, LI-COR, Lincoln, NE, USA) unless it was required for additional measurements (Sections 2.2.4 & 2.2.5). The difference in pot weight in g was converted to mg, normalized to leaf area (m^2) and time (min) to calculate transpiration rate (TR) in mg H_2O/ m^2/min. VPD was calculated as a function of air temperature and relative humidity using the online VPD calculator provided by the College of Agriculture and Life Sciences, Arizona University (https://cals.arizona.edu/vpdcalc/).

2.2.4 Measuring single leaf transpiration response to VPD.

An infra-red gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA) measured individual leaf transpiration response to VPD between 09:10 h and 18:45 h of the same plants previously measured with the balance. Preliminary experiments varied light intensity between 100 and

1600 µmol m^{-2} s^{-1} PPFD in 100.0 µmol m^{-2} s^{-1} PPFD intervals inside the cuvette to establish a light intensity inside the IRGA cuvette that was saturating for single leaf transpiration and photosynthesis when measured at a constant CO_2 concentration (400 ppm), (Fig. B-2 A & B). While the plants were exposed to fluctuating greenhouse light conditions, the youngest fully expanded leaf (usually the 4th one from the base of the plant) was selected and placed in the LI-6400XT cuvette (2 × 3 cm^2) (Fig. B-1 B), which was held at ambient air temperature, flow rate (500 µmol s^{-1}), ΔCO_2 (-5 µmol mol^{-1}), ΔH_2O (-0.5 µmol mol^{-1}) and a constant light intensity of 800 µmol m^{-2} s^{-1} PPFD. By adjusting the desiccant valve in both bypass (high relative humidity, low VPD) and scrub (low relative humidity, high VPD) directions, VPD could be rapidly altered in ~ 0.3-0.5 kPa intervals depending to some extent on the air temperature inside the glasshouse. Transpiration, measured in mmoles of water, was converted into mg (1 mmol H_2O = 18 mg) to allow comparison with the other systems. Three plants were measured per day, with six VPD levels typically requiring 3-4 hours for each plant. After these measurements, and on the same day if possible, the plant was taken to the whole-plant gas exchange chamber to measure its whole transpiration response to VPD.

2.2.5 Measuring transpiration response to VPD in the whole plant gas exchange chamber.

The whole-plant gas exchange system (Fig. B-1 C & D) is a 30 L (25 × 20× 60 cm) perspex chamber, with a nominal thickness of 3.5 mm connected to an infra-red gas analyzer (LI-6400XT, LI-COR, Lincoln, NE, USA). A sealable slot exists in the base of the chamber that fits

with the 21 mm diameter sleeving, to fully isolate the plant from the surrounding atmosphere. A compressor (OF1202-40MQ3, Junk Air, USA) provides a stable airflow, while homogenous airflow is ensured by four internal fans. Light is supplied by two Son-T high-pressure sodium lamps (Philips, Netherlands) providing 450 µmol m^{-2} s^{-1} PPFD at the top of the canopy ~ 1 m below the lamp. Air is humidified by a water bath containing an Ultrasonic humidifier (Growell, UK) and manually operated low-pressure valves (Swagelok, UK) that are used to control the amount of air passing through the water bath.

Whole-plant transpiration response to VPD was measured as described by Jauregui et al., (2018) from 09:00 h to 20:00 h under six VPD levels within the range of ~ 1.5-3.5 kPa, with three plants were measured per day. The same plants measured with the previous systems were sequentially inserted into the chamber which was sealed carefully with 1 cm wide neoprene sponge rubber and closed using eight metal clips to prevent leaks. A neoprene sponge rubber ensured a tight fit of the plant into the chamber. The equipment is operated in the laboratory, allowing the temperature to remain stable at 26.6°C ± 0.3°C (mean ± SE, n= 36, comprising 6 plants x 6 VPD levels) when the fans are on. Temperature and relative humidity inside the chamber remains comparable. Airflow was set at 98 ± 1.3 µmol s^{-1} (mean ± SE, n= 36) to allow a reasonable CO_2 differential across the chamber (between − 18 to − 25 µmol CO_2 m^{-2} s^{-1}). Differences in CO_2 (ΔCO_2) and H_2O (ΔH_2O) between the air entering and leaving the chamber were measured and recorded using an infra-red gas analyzer (IRGA). If necessary (at high VPDs or with large plants), the flow rate was increased as long as ΔCO_2 and ΔH_2O within their optimal ranges. Since there were minor changes in the temperature, VPD within the chamber was controlled by manipulating relative humidity by supplying ambient, dried, or humidified air. Humidified air was achieved by passing air over a heated

water bath while passing air through a 2 L plastic bottle containing silica gel desiccant dried the air.

The measurement sequence starts by inserting the plant into the chamber and sealing it with eight metal clips, then relative humidity was increased to ~ 65 % to generate the first VPD. Once CO_2 and H_2O exchange was steady for at least 5 min (steady-state), averaged values were logged every minute for 5 min. Then, relative humidity was dropped by about 10-15 % intervals by introducing a mixture of humidified and dry air to the chamber to generate a range of six different VPDs (~1.5-3.5 kPa, each about 0.4 kPa intervals). It usually takes 30-45 min to reach a new steady-state at each VPD, after which a five minutes average was recorded. 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). Different cultivars were alternated between measurement occasions to ensure that plants from each genotype were measured on different days and at different times of the day. Data were then downloaded from the LI-6400 comprising records of transpiration in mg, VPD, and other physiological parameters.

2.2.6 Time of year effects on TR response to VPD

After Experiment 1 in June 2018 (which compared the 3 measurement approaches), two additional batches of plants were grown as Experiment 2 in mid-June & mid-September 2018 to test whether the time of the year the plants were measured affected transpiration response to VPD (Table B-2). Plants were grown under glasshouse conditions as described in Section 2.2.1 for four weeks before they were measured in July and October. 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 was 655 ± 24 and 627 ± 5 µmol m^{-2} s^{-1} PPFD (mean ± SE, n= 336) in June and September, respectively. In June, day/night temperature ranges were 29.6 ± 0.21 °C and 19.9 ± 0.16°C (mean ± SE, n= 336), while day/night relative humidity ranges were and 33 ± 0.43 % and 55 ± 0.55 % (mean ± SE, n= 336), respectively. In September, day/night temperature ranges were 28.5 ± 0.12 °C and 17.8 ± 0.08°C (mean ± SE, n= 336), respectively while relative humidity ranges were 39 ± 0.48 and 58 ± 0.62, (mean ± SE, n= 336) at day and night, respectively across the four weeks growing period. Homogenous plants with 6-7 fully expanded leaves (leaf area= 449 ± 14 & 413 ± 12 cm^2 in July and 482 ± 7 & 427 ± 6 cm^2 in October for cv. Robin Hood and cv. Masterpiece, respectively, (mean ± SE, n= 3) were assigned to measure transpiration response to VPD with the single balance system (Table B-2). Transpiration response of each plant to VPD was measured over 24 hours, with 60-minute averages aligned with the Hortimax records over the photoperiod (12 h) with different cultivars measured on alternate days for six consecutive days.

2.2.7 Time of day effects on TR response to VPD

After comparing the response between two contrasting months (July & October), an additional batch of plants was grown as in Section 2.2.1 in October 2019 to test whether the time of the day the plants were measured affected transpiration response to VPD. Supplementary lighting maintained the photoperiod at 12 hours (08:00-20:00 h.) across the four weeks growing period. The light intensity during the growing period was 585 ± 2

µmol m^{-2} s^{-1} PPFD (mean ± SE, n= 336). Day/night temperatures were 28 ± 0.07 °C and 18.60 ± 0.07°C (mean ± SE, n= 336). While relative humidity was 26 ± 4 and 46 ± 1 %, (mean ± SE, n= 336) at day and night, respectively. Homogenous plants with 6-7 fully expanded leaves (leaf area= 552 ± 15 cm^2 and 482 ± 20 cm^2 for cv. Robin Hood and cv. Masterpiece, respectively, mean ± SE, n= 9) were selected to measure their transpiration response to VPD with the whole-plant gas exchange chamber in November 2019. Three plants were measured per day under six ascending VPD levels and transpiration response to VPD was compared between morning, afternoon, and late afternoon to determine if it differs over the three periods of the day (Table B-3).

2.2.8 Statistical Analysis

ANCOVA was used to determine the effects of genotype and experimental technique (Experiment 1), times of the year (Experiment 2), and day (Experiment 3) on the response using SPSS 27.0 for Windows statistical software package (SPSS, Inc., Cary, NC). Analysis of TR response to VPD was performed using the segmented linear regression model of GraphPad Prism 9.3.1 (GraphPad Software Inc., San Diego, CA, 2007), which provides a 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. Significant ($P< 0.05$) genotypic differences in regression parameters (slopes and BPs) were discriminated against Student's T-test.

2.3 Results

Within the glasshouse, plants were placed on a balance at 08:30 h for 24 h. For a representative Robin Hood plant (RH1), from 08:45 h, not long after the supplementary lights were switched on (Fig. 2.1 A), VPD increased steadily to 1.28 kPa at 10:00 h, then increased to 2.42 kPa at 12:00 h (Fig. 2.1 B) at the maximum light intensity (1222 µmol m^{-2} s^{-1} PPFD). Thereafter, it rapidly increased to its maximum (3.47 kPa) at 13:00 h when the maximum temperature (29.9°C) and the lowest relative humidity (18 %) were recorded while the light intensity was 1157 µmol m^{-2} s^{-1} PPFD, before dropping rapidly by about 60 % (1.39 kPa) in the late afternoon (17:45 h). Then it steadily decreased to be 1.07 kPa at the end of the photoperiod (20:00 h) and remained reasonably stable at ~ 0.55 kPa at night. Similarly, transpiration followed a diurnal response pattern where it remained reasonably stable at ~ 5.7 mg $H_2O/m^2/min$ when the supplementary lighting was turned off at night and started to increase steadily from 08:45 h to 27 mg $H_2O/m^2/min$ at 10:00 h (Fig. 2.1 C). Thereafter, it rapidly increased to reach its maximum (49 mg $H_2O/m^2/min$) at 12:15 h when light intensity was maximal (1222 µmol m^{-2} s^{-1} PPFD), before dropping rapidly by about 50 % (23.9 mg $H_2O/m^2/min$) in the late afternoon (17:45 h). Then it steadily decreased to 17.8 mg $H_2O/m^2/min$ at the end of the photoperiod (20:00 h) (Fig. 2.1 C).

Evaporative demand in the greenhouse (VPD) and transpiration (TR) were correlated with light intensity, with considerable scatter at the higher light intensities (Fig. 2.1 D & E). For this plant, photoperiod transpiration between 08:00 h and 20:00 h was plotted against VPD, generating the blue symbols in Fig. 2.1 F. TR increased linearly with increasing VPD to reach its maximum (49 mg $H_2O/m^2/min$) at 2.58 kPa (BP), after which there was a remarkable decrease in TR under increased VPD. Two other Robin Hood plants (RH2 and RH3) showed similar diurnal patterns of TR and VPD, with TR ranging from 21.13 to 35.09 mg $H_2O/m^2/min$

and VPD from 1.22 to 2.74 kPa for the second plant. The third plant had a TR range of 19.3 to 40.8 mg $H_2O/m^2/min$ and VPD ranged from 1.39 to 3.07 kPa. VPD significantly ($P< 0.001$) affected TR with no significant Plant x VPD interaction ($P= 0.35$). Despite measurements on sequential days, the three plants showed a consistent whole-plant transpiration response to VPD when measured on balance in the glasshouse (Fig. 2.1 F).

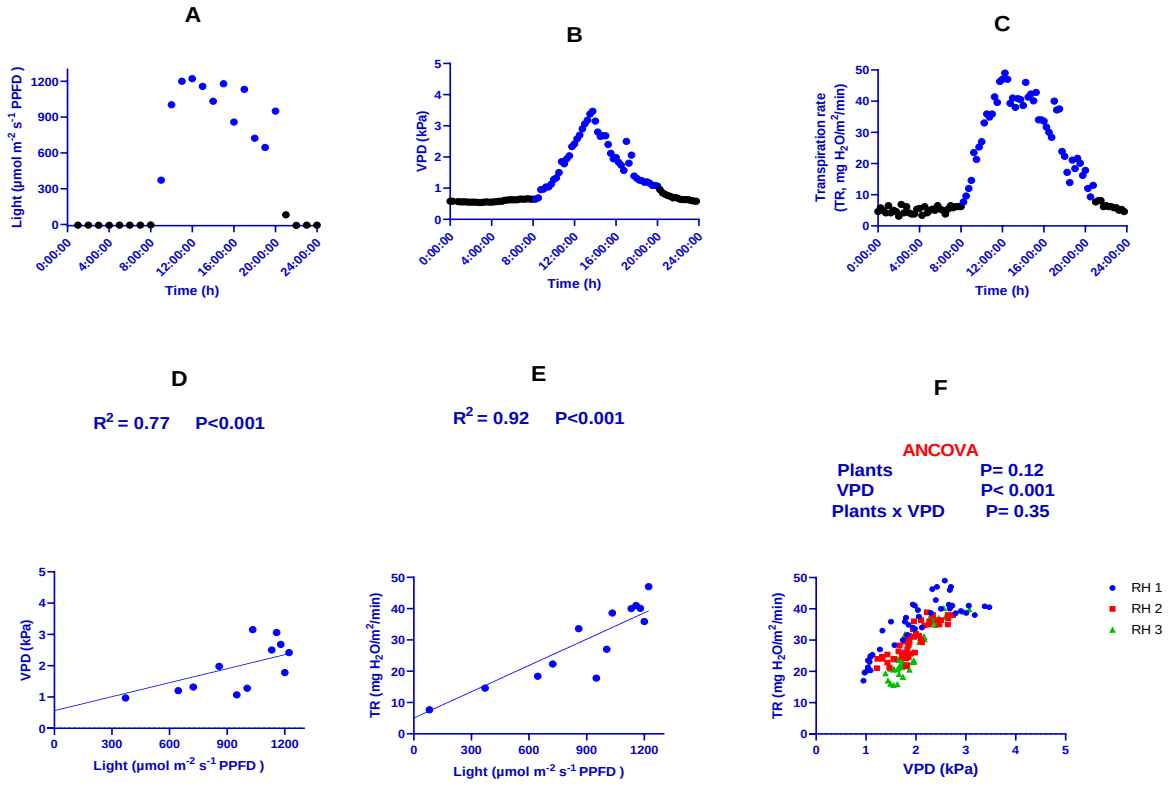


Figure 2.1 Light intensity (A), VPD (B), and TR (C) fluctuations within the glasshouse for a single plant of *Vicia faba* cv. Robin Hood, with black and blue symbols representing the night and day periods respectively. Using the daytime values, VPD (D) and TR (E) are plotted against light intensity. TR is plotted against VPD of three Robin Hood plants (F), with the blue symbols from panels B and C. Light intensity (A) is represented by 60 minutes average recorded by a quantum sensor, while each VPD & TR point represents an average of 15 minutes recorded by an Omega sensor probe (B) and balance (C) respectively. The 15-minute averages of VPD

and TR from (B) and (C) were averaged hourly in (D) and (E) to allow comparison with hourly light intensity measurements, with R^2 and P values from Pearson correlations indicated in the top of panels D and E. P values from ANCOVA for the effects of the plant, VPD, and their interaction are indicated in the top of panel F.

With single leaf measurements, TR was measured between 09:10-18:45 under a VPD range of 1.33-3.60 kPa with three plants measured per day (Fig. 2.2 A). The three plants had a TR of 22 - 41 mg $H_2O/m^2/min$ (Fig. 2.2 B). For the three plants, single leaf transpiration was plotted against VPD (Fig. 2.2 C). Transpiration rate (TR) increased linearly with increasing VPD until 2.3 kPa (BP) for the first plant after which there was stability in TR under increased VPD. For the second plant, VPD ranged from 1.42-3.57 kPa and resulted in a TR of 23.88-38 mg $H_2O/m^2/min$. The third plant had a TR range of 22.44 to 34.42 mg $H_2O/m^2/min$ under a VPD range of 1.5 to 3.6 kPa. In the three plants, leaf transpiration increased linearly with elevated VPD until 2.35 kPa after which it either stabilized or declined (Fig. 2.2 C). Again, VPD significantly (P= 0.04) affected TR with no significant Plants x VPD interaction (P= 0.62). Despite variation in the time of day, the three plants showed a consistent single leaf TR when measured with the Infra-red gas analyzer in the glasshouse.

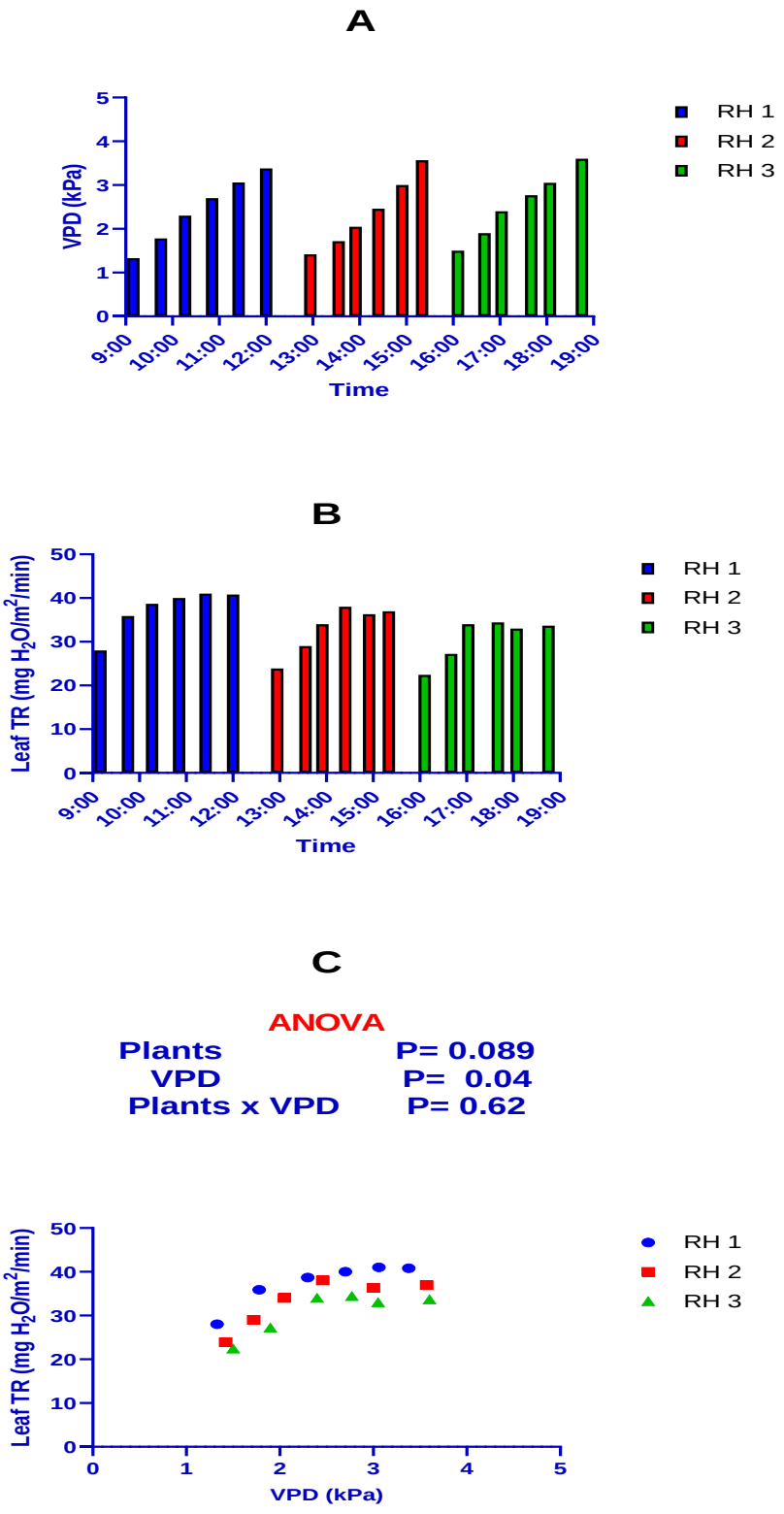


Figure 2.2 Cuvette VPD (A), leaf TR (B) fluctuations during the day of three Robin Hood plants measured with the LI-6400XT over 11 hours (the same 3 plants in Figure 1), and TR response

to VPD (C). Each column/point represents an average of 5 minutes for both parameters after 15 minutes of steady-state. P values from ANCOVA for the effects of the plant, VPD, and their interaction are indicated at the top of panel C.

Within the whole-plant gas exchange chamber, TR was measured between 09:00 and 20:00 h under a VPD range of 1.40-3.79 kPa with three plants measured in a single day (Fig. 2.3 A) that had a TR range of 21.3-35.0 mg $H_2O/m^2/min$ (Fig. 2.3 B). For the three plants, whole-plant transpiration was plotted against VPD (Fig. 2.3 C). Transpiration rate (TR) increased steadily with increasing VPD to have a maximum TR (33.8 mg $H_2O/m^2/min$) at 2.47 kPa (BP) for the first plant. Thereafter, TR was relatively stable despite increases in VPD, to be 33 mg $H_2O/m^2/min$ at 3.79 kPa. For the second plant, VPD ranged from 1.44-3.40 kPa and resulted in a TR of 23.30-35.66 mg $H_2O/m^2/min$. The third plant had a TR range of 23.44 to 35.80 mg $H_2O/m^2/min$ under a VPD range of 1.40 to 3.34 kPa. Again, VPD significantly (P= 0.046) affected TR with no significant Plants x VPD interaction (P= 0.3) when whole-plant transpiration response to VPD was measured with the whole-plant gas exchange chamber (Fig. 2.3 C).

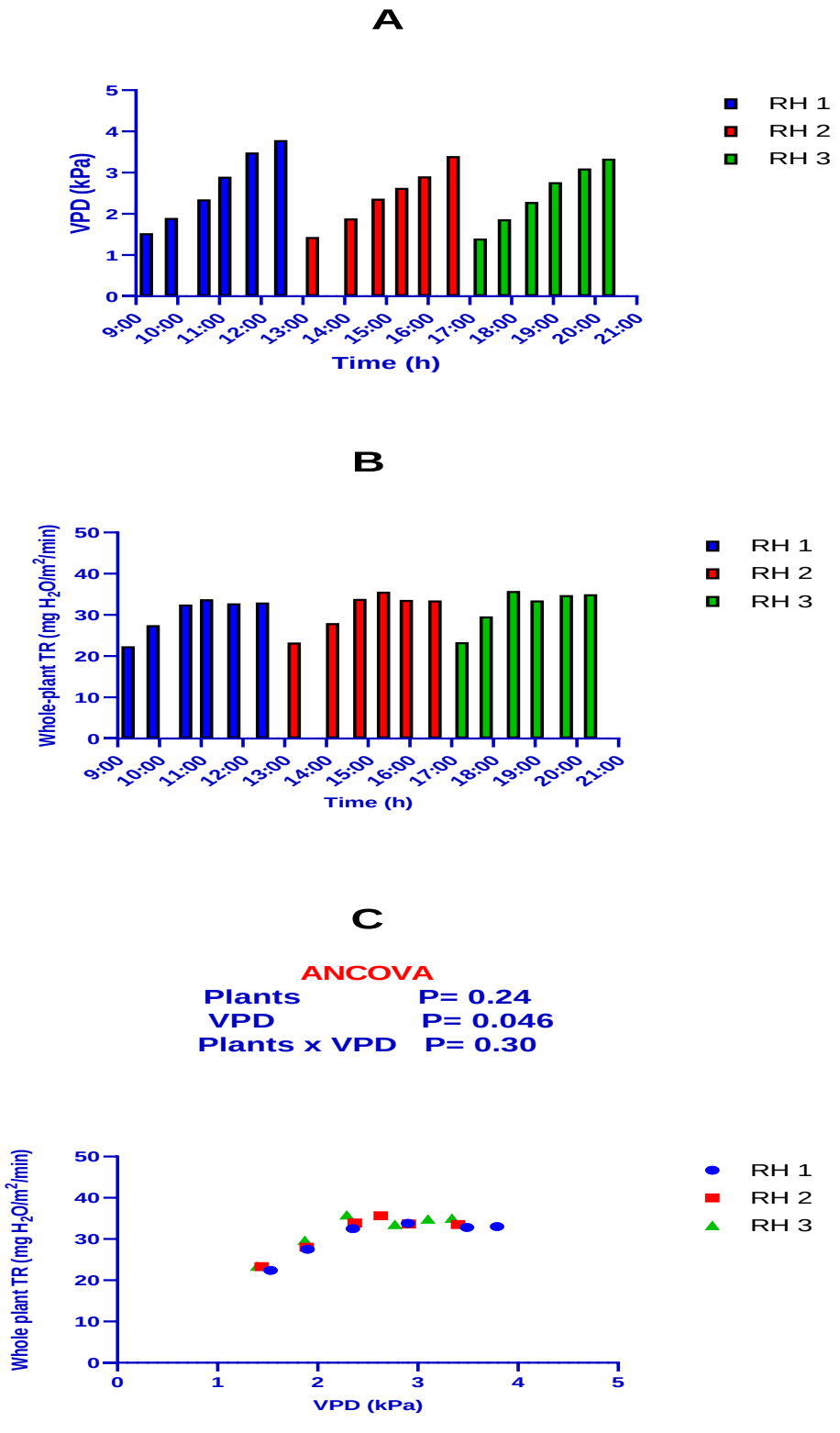


Figure 2.3 Chamber VPD (A) and whole-plant TR (B) fluctuations in the whole-plant gas exchange chamber for three Robin Hood plants over 11 hours (the same 3 plants in Figure 1),

and transpiration response to VPD (C). Each column/point represents an average of 5 minutes for both parameters after 15 minutes of steady-state. P values from ANCOVA for the effects of the plant, VPD, and their interaction are indicated at the top of panel C.

For the three tested systems, segmented regression analysis identified a significant break-point (BP) in both cultivars, which averaged 3.08 ± 0.08 and 2.39 ± 0.06 kPa (means ± SE, n= 3), for Masterpiece and Robin Hood, respectively (Fig. 2.4 & Table 2.1). The initial slope of the TR *versus* VPD relationship (Slope 1), the BP, and the slope above the BP (Slope 2) differed significantly between cultivars across all three measurement approaches. Moreover, the measurement method did not reveal any significant effect on the transpiration response to VPD as indicated by no significant system x VPD interaction (P= 0.31) (Table 2.1). The slope of the linear regression below the BP (Slope 1) averaged 10.08 ± 0.48 mg H_2O/ m^2/min/kPa for Masterpiece, while it was 37 % higher in Robin Hood and averaged 13.79 ± 0.88 mg H_2O/ m^2/min/kPa (means ± SE, n= 3) across the three measurement approaches. The slope of the linear regression above the BP (Slope 2) averaged -1.54 ± 0.9 mg H_2O/ m^2/min/kPa for Masterpiece and it was 5-fold higher in Robin Hood (-0.25 ± 1.5 mg H_2O/ m^2/min/kPa, means ± SE, n= 3) across the three measurement approaches. Transpiration of both cultivars was relatively stable above the BP (averaging 36.5 ± 0.71 mg H_2O/ m^2/min, means ± SE, n= 3) in both cultivars. Across cultivars and systems, break-point values were not correlated with either Slope 1 or Slope 2 (Fig. B-3). Thus, significant genotypic differences in transpiration response to VPD were consistent across the three systems.

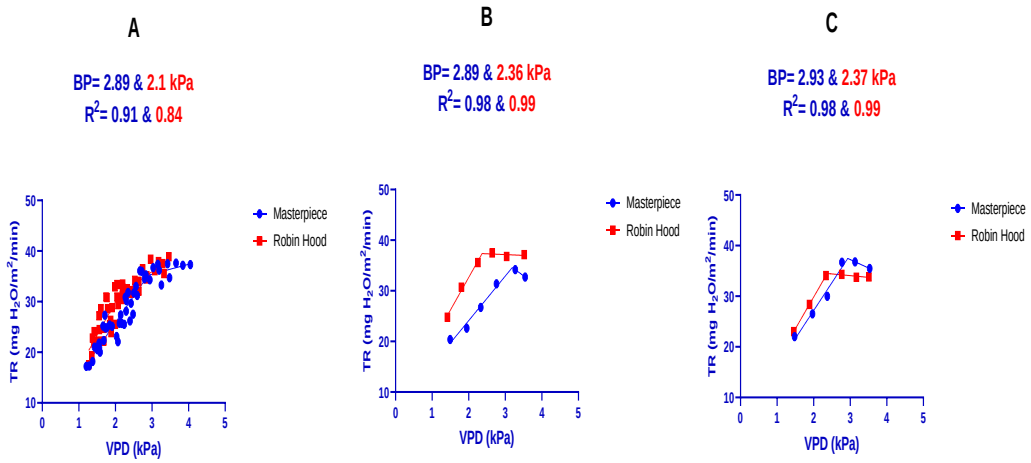


Figure 2.4 Transpiration response of Masterpiece plants and Robin Hood to VPD on a single balance system (A), where each point represents 15 minutes of transpiration during the photoperiod, single leaf cuvette (B), and whole-plant gas exchange chamber (C), where each point represents 5 minutes transpiration rate after 15 minutes of steady-state. Symbols represent the means of three plants with error bars omitted for clarity. Broken-stick regression lines ($P< 0.01$) were fitted in Prism where the slopes of the fitted regression differ significantly. The BP and R^2 values for each cultivar are represented on the top of each panel.

Table 2.1 Regression variables describing the relationships between TR and VPD for Masterpiece and Robin Hood over the three systems, comprising Slopes 1 and 2 and break-point values obtained from data in Figure 2.4. Data reported are means ± SE of the same three plants per cultivar, with different letters within a column indicating significant differences between cultivars. P values from ANCOVA are reported, with significant P values italicised.

System	Genotype	Slope 1	BP	Slope 2	P (ANCOVA)					
					Genotype	VPD	Genotype x VPD	System	VPD	System x VPD
Balance	Masterpiece	10.41 ± 0.63 d	3.08 ± 0.15 a	0.68 ± 1.75 a	*<0.001*	*<0.001*	*0.005*	*<0.001*	*0.003*	0.31
	Robin Hood	16.03 ± 1.67 a	2.43 ± 0.06 b	0.60 ± 3.21 a						
Single leaf cuvette	Masterpiece	8.48 ± 0.76 e	3.16 ± 0.10 a	-1.99 ± 0.64 d	0.06	*0.034*	*0.046*			
	Robin Hood	13.27 ± 1.5 b	2.32 ± 0.04 b	-0.08 ± 0.65 b						
Whole-plant gas	Masterpiece	10.98 ± 0.78 d	2.99 ± 0.06 a	-3.33 ± 1.44 e	*<0.001*	0.97	*0.037*			
exchange chamber	Robin Hood	12.06 ± 0.33 c	2.42 ± 0.06 b	-1.26 ± 0.66 c						

Experiment 2 conducted similar measurements at two times of the year when environmental conditions in the glasshouse differed. Although absolute values differed, ambient VPD revealed the same diurnal pattern in both July and October 2018: lower in the morning increasing to maximum values in the afternoon before declining to low values in the late afternoon and night. Both cultivars showed a segmented linear TR response to high VPD in both months with a BP range of 3.05 ± 0.03 and 2.33 ± 0.08 kPa (means ± SE, n= 3) for Masterpiece and Robin Hood, respectively with genotypic differences as indicated by significant genotypes x VPD interaction (P= 0.041 & 0.037) in July and October, respectively (Fig. 2.5 & Table 2.2). For the two tested periods, BPs of the TR *versus* VPD relationships did not show any significant (P> 0.05) difference within a cultivar, while the first and the second slopes were significantly lower in October than in July in both cultivars. Slope 1 averaged 10.45 ± 0.89 mg H_2O/ m^2/min/kPa for Masterpiece, while it was 31 % higher in Robin Hood and averaged 13.67 ± 1.97 mg H_2O/ m^2/min/kPa across the two times of the year. Slope 2 averaged -1.74 ± 1.13 mg H_2O/ m^2/min/kPa for Masterpiece, while it was 33 % higher in Robin Hood and averaged -1.31 ± 0.68 mg H_2O/ m^2/min/kPa across the two periods. Transpiration of both cultivars was relatively stable above the BP (averaging 33.86 ± 0.41 mg H_2O/ m^2/min). While VPD significantly (P< 0.001) affected TR, the time of the year and the interaction between VPD and the time of the year were not significant (P> 0.05). Thus, whole-plant transpiration response to VPD, when measured gravimetrically with a single balance was reasonably consistent at different times of the year (Table 2.2) although environmental conditions varied in the greenhouse.

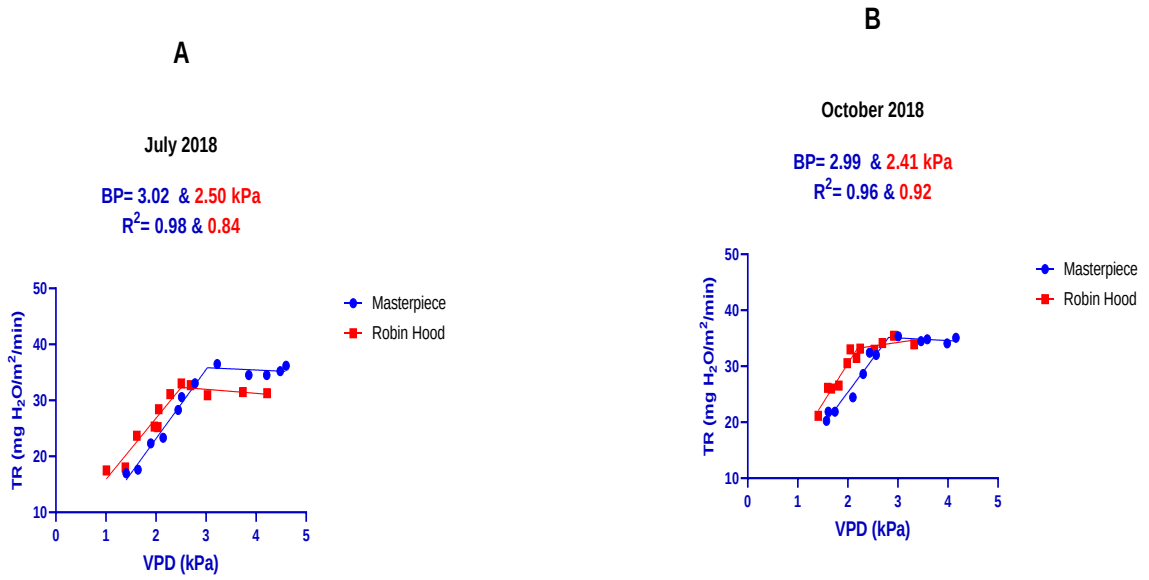


Figure 2.5 Transpiration response of Masterpiece and Robin Hood plants to VPD in July (A) and October (B) 2018, recorded on a balance. Symbols represent the means of three plants for each cultivar, while each point represents an average of 60 minutes recorded by the Hortimax system with error bars omitted for clarity. Broken-stick regression lines ($P< 0.01$) were fitted in Prism where the slopes of the fitted regression differ significantly. BPs and R^2 for both cultivars are indicated at the top of each panel.

In Experiment 3, when three plants were measured per day within the whole-plant gas exchange chamber, with a VPD range of 1.28-3.84 kPa for each plant, again Masterpiece and Robin Hood exhibited a segmented TR model to high VPD with a BP range of 3.01 ± 0.06 and 2.34 ± 0.04 kPa for Masterpiece and Robin Hood, respectively with genotypic differences as indicated by significant genotypes x VPD interaction ($P= 0.033$) (Fig. 2.6 & Table 2.3). In comparing the three times of day, BP values of the TR *versus* VPD relationships did not show any significant ($P> 0.05$) difference within a cultivar, while there was limited cultivar variation in the slopes. Morning measurements of Slope 1 below the BP and Slope 2 above the BPD

were 10 % and 30 % and higher in Masterpiece and Robin Hood, respectively than afternoon and late afternoon measurements, which had similar slopes. Similarly, Slope 2 of morning measurements was ~ 5-fold higher than afternoon and late afternoon measurements in both cultivars. Slope 1 averaged 9.77 ± 0.43 mg H_2O/ m^2/min/kPa for Masterpiece, while it was 20 % higher in Robin Hood and averaged 11.39 ± 1 mg H_2O/ m^2/min/kPa across the three times of the day. Slope 2 averaged -1.55 ± 0.79 and 0.02 ± 0.23 mg H_2O/ m^2/min/kPa for Masterpiece and Robin Hood, respectively across the three periods. Transpiration of both cultivars was relatively stable above the BP (averaging 30.12 ± 0.47 mg H_2O/ m^2/min). While VPD significantly ($P< 0.001$) affected TR, the time of the day and the interaction between VPD and the time of the year were not significant ($P> 0.05$), (Table 2.3). Thus, whole-plant transpiration response to VPD, when measured in the whole-plant gas exchange chamber was reasonably consistent at different times of the day.

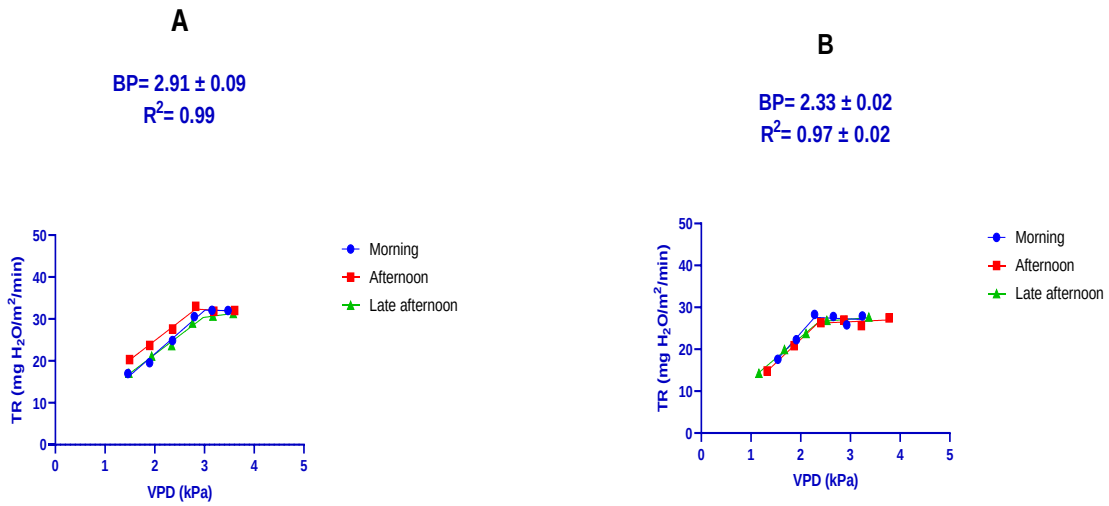


Figure 2.6 Whole-plant transpiration rate response to VPD of Masterpiece (A) and Robin Hood (B) at different times of the day in the whole-plant gas exchange chamber. Transpiration was measured for 5 minutes at each VPD, with symbols representing the combined response of three plants, with error bars omitted for clarity. Broken-stick regression lines ($P< 0.01$) were fitted in Prism where the slopes of the fitted regression differ significantly.

Table 2.2 Regression variables describing the relationships between transpiration and VPD for Masterpiece and Robin Hood grown at different times of the year in Experiment 2, comprising Slopes 1, 2 and break-point values obtained from data in Figure 2.5. Data reported are means ± SE of three replicates, with different letters within a column indicating significant differences between cultivars with P-value from ANCOVA reported. Significant P values italicised.

Genotype	**Year time**	**Slope 1**	**BP**	**Slope 2**	**P (ANCOVA)**					
					Genotype	**VPD**	**Genotype x VPD**	**Year time**	**VPD**	**Year time x VPD**
Masterpiece	July	12.0 ± 0.57 b	3.03 ± 0.03 a	0.07 ± 1.01 a	*0.004*	*0.039*	0.041	0.07	*0.001*	0.36
	October	8.90 ± 1.11 c	3.06 ± 0.07 a	-3.56 ± 1.45 d	*0.011*	*0.028*	0.037			
Robin Hood	July	14.33 ± 4.33 a	2.30 ± 0.17 b	-0.07 ± 0.61 b				0.95	*0.045*	0.74
	October	13 ± 0.57 b	2.36 ± 0.03 b	-2.56 ± 0.61 c						

Table 2.3 Regression variables describing the relationships between transpiration and VPD for Masterpiece and Robin Hood over different times of the day in Experiment 3, comprising Slopes 1, 2, and break-point values obtained from data in Figure 2.6. Data reported are means ± SE of 3 replicates, with different letters within a column indicating significant differences Within a cultivar, P-value from ANCOVA is reported. Significant P values italicised.

Genotype	Day time	Slope 1	BP	Slope 2	P (ANCOVA)					
					Genotype	VPD	Genotype x VPD	Day time	VPD	Day time x VPD
Masterpiece	Morning	10.41 ± 1.3 b	3.02 ± 0.11 a	-0.49 ± 0.69 c	*0.047*	*<0.001*	*0.033*	0.13	*0.023*	0.36
	Afternoon	9.41 ± 0.22 c	2.94 ± 0.03 a	-2.09 ± 0.87 d						
	Late afternoon	9.50 ± 0.78 c	3.08 ± 0.1 a	-2.08 ± 2.33 d						
Robin Hood	Morning	12.80 ± 2.15 a	2.37 ± 0.09 b	0.44 ± 0.88 a				0.34	*<0.001*	0.29
	Afternoon	10.99 ± 2.1 b	2.45 ± 0.05 b	-0.12 ± 0.42 b						
	Late afternoon	10.38 ± 0.45 b	2.39 ± 0.04 b	-0.27 ± 0.03 b						

2.4 Discussion

While previous work with other legume species identified genetic variation in transpiration response to VPD, this work appears to be the first such report in faba bean. This variation was consistently detected using different methodologies (gravimetric measurements and infra-red gas analysis) in both leaves and whole plants. While gravimetric phenotyping of transpiration response to VPD seems appropriate to screen many cultivars in a short period (100 plants/week) (Ryan et al., 2016), co-variation of light and VPD in the greenhouse made it difficult to discriminate the cause of these responses. Thus, subsequent measurements (with the same plants) regulated VPD within a leaf cuvette or whole-plant chamber while maintaining a constant light intensity using infra-red gas analysis.

Consistent cultivar variation in transpiration responses to VPD over the three measurement approaches under both constant (infra-red gas analysis) and fluctuating (gravimetric measurements) light conditions supports the possibility that those responses also occur in fluctuating field conditions as in soybean (Gilbert et al*.,* 2011). Unlike the usual model of transpiration rate increases linearly with increasing VPD (Sinclair and Bennett, 1998), both faba bean cultivars restricted or maintained transpiration as VPD increased (Fig. 2.4) resulting in a break-point (BP) of transpiration. Most of the BP values reported for both cultivars (2.32-3.08 kPa) are within the range previously reported in other legumes including soybean (Sadok and Sinclair, 2009 a & b), peanut (Devi et al., 2010), chickpea (Zaman-Allah et al., 2011 a), and cowpea (Belko et al., 2012), which varied from 1.1 to 2.92 kPa as identified in both controlled environments and field conditions. Variation in BP between cultivars may allow genotypes to be developed with specific BP for differing water-deficit environments. This genetic variation

suggests that much larger variability could be expected in bigger populations as well as complex inheritance of this trait, which suggests more than one controlling mechanism. Restricted transpiration only under higher atmospheric VPD is considered an effective physiological strategy to avoid water deficits. Although both cultivars are grown in the UK, this trait may be particularly useful for breeding faba bean cultivars specifically targeted to environments with high VPD and low water availability.

Statistical analysis confirmed that a double segmented model best represented the transpiration response to VPD in both cultivars, with the two slopes (either side of a break-point value) significantly differing (Table 2.1). These responses were stable across different times of the day in plants grown at different times of the year (Fig. 2.5 & 2.6, Table 2.2 & 2.3), suggesting these traits can be measured whenever VPDs are sufficiently high. Although gravimetric whole-plant transpiration measurements in the glasshouse spanned a 24 h period with typical diurnal variation in VPD, with no variation between morning and afternoon measurements (Fig. 2.1 F). This lack of hysteresis in the transpiration response of VPD suggests that transiently high VPD did not result in sufficient soil drying to impose an additional restriction on transpiration since plants were well-watered before the measurements.

As the two cultivars showed consistent responses over the three systems, using a gravimetric phenotyping platform system for measuring transpiration response to VPD seems the most appropriate when screening a large population. Its ability to phenotype transpiration response to VPD in many cultivars in a short period (100 plants/week) (Ryan et al., 2016) can increase replication if sufficiently high VPDs can be achieved in the glasshouse, which may be restricted to the summer months. Although the individual leaf and whole-plant infra-red gas

analyses offer greater control in establishing precise VPDs, each plant needs 3-4 hours to be measured, limiting throughput to 15-18 plants/week. Thus, the choice of phenotyping system will be determined by the size and genetic diversity of the populations to be measured. Since this is the first report of limited transpiration response to VPD in faba bean, further experiments are needed to determine the possible regulatory physiological mechanisms.

Various investigators have provided physiological explanations for the mathematical description of the break-point response of transpiration to VPD (Sadok and Sinclair 2009 a). The initial increase in TR response to VPD (Slope 1) was interpreted as the maximum rate of stomatal opening in response to elevated VPD, while the later stabilized/declined Slope 2 reflects a decrease in stomatal conductance to match transpiration rate with the hydraulic conductance of the plant. Higher Slope 1 values for Robin Hood than Masterpiece (Table 2.1 & 2.2) indicates that Robin Hood plants can transport water at higher rates at low VPD to reach their maximum TR more rapidly as VPD increases (Sinclair et al., 2010). Since this hypothesis is based on differences in plant hydraulics, measuring the hydraulic conductance of these cultivars is necessary to determine possible regulation of plant water fluxes. While some studies have emphasized the importance of leaf hydraulic conductance in restricting transpiration at high VPD in soybean (Sinclair et al., 2008), others have suggested the roots represent the major limitation to sustaining transpiration at high VPD (Sivasakthi et al., 2020) in chickpea. Also, differential ABA accumulation and variation in hydraulic conductance at the leaf and/or root levels (Parent et al*.,* 2009; Mahdid et al., 2011) might determine transpiration response to VPD. Conservation of the transpiration response to VPD in individual leaves and whole plants (Fig. 2.2 & 2.3) provides no further insights as all plants measured were intact, with limitations possible in all roots, stems, and leaves. In this regard, whole-plant hydraulic

conductance and ABA concentrations were measured in both faba bean cultivars in Chapter 3, assuming that restrictions in hydraulic conductance at high VPDs caused leaf water deficits and stimulated ABA accumulation, thus limiting transpiration.

2.5 Conclusion

As in other species, faba bean shows genetic variation in transpiration response to evaporative demand. Although both cultivars restricted transpiration at high VPD, the significantly lower BP values of cv. Robin Hood suggests more water-conservative behaviour. Whether these differences translate into better drought tolerance requires further work, that is timely since lower faba bean yields in the UK have been associated with drier summers.

Chapter 3: Low hydraulic conductance & ABA accumulation restrict transpiration under high VPD in faba bean

3.1 Introduction

For plants to replace transpirational losses, the soil needs to continuously supply water to the roots, otherwise plant transpiration will be restricted under unfavourable conditions such as high vapour pressure deficit (VPD). Despite many reports on transpiration response to VPD in several crop species, the mechanism of stomatal closure under high VPD remains largely un-understood (Damour et al., 2010). Currently, there is evidence supporting the idea that plant TR response to VPD involves an interaction between 'local' (i.e., leaf-based) and 'non-local' (root-based) mechanisms mobilizing long-distance hydraulic signals (Vadez, 2014; Vandeleur et al., 2014; Maurel et al., 2016; Sivasakthi et al., 2017).

The positive relationship between maximum transpiration rate (TR) and maximum whole-plant hydraulic conductance (K_{plant}) (Tsuda and Tyree, 2000) suggests that hydraulic conductance of different plant organs such as leaves (Sadok and Sinclair, 2010) and roots (Sinclair et al., 2014; Sivasakthi et al., 2020) can constrain transpiration at high VPDs, but 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 (*Glycine max* (L.) Merr.), (Sadok and Sinclair, 2010) and sorghum (*Sorghum bicolor* L.) (Choudhary et al., 2013), while limited root hydraulic conductance restricted TR at high VPD in chickpea (*Cicer arietinum* L.) (Sivasakthi et al., 2020). In maize (*Zea mays* L.), limited TR at high VPD results from limitations in both leaf and root hydraulic conductance (Choudhary et al., 2014). Thus, plant hydraulic conductance seems to play a vital role in stomatal response to changes in VPD (Sperry et al., 2002).

Other investigations highlighted the role of abscisic acid (ABA) in coordinating hydraulic conductance and transpiration responses to VPD. To explain the diurnal movements of stomata that many plants show in temperate or dry conditions, ABA production has been proposed to follow a diurnal pattern as well (Tallman, 2004). In this model, (i) endogenous guard cell ABA declines in the morning; (ii) root-sourced ABA is transferred to the guard cell apoplast via the transpiration stream at midday, and (iii) ABA increases in the guard cells at night. In tobacco, light/dark transition induced leaf ABA accumulation to reach its maximum after 3 h of dark initiation (Nováková et al., 2005). In Arabidopsis, high VPD rapidly (within 20 minutes) triggers leaf ABA synthesis (McAdam et al., 2016) which elicits stomatal closure (Kholova et al., 2010; Bauer et al., 2013 a). Subsequent export of some of this ABA to the roots increased root hydraulic conductance (Kudoyarova et al., 2011; Veselov et al., 2018), thereby helping to maintain leaf hydration under high VPD.

In *Arabidopsis thaliana*, exogenous ABA application decreased stomatal conductance and down-regulated leaf hydraulic conductance in both wild-type and ABA-insensitive mutants (Pantin et al., 2013). They proposed that ABA has a dual effect in promoting stomatal closure via its well-known biochemical (direct) effect on the guard cell as well as its indirect effect on leaf hydraulic conductance via decreasing water permeability within leaf vascular tissues. Moreover, guard cells can autonomously produce ABA and close stomata in response to elevated VPD (Bauer et al., 2013 a). However, ABA-deficient mutants did not show appreciable stomatal closure in response to elevated VPD (McAdam et al., 2016; Jauregui et al., 2018). Thus, whether or not ABA participates in the direct stomatal closure under high VPD is still controversial since studies with *Arabidopsis thaliana* ABA-deficient and/or insensitive mutants have not provided consistent information on the involvement of ABA in

causing stomatal closure in response to high VPD (Assmann et al., 2000; Xie et al., 2006; Merilo et al., 2018).

Stomatal sensitivity to ABA depends on current leaf water status. Greater stomatal sensitivity to xylem ABA concentration in the afternoon in maize was associated with lower leaf water potential (Ψ_{leaf}). Greater stomatal sensitivity to ABA in the afternoon could be due to the increased VPD at that time or related to the increased ABA delivery from the xylem stream to the stomata. If xylem sap ABA concentration is constant, increased transpiration rates at higher VPDs later in the day would enhance ABA flux into the leaf (Tardieu and Davies, 1992) However, genetic variation in transpiration response to VPD has not always been associated with ABA accumulation. In peanut, genetic variation in TR to VPD was associated with lower leaf hydraulic conductance resulting from a lower population of water channel proteins (AQPs) that are critical for transport between cells (Shekoofa et al., 2013).

Most studies use the evaporative flux method (Cochard et al., 1996) to determine whole-plant hydraulic conductance (K_{plant}) and its components i.e., root and stem hydraulic conductance, K_{root} and K_{stem}, respectively. Transpiration rate is divided by the water potential gradient, thus $K = TR / \Delta\Psi$, where K is hydraulic conductance, TR is plant transpiration rate when plant water potential is determined and $\Delta\Psi$ is the difference in water potential between the two considered points (e.g., between soil water potential and leaf water potential) (Tsuda and Tyree, 2000). Alternatively, root hydraulic conductance can be measured by pressurizing de-topped roots in the pressure chamber (Jackson et al., 1996).

Statement of Research Objectives

Understanding how plants coordinate hydraulic conductance and stomatal regulation in response to fluctuating VPD might help breeders select genotypes that are more suited to specific environments. However, to the best of our knowledge, no studies in faba bean have studied the possible physiological mechanisms underlying plant transpiration phenotypes under high VPD. Thus, this work aims to understand why Masterpiece and Robin Hood faba bean genotypes vary in their TR response to VPD (Chapter 2). Whole-plant, root and stem hydraulic conductance were determined at different VPD levels. Furthermore, leaf, root, leaf xylem sap and root xylem sap ABA levels were measured after plants were placed in a whole-plant gas exchange chamber (Jauregui et al., 2018) which tightly controlled VPD around the shoot. It was hypothesised that limited root or stem hydraulic conductance and/or ABA accumulation restricted TR under high VPD.

3.2 Material and Methods

3.2.1 Growth conditions and plant material

Commonly grown broad bean *(Vicia faba* cvs. Masterpiece and Robin Hood) cultivars were used in multiple experiments that measured transpiration, leaf water potential, hydraulic conductance and ABA accumulation under a range of VPDs (Table C-1). Seeds of the two faba bean cultivars were planted as described in Section 2.1 under the glasshouse conditions in January 2019. Supplementary lighting (high-pressure sodium lamps, Osram Plantastar 600 W, Munich, Germany) maintained the photoperiod at 12 hours (08:00-20:00 h.) providing 588 ± 9 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (mean ± SE, n= 336, comprising 12 h x 28 days) after sunset. Actual air temperature and relative humidity in the centre of the

glasshouse were recorded with a Hortimax system (HortiMax Ektron III, hortisystems.co.uk). Day/night temperature ranges were 24.70 ± 0.28°C and 19.9 ± 0.18°C (mean ± SE, n= 336), respectively. Relative humidity day/night ranges were 34 ± 1 % and 42 ± 1 % (mean ± SE, n= 336), respectively. Homogenous plants with 6-7 fully expanded leaves (leaf area = 448 ± 12 & 401 ± 4 cm^2 for cv. Robin Hood and cv. Masterpiece, respectively, mean ± SE, n= 15, P> 0.05) were assigned to measure hydraulic conductance response to VPD using the whole-plant gas exchange chamber (Table C-1).

3.2.2 Measuring whole-plant hydraulic conductance and its components

In Experiment 1, Masterpiece and Robin Hood faba bean plants were grown as described in Section 3.2.1 for four weeks. On the measuring day, the plant was watered to maximum pot drained capacity at 08:30 to ensure negligible soil water potential values. Afterwards, one leaf per plant (normally the first fully expanded one) was covered with aluminium foil to estimate stem water potential (Ψ_{stem}) by measuring its water potential. The plant was then inserted into the whole-plant gas exchange chamber to measure its transpiration at the targeted VPD as described in Section 2.2.6. Measurements were done over six consecutive days between 9:30 h and 19:30 h under three VPD levels i.e., 1.39, 2.42, 3.57 kPa with five individual plants measured at each VPD, so ultimately each genotype was represented by 15 plants over the applied VPD range (Table C-1).

After inserting the plant into the chamber, it was sealed with eight metal clips, and then RH was adjusted to generate the desired VPD. It usually takes 30-45 min for CO_2 and H_2O to reach a steady-state. Once CO_2 and H_2O exchange were steady for at least 5 min (steady-state),

averaged values were logged every minute for 5 minutes. The xylem pressure potentials of the aluminium foil-enclosed (Ψ_{stem}) and transpiring leaf (Ψ_{leaf}) (15-20 % of total leaf area) across both leaves were determined in a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). The leaf was excised from the plant and directly sealed into the pressure chamber. The pressure was gradually increased until water appeared on the surface of the midrib, and this was taken as the water potential. After measuring whole-plant gas exchange at the targeted VPD, Ψ_{leaf} and Ψ_{stem}, the plant was taken out of the chamber and harvested to determine its leaf area using a leaf area meter (Model LI-3100C, LI-COR, Lincoln, NE, USA). To accurately determine transpiration rate per unit leaf area, the area of the aluminium foil-enclosed leaf (used to measure Ψ_{stem}) was excluded from the total leaf area, while the area of the leaf was used to measure Ψ_{leaf} was included in the whole-plant leaf area. At each target VPD, this process was repeated to ensure five replicate plants of each genotype. Whole- plant, root and stem hydraulic conductance (mg $H_2O/m^2/min/MPa$) were measured at each VPD by dividing transpiration rate (TR) by the $\Psi_{gradient}$ as described by Tsuda and Tyree, 2000. as follows:

$K_{plant} = TR/ (\Psi_{soil}–\Psi_{leaf})$

$K_{root} = TR/ (\Psi_{soil}–\Psi_{stem})$

$K_{stem} = TR/ (\Psi_{stem}–\Psi_{leaf})$

Since the plants were well watered, Ψ_{soil} was considered to equal zero.

3.2.3 Measuring root hydraulic conductance by pressurizing the roots

In Experiment 2, pre-germinated seeds were planted in the same substrate as described in Section 3.2.1, but in one-litre cylindrical pots (20 cm high × 9 cm diameter) that snugly fit in the pressure chamber. Plants were grown for four weeks, with homogenous plants with 6-7 fully expanded leaves (leaf area= 374 ± 12 & 360 ± 8 cm^2 for cv. Robin Hood and cv. Masterpiece, respectively, mean ± SE, n= 4, P> 0.05) assigned to measure hydraulic conductance with both the evaporative flux and root pressurization methods (Table C-1). Immediately after measuring hydraulic conductance with the evaporative flux method as described in Section 3.2.2 at the highest VPD, the chamber can achieve (3.6 ± 0.02 kPa), root hydraulic conductance was measured in the same plants according to methods previously described (Siemens and Zwiazek, 2004). Root hydraulic conductance was measured using a Scholander-type pressure chamber after measuring the xylem pressure potentials of both the aluminium foil-enclosed leaf (Ψ_{stem}) and transpiring leaves (Ψ_{leaf}). Then the whole pot was taken out of the whole-plant gas exchange chamber and inserted into the pressure chamber. The stem was cut ~2.5 cm above the root collar and pneumatic pressure was applied in 0.1 MPa intervals, where 0.45 ± 0.03 MPa (n= 8) was the first pressure that allowed xylem sap to exude from the cut surface. Sap exudation rate was estimated by attaching water-absorbing paper (within a pre-weighed Eppendorf) to the cut surface of the root system for a minute. The root exudate was then weighed quickly on a 4-point electronic balance. Roots were gradually pressurized, the flow rate was allowed to stabilize for 1 min, and the steady-state flow rate was measured for another 1 min. These experiments occurred over two consecutive days with 4 plants measured per day from around 10:30 to 17:00 h. Root hydraulic conductance, K_{root} (mg $H_2O/m^2/min/MPa$) was calculated as the slope of the regression line

of the flow rate (F) plotted against hydrostatic pressures according to the following equation: (K_{root} =F/P), (Miyamoto et al., 2001), with the slopes normalized according to leaf area (Toca et al., 2020).

3.2.4 ABA response to different VPD levels

In Experiment 3, pre-germinated seeds were planted in the same substrate as described in Section 3.2.1 also in one-litre cylindrical pots (20 cm high × 9 cm diameter) that snugly fit in the pressure chamber. Plants were grown for four weeks with homogenous plants with 6-7 fully expanded leaves (leaf area = 445 ± 5 & 428 ± 7 cm^2 for cv. Robin Hood and cv. Masterpiece, respectively, mean ± SE, n= 4, P> 0.05) assigned to ABA sampling along with TR and hydraulic conductance (Table C-1). TR was measured in the whole-plant gas exchange chamber as described in Section 2.4 under three VPD levels i.e., 1.42, 2.42, and 3.55 kPa with five plants measured at each VPD, so ultimately each genotype was represented by 15 plants over the applied VPD range. After measuring whole-plant gas exchange response at the targeted VPD, the chamber was opened and a tissue sample of about 4 cm^2 of young, developing leaf (part of a single leaflet) was taken, directly placed in a pre-weighed Eppendorf and kept in liquid nitrogen until storage at -20°C. This 4 cm^2 was later excluded from the total leaf area to ensure accurate TR values at the targeted VPD. Afterwards, the xylem pressure potentials of the aluminium foil-covered leaf (Ψ_{stem}) and transpiring leaf (Ψ_{leaf}), which was also sampled for ABA analysis, were determined at the targeted VPD in a Scholander-type pressure chamber as described in Section 3.2.2 to estimate K_{plant}, K_{root}, and

K_{stem}. After measuring Ψ_{leaf}, the pressure was increased by ~ 0.1-0.2 MPa and leaf xylem sap was collected using a micropipette until sufficient volume (< 50 µl) for the ABA assay was achieved. The pot was then removed from the whole-plant gas exchange chamber and inserted into the pressure chamber. The stem was cut ~ 2.5 cm above the root collar and the pressure was increased until xylem sap exuded from the cut surface. Root xylem sap was also collected for about five minutes. All sap samples were directly placed in liquid nitrogen until storage at -20°C.

Then the root system was removed from the pot and gently washed under tap water for about five minutes to remove the adhering substrate, then a fresh mass of ~ 1 g roughly from the middle of the root system was placed in a pre-weighed Eppendorf and kept in liquid nitrogen until storage at -20°C. Leaf area was measured using a leaf area meter (LI-3100C Area Meter, LI-COR, Lincoln, NE, USA).

All tissue samples were freeze-dried, chopped to a fine powder with scissors, extracted in de-ionized (DI) water at a ratio of 1:25 (leaf tissue[µg]: water [µl]), shaken overnight at 5°C and thereafter kept frozen at -20°C until measured, while sap samples were directly used in the assay. Samples were centrifuged and the supernatant removed for analysis by radioimmunoassay as described by Quarrie et al., (1988).

3.2.5 Statistical Analysis

ANCOVA (for main effects of genotypes, VPD, and their interaction in all studied variables were carried out separately on each measurement occasion with SPSS 27.0 for Windows statistical software package (SPSS, Inc., Cary, NC). Regression coefficients were carried out using the linear regression model of GraphPad Prism 9.3.1 (GraphPad Software Inc., San

Diego, CA, 2007). Significant (P< 0.05) genotypic differences in all studied characters were discriminated against using Student's T-test.

3.3 Results

3.3.1 Hydraulic conductance measured with the evaporative flux method (EF)

Since transpiration depends on hydraulic flow from the site of water uptake (root surfaces) to the site of water loss in the leaves, measuring hydraulic conductance was essential to determine whether it restricted maximum transpiration at high VPD. Whole-plant hydraulic conductance (K_{plant}) was independent of the time of the day it was measured, as there were no significant differences between Masterpiece K_{plant} measured between afternoon (12:45) and late-afternoon (16:45-19:30) and that of Robin Hood measured in the morning (10:30 & 10:45) and afternoon (14:30 & 15:00) (Fig. C-1).

The cultivars significantly differed in their TR response to VPD, as indicated by a significant genotype x VPD interaction (P= 0.023). At the two first VPDs (1.39 & 2.42 kPa), Robin Hood TR was 22 % and 14 % higher than Masterpiece TR. As VPD increased from 1.39 to 2.42 kPa, TR increased by 37 % and 28 % in Masterpiece and Robin Hood, respectively. Whereas Robin Hood TR declined by 14 % at the highest VPD (3.57 kPa), Masterpiece TR was stable at the two highest VPDs (Fig. 3.1 A).

Stem water potential (Ψ_{stem}) was always higher than Ψ_{leaf} by 11-34 % and 15-24 % in Masterpiece and Robin Hood, respectively across the applied VP with no significant difference

between both cultivars (Fig. 3.1 C). Overall, Ψ_{leaf} and Ψ_{stem} responded similarly to the VPD changes in both cultivars (no significant genotype x VPD interaction – Fig. 3.1 B & C).

At the two lowest VPDs, leaf water potential (Ψ_{leaf}) of Masterpiece was significantly lower by 5-12 % than Robin Hood, while both cultivars displayed similar Ψ_{leaf} at the highest VPD. Ψ_{leaf} declined by 22 % and 14 % in Masterpiece and Robin Hood, respectively as VPD increased from 1.39 to 2.42 kPa. While Robin Hood Ψ_{leaf} further decreased by 15 % at the highest VPD (3.57 kPa), Masterpiece Ψ_{leaf} revealed stability with no significant difference between both cultivars. Ψ_{leaf} of both genotypes changed similarly as the VPD increased (no significant genotype x VPD interaction, P= 0.11) (Fig. 3.1 B).

Hydraulic conductance significantly differed between genotypes at each VPD, with Masterpiece lower than Robin Hood by ~ 30 % at the two first VPDs, but 7 % higher at the final VPD. As VPD increased from 1.39 to 2.42 kPa, K_{Plant} increased by 14 and 12 % in Masterpiece and Robin Hood, respectively. In contrast, it stabilized and declined by 25 % as VPD increased from 2.42 to 3.57 kPa in Masterpiece and Robin Hood, respectively. The cultivars differed in their K_{plant} response to VPD, as indicated by a significant genotype x VPD interaction (P< 0.001) (Fig. 3.1 D). Thus, Masterpiece better regulated its leaf water status across the applied VPD associated with its higher TR and K_{Plant} at the highest VPD than Robin Hood.

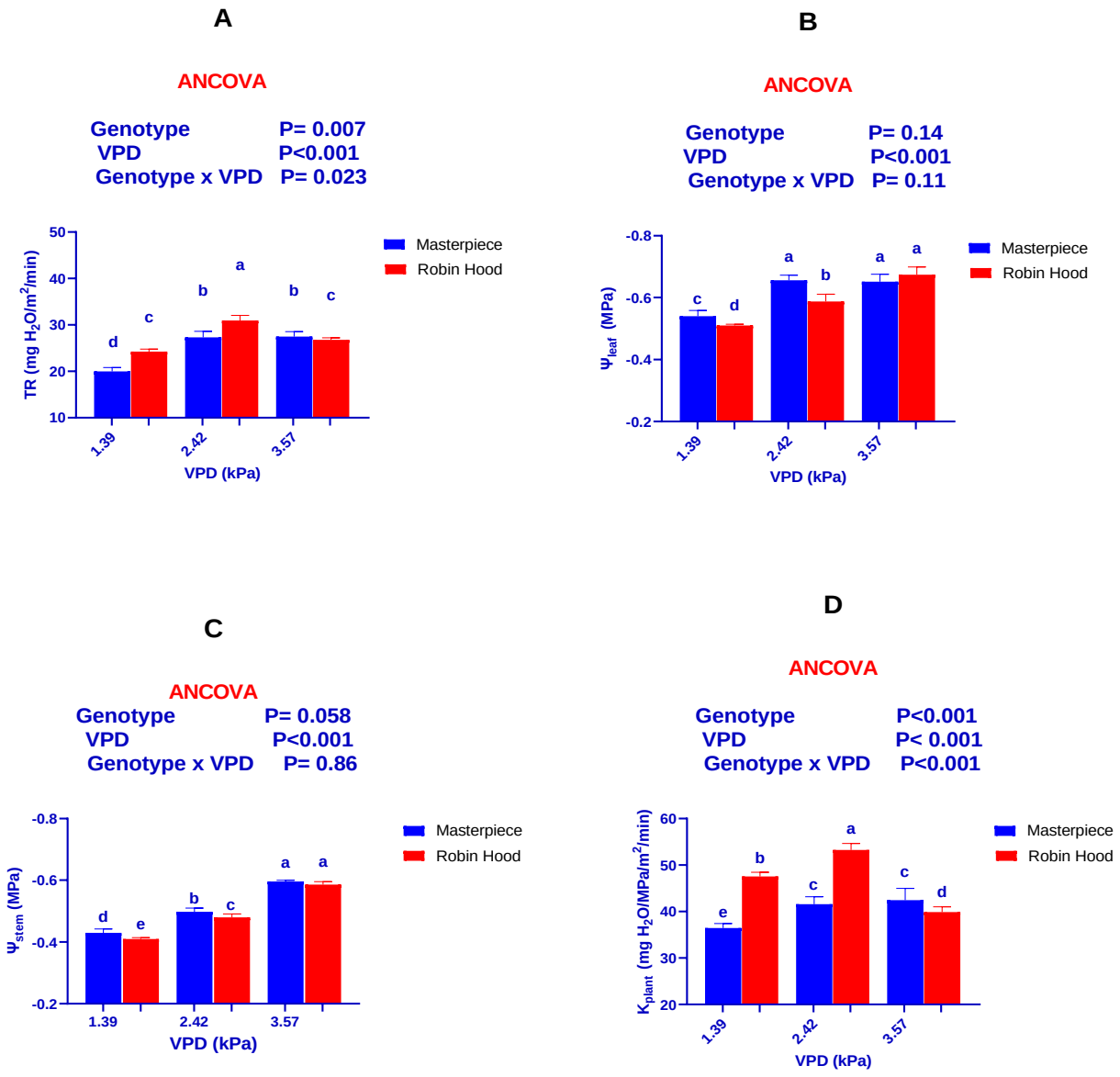


Figure 3.1 TR (A), Ψ_{leaf} (B), Ψ_{stem} (C), and K_{plant} (D) of Masterpiece and Robin Hood plants at different VPD levels in Experiment 1. Data are means ± SE of five replicates with different letters above the bars indicating significant ($P< 0.05$) differences according to T-test. P values from ANCOVA are indicated above each panel.

While Ψ_{leaf} decreased as transpiration rate increased in Masterpiece, (P= 0.007), there was no significant relationship in Robin Hood (P= 0.16) (Fig. 3.2 A). Whereas K_{plant} significantly (P< 0.001) increased similarly in both cultivars as TR increased (Fig. 3.2 B), it was unrelated (P= 0.31 & 0.15) to changes in Ψ_{leaf} (Fig. 3.2 C). Thus, variation in K_{plant} was better explained by variation in transpiration rate than variation in Ψ_{leaf}.

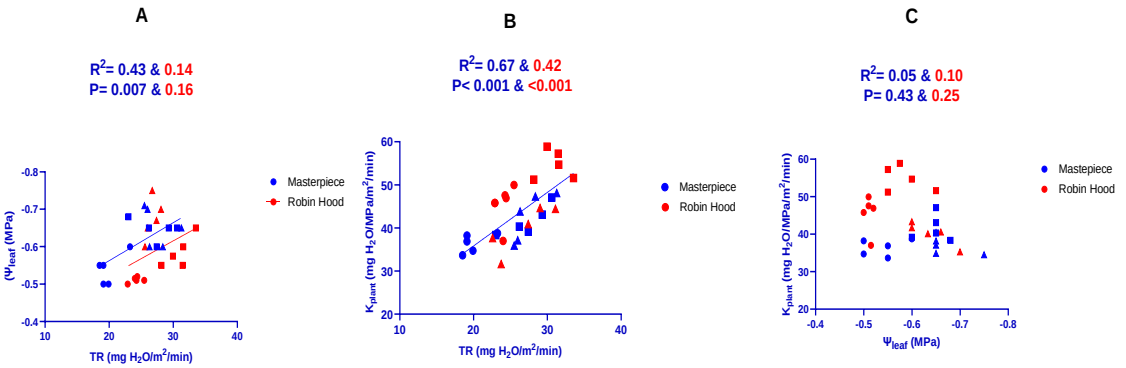


Figure 3.2 Relationships between Ψ_{leaf} & TR (A), K_{plant} & TR (B), and K_{plant} & Ψ_{leaf} (C) of Masterpiece and Robin Hood in Experiment 1. Points are individual plants measured at the lowest (●), intermediate (■) and the highest VPD (▲). P and R^2 values are indicated above each panel.

To determine where hydraulic conductance is limited, Experiment 1 measured root and stem hydraulic conductance at different VPDs. In both genotypes, stem hydraulic conductance (K_{stem}) was significantly higher than root hydraulic conductance (K_{root}) by about 3-fold (Fig. 3.3 A & B). Both K_{stem} and K_{root} had their maximal values at the intermediate VPD (2.42 kPa) before either declining or slightly increasing at the highest VPD. K_{stem} of Robin Hood was 22 % higher than that of Masterpiece at the lowest VPD, while it was 8 % lower at the

intermediate and the highest VPD with a genotypic difference at each VPD (Fig. 3.3 A). While K_{stem} increased by 37 % and 6 % as VPD increased from 1.39 to 2.42 kPa in Masterpiece and Robin Hood, respectively, both cultivars revealed stability in K_{stem} as VPD increased from 2.42 to 3.57 kPa. In contrast, Robin Hood K_{root} was 33 % and 39 % higher than that of Masterpiece at the lowest and the intermediate VPD, respectively, while it was 4 % lower than Masterpiece at the highest VPD, with a genotypic difference at each VPD (Fig. 3.3 B). As VPD increased from 1.39 to 2.42 kPa, K_{root} increased by 10 % and 15 % in Masterpiece and Robin Hood, respectively. Whereas Robin Hood K_{root} declined by 41 % as VPD increased from 2.42 to 3.57 kPa, Masterpiece K_{root} revealed stability. At the highest VPD, K_{root} of Masterpiece was ~ 5 % higher than K_{root} of Robin Hood. Both cultivars revealed high genotypic differences in K_{stem} and K_{root} as indicated by significant genotype x VPD interactions ($P= 0.036$ & <0.001 for K_{stem} and K_{root} respectively). Taken together, both cultivars revealed similar responses in K_{plant} and its components at the lowest VPD, while K_{root} better explains variation in K_{plant} at the two highest VPDs than K_{stem}.

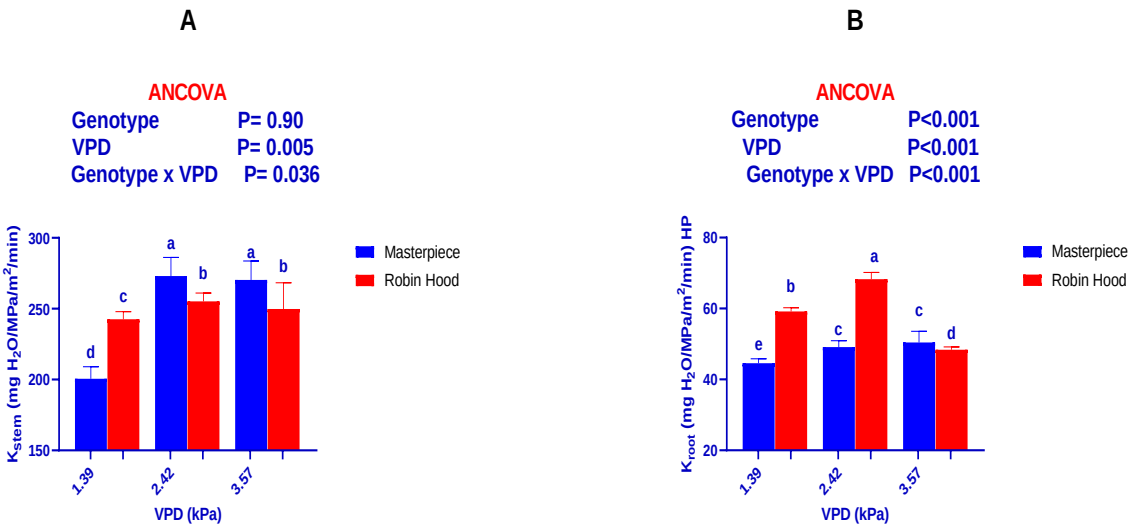


Figure 3.3 K_{stem} (A) and K_{root} (B) of Masterpiece and Robin Hood plants at different VPD levels in Experiment 1. Data are means ± SE of five replicates with different letters above the bars indicating significant (P< 0.05) differences according to the T-test. P values from ANCOVA are indicated above each panel.

3.3.2 Root hydraulic conductance by root pressurization (RP)

To corroborate these genotypic differences and independently verify K_{root} values using a different measurement technique, Experiment 2 determined K_{root} of the same plants using both the evaporative flux (EF) method and root pressurization technique (RP). In both genotypes, increasing the pressure applied to de-topped root systems increased the sap flow rate, with the slope of this relationship equalling K_{root}. At any specific pressure, sap flow rate was higher in Masterpiece than in Robin Hood (Fig. 3.4 A), and these differences diverged as the pressure increased, as indicated by a significant genotype x pressure interaction (P= 0.038). Irrespective of the measurement method, Masterpiece K_{root} values were always ~ 20

% higher than those of Robin Hood with similar values obtained with both methods (Fig. 3.4 B) and highly significant (P< 0.001) positive correlation between both measurement approaches (Fig. 3.4 C).

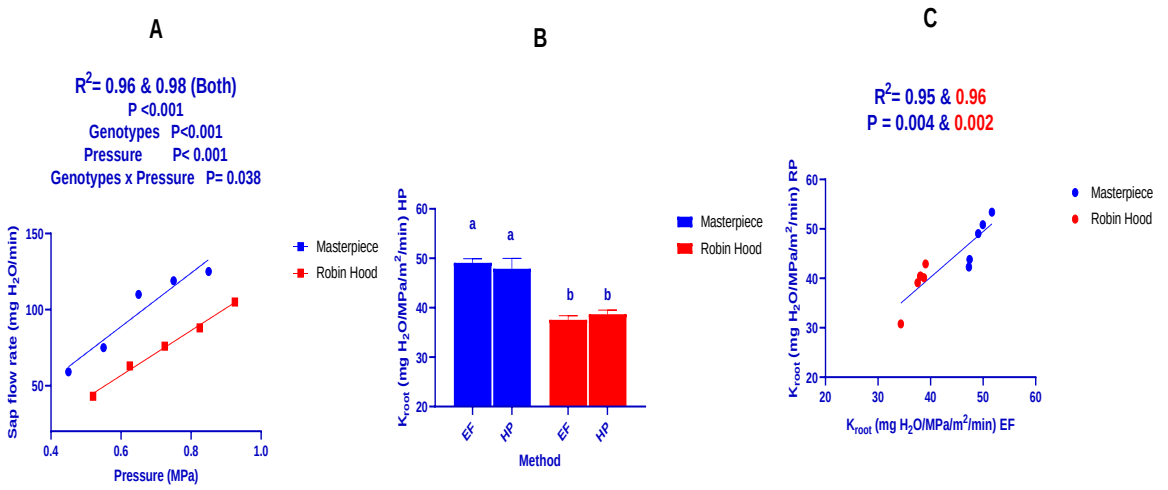


Figure 3.4 Relationship between sap flow rate and applied pressure (A), K_{root} values measured by the evaporative flux (EF) and root pressurisation (RP) methods (B) and the relationship between K_{root} measured by the root pressurisation (RP) and evaporative flux (EF) methods (B) of Masterpiece and Robin Hood in Experiment 2. Symbols and columns are means ± SE of four plants for each genotype with different letters above the bars of (B) indicating significant (P< 0.05) differences according to the T-test, with error bars omitted from A and C for clarity. R^2 and P values for regression coefficient analysis are indicated above panels A and C, while P values from ANCOVA are indicated above panel A.

3.3.3 Leaf & root & xylem saps ABA concentration under different VPD levels

To determine whether plant ABA concentrations were regulating plant water relations, Experiment 3 measured ABA concentrations of leaves, roots and xylem saps collected from these organs, alongside the preceding measurements at different VPDs. As before, both cultivars revealed significant differences in their TR, Ψ_{leaf}, K_{plant} and K_{root} responses to VPD as indicated by significant genotype x VPD interactions (Fig. C-2 A:D). Both genotypes limited their TR, K_{Plant} and K_{root} at the highest VPD with a greater limitation in Robin Hood than Masterpiece. Whereas Ψ_{leaf} of Robin Hood progressively decreased as VPD increased from 2.42 to 3.53 kPa, Masterpiece Ψ_{leaf} stabilised at the highest VPD, after declining by 0.09 MPa from 1.42 to 2.42 kPa. Masterpiece always had significantly lower Ψ_{leaf} values than Robin Hood at the two first VPDs, while similar Ψ_{leaf} values occurred at the final VPD. (Fig. C-2 B).

As in the previous experiment, changes in transpiration significantly affected Ψ_{leaf} only in Masterpiece ($P= 0.013$) (Fig. C-3 A). Whereas K_{plant} increased linearly with TR ($P< 0.001$) similarly in both cultivars (Fig. C-3 B), it was unrelated to changes in Ψ_{leaf} ($P= 0.80$ & 0.77) (Fig. C-3 C).

Masterpiece leaf ABA concentrations were 15 % and 8 % higher than that of Robin Hood at the lowest and the highest VPD (1.42 & 3.55 kPa) with no significant differences at the intermediate VPD (2.42 kPa). When VPD increased from 1.42 kPa to 2.42 kPa, leaf ABA concentration increased by 15 & 36 % before declining by 35 & 47 % in Masterpiece and Robin Hood, respectively at the highest VPD. Although genotype altered leaf ABA concentration,

both genotypes responded similarly to the VPD changes, as indicated by no significant genotype x VPD interaction (Fig. 3.5 A).

Although both cultivars had a similar leaf xylem sap ABA concentration at the intermediate VPD, Robin Hood xylem sap ABA concentrations were ~ 11 % higher than that of Masterpiece at the lowest and the highest VPD resulting in a significant ($P< 0.001$) genotypic effect. When VPD increased from 1.42 kPa to 2.42 kPa, leaf xylem sap ABA concentration doubled in both cultivars before declining by 22 & 18 % in Masterpiece and Robin Hood, respectively at the highest VPD (Fig. 3.5 C). Thus, leaf xylem sap ABA concentration responded similarly to VPD in both cultivars, as indicated by no significant genotype x VPD interaction (Fig. 3.5 C).

Root ABA concentrations were ~ 2-fold lower than leaf ABA concentrations in both cultivars. Masterpiece root ABA concentrations were 26 % higher and 10 % lower than that of Robin Hood at the lowest and the highest VPD, respectively with no genotypic difference at the intermediate VPD. Although root ABA concentrations of both cultivars increased as VPD increased, the greater response of Robin Hood than Masterpiece resulted in a significant genotype x VPD interaction ($P< 0.001$). As VPD increased from 1.42 kPa to 2.42 kPa, root ABA concentration increased by 8 and 34 % in Masterpiece and Robin Hood, respectively. Root ABA concentrations progressively increased at the highest VPD by 10 % and 22 % as VPD increased from 2.42 to 3.55 kPa in Masterpiece and Robin Hood, respectively (Fig. 3.5 B).

Across the applied VPD levels, root xylem sap ABA concentrations were 8-36 % and 1.4 -2-fold higher than leaf xylem sap ABA concentrations in Masterpiece and Robin Hood, respectively. Robin Hood root xylem sap ABA concentrations were ~ 1.5-fold higher than that of Masterpiece across the applied VPD range, resulting in a highly significant ($P< 0.001$) genotypic effect. When VPD increased from 1.42 kPa to 2.42 kPa, root xylem sap ABA concentration doubled in both cultivars before declining by 18 & 14 % in Masterpiece and

Robin Hood, respectively at the highest VPD (3.55 kPa). (Fig. 3.5 D). Thus, root xylem sap ABA concentration response to VPD varied between cultivars, as indicated by significant genotype x VPD interaction (P< 0.001) (Fig. 3.5 D).

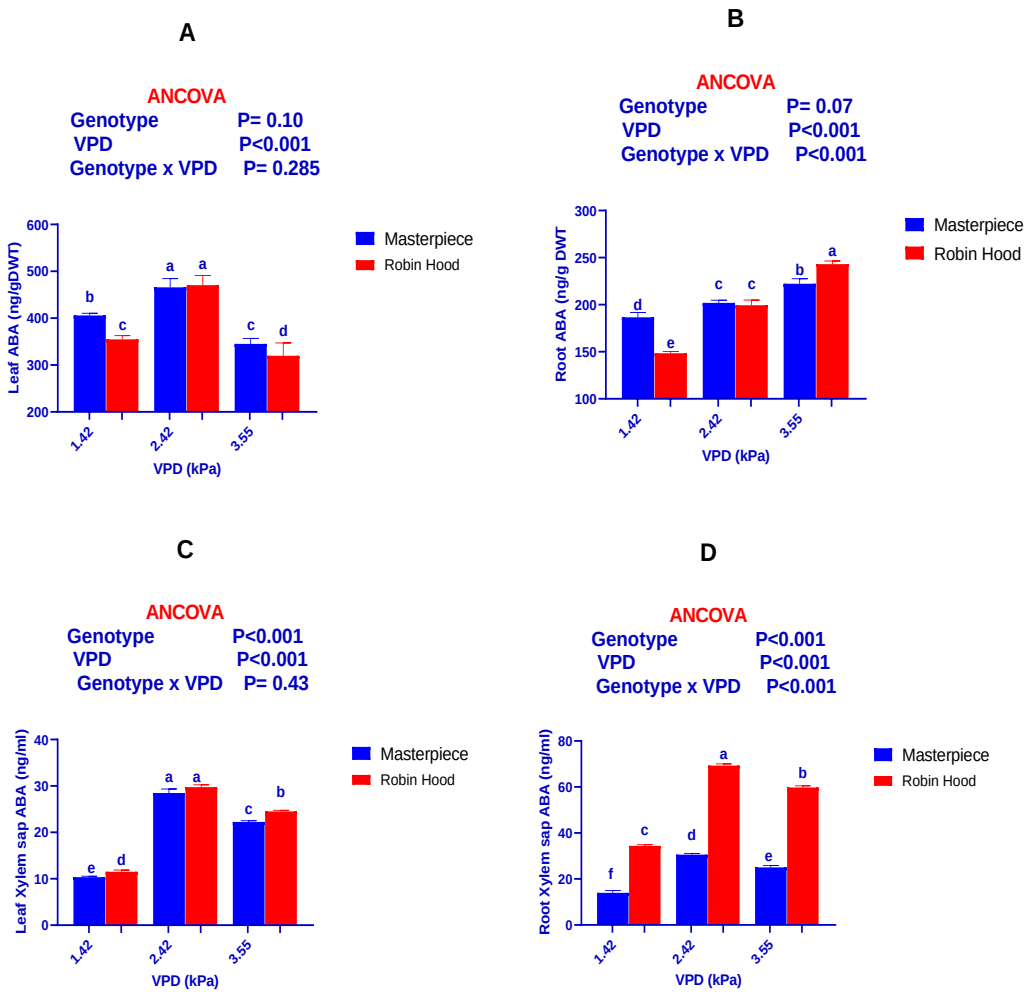


Figure 3.5 Leaf (A), root (B), leaf xylem sap (C), and root xylem sap (D) ABA concentrations of Masterpiece and Robin Hood at different VPD levels. Data are means ± SE of five replicates. P values from ANCOVA are indicated above each panel.

Changes in leaf ABA concentration were significantly and positively associated with TR, K_{plant} and K_{root} in Robin Hood, but not in Masterpiece. Apart from the significant negative correlation between Robin Hood ψ_{leaf} and root ABA concentrations, root ABA concentrations were not related to TR or hydraulic conductance of either cultivar. Changes in leaf xylem sap and root xylem ABA concentrations were significantly and positively associated with TR and K_{plant} in both cultivars (Table 3.1).

Table 3.1 Pearson's correlation coefficients between tissue and xylem sap ABA, TR, ψ_{leaf}, K_{root} and K_{plant} in Masterpiece and Robin Hood.

Significance of P values reported thus: * P< 0.05, ** P< 0.01, *** P< 0.001. significant correlations involving TR, K_{root} and K_{plant} are bolded italicised

Masterpiece **Robin Hood**	Leaf xylem sap ABA	Root ABA	Root xylem sap ABA	TR	ψ_{leaf}	K_{root}	K_{plant}
Leaf ABA	0.06 0.20	0.21 0.025	0.07 0.20	0.06 ***0.53*** **	-0.015 - 0.023	0.06 ***0.76*** ***	0.06 ***0.76*** ***
Leaf xylem sap ABA		0.29* 0.49**	0.94*** 0.98**	***0.87*** *** ***0.71*** ***	- 0.27* - 0.53**	0.17 0.19	***0.56*** ** ***0.31*** *
Root ABA			0.29* 0.52**	***0.27*** * 0.06	- 0.07 - 0.74***	0.04 0.05	0.08 0.02
Root xylem sap ABA				***0.89*** *** ***0.68 *** ***	- 0.22 - 0.56**	0.22 0.17	***0.54*** ** ***0.29*** *
TR					*- 0.30** * - 0.13	0.17 ***0.65*** ***	***0.47*** ** ***0.76*** ***
ψ_{leaf}						0.24 0.046	0.004 0.006
K_{root}							***0.69*** *** ***0.98*** ***

Despite the varied effect of tissue and xylem sap ABA concentration on the hydraulic variables in both cultivars (Table 3.1), canopy conductance (GC) calculations (Whitehead and Jarvis, 1981) revealed that GC of both cultivars consistently declined across the applied VPD range, as indicated by no significant genotype x VPD interaction (Fig. 3.6). GC was inversely correlated with leaf ABA concentration only at the lowest (1.42 kPa) and the intermediate (2.42 kPa) VPDs, with no significant relationship at the highest VPD (Fig. 3.7 A). Moreover, root ABA concentration was significantly and negatively associated with GC across the entire VPD range in both cultivars (Fig. 3.7 B). On the other hand, root and leaf xylem sap ABA concentrations were significantly and negatively associated with GC in Masterpiece ($P= 0.024$ & 0.029) with a tendency towards significance in Robin Hood ($P= 0.06$ & 0.059) (Fig. 3.7 C & D). Thus, stomatal closure at high VPD was most consistently associated with increased root ABA accumulation in both cultivars, even if root ABA export and foliar ABA accumulation were more weakly related to stomatal closure.

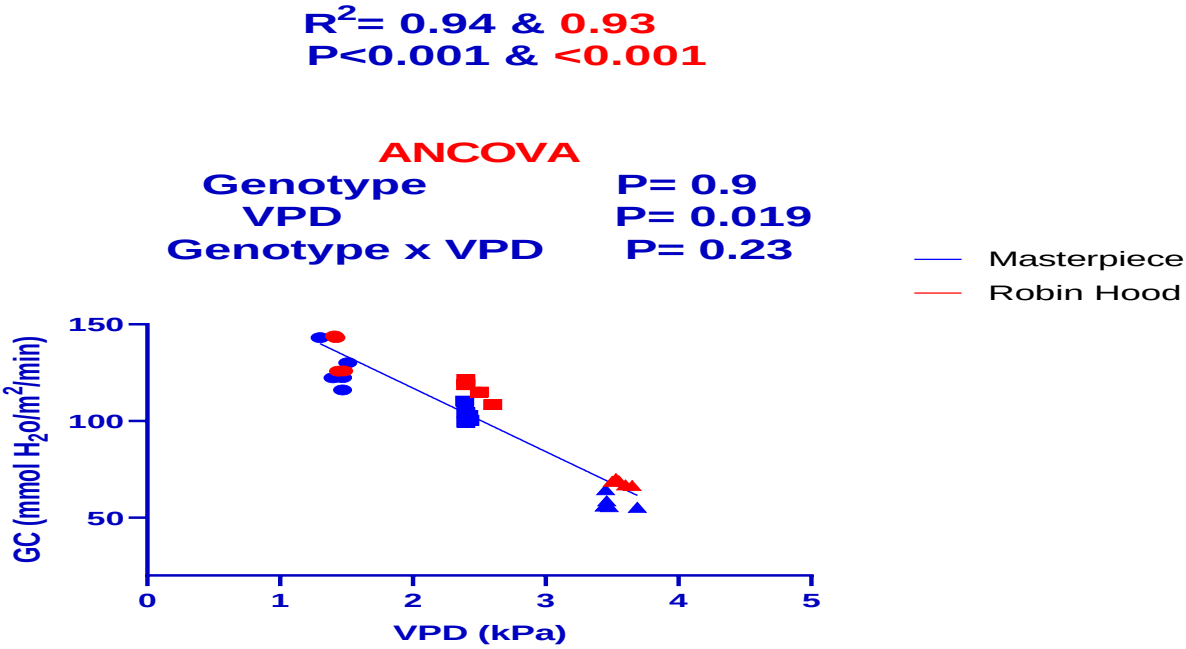


Figure 3.6 GC response to VPD in Masterpiece and Robin Hood. Points are individual plants measured at the lowest (●), intermediate (■) and the highest VPD (▲). P and R^2 values for the regression and P values from ANCOVA are indicated above each panel.

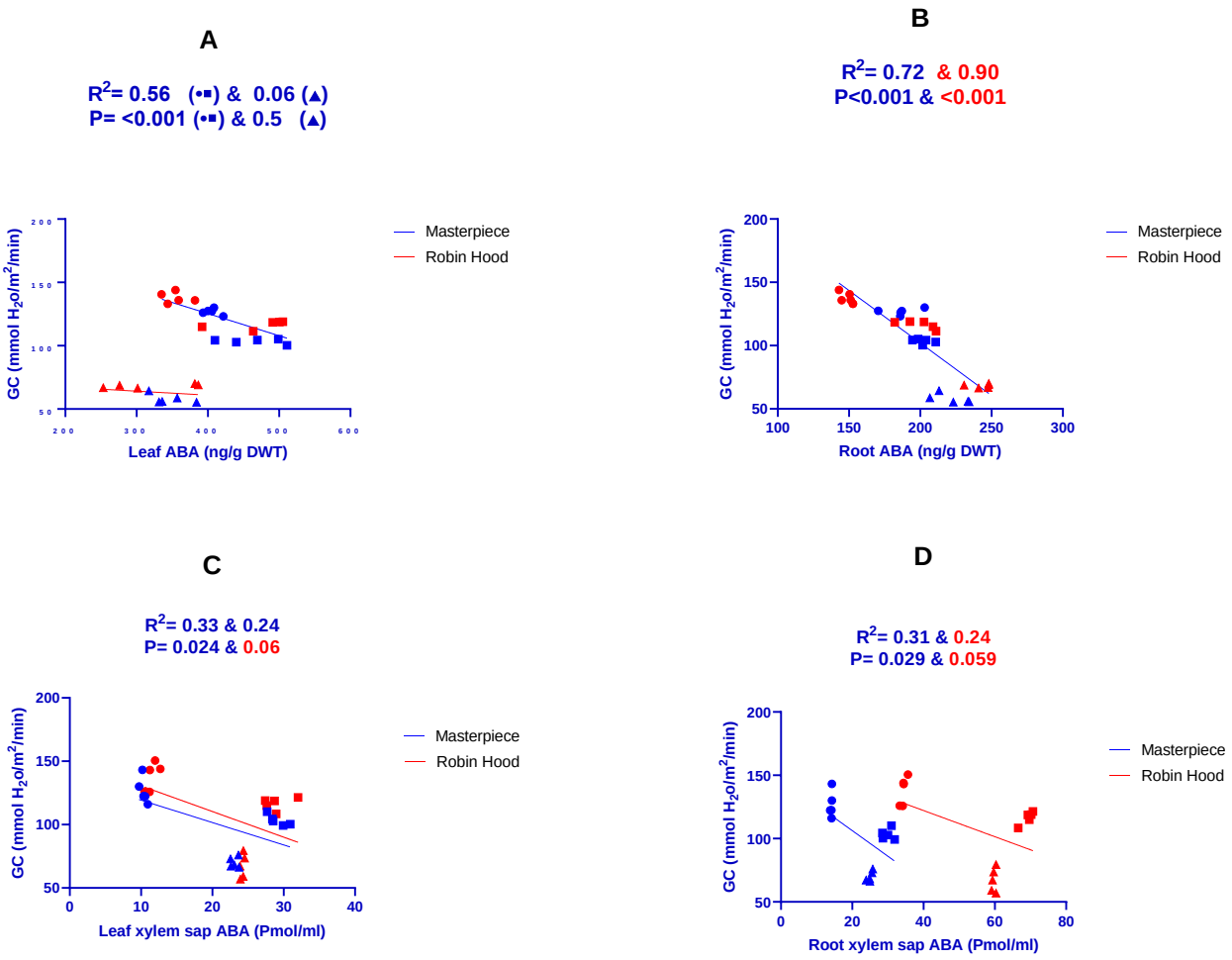


Figure 3.7 Relationship between GC and leaf (A), root (B), leaf xylem sap (C) and root xylem sap (D) ABA concentrations in Masterpiece and Robin Hood. Points are individual plants measured at the lowest (●), intermediate (■) and the highest VPD (▲). P and R^2 values are indicated above each panel.

3.4 Discussion

This is the first report identifying possible physiological mechanisms regulating the limited transpiration response under high VPD in faba bean. The basic hypothesis investigated was that variation in plant hydraulic conductance and ABA levels maintain plant water status by

restricting TR at high VPD. Whereas Masterpiece TR did not increase at the highest VPD, Robin Hood TR significantly decreased, reflecting similar changes in root hydraulic conductance.

The higher whole-plant hydraulic conductance (K_{plant}) in Robin Hood than Masterpiece at the two first VPDs (Fig. 3.1 D) supports the hypothesis that the low BP (~ 2.3 kPa) cultivar maximizes its hydraulic conductance at low VPD then shifts to the water conservation strategy as VPD increases by decreasing hydraulic conductance. K_{plant} significantly increased with transpiration in both cultivars ($P< 0.001$, Fig. 3.2 B), as described earlier (Weatherley 1982; Else et al., 1995; Steudle and Peterson 1998) which supports the hypothesis that low hydraulic conductance somewhere in the plant limits transpiration under high VPD (Zwieniecky et al., 2001; Sperry et al., 2002; Sinclair et al., 2008).

Maintenance of TR at the highest VPD in Masterpiece helped to maintain a stable Ψ_{leaf} (Fig 3.1 B), with Ψ_{leaf} declining as TR increased across the applied VPD range in this genotype (Fig 3.2 A). Paradoxically, even though Ψ_{leaf} declined with VPD in Robin Hood (Fig. 3.1 B), there was no significant relationship between Ψ_{leaf} and TR in this genotype (Fig. 3.2 A). Consequently, higher K_{plant} in Masterpiece than Robin Hood at higher VPD is likely to be a consequence of Ψ_{leaf} stability. By restricting transpiration at high VPD, Masterpiece stabilised Ψ_{leaf} and permitted leaf cells to rehydrate, thereby increasing hydraulic conductance at high VPD. According to Jones, 1983, $\psi_{leaf} = \psi_{soil} - TR*R$ (R is the hydraulic resistance on the way of water from the soil), the decline in leaf water potential equals TR* R, since plants were well-watered so, ψ_{soil} equals zero. Since hydraulic conductance is 1/R, an increase in hydraulic conductance should compensate for the increase in transpiration thereby maintaining ψ_{leaf}

and leaf hydration. This possibility was confirmed by measuring water flow from detached roots and calculating root hydraulic conductivity. Roots can contribute up to approximately half of the whole-plant hydraulic resistance (Martre et al., 2001). Hence, increased hydraulic conductance should enhance root water uptake at any given level of VPD.

In wheat, air warming did not result in stomatal closure in young plants (Vysotskaya et al., 2004 a) and high Ψ_{leaf} was maintained by an increase in K_{root} that reduces the negative impact on shoot hydration that faster water loss from the leaves would otherwise bring about (Vysotskaya et al., 2004 b). However, maintaining higher stomatal conductance is advantageous if only it can be matched by a higher water supply from the roots to the shoot to compensate for the transpirational losses otherwise an increase in VPD may create leaf water deficit. Wheat genotypes revealed genotypic differences in their response to elevated VPD where some closed their stomata at elevated VPD, while others did not due to differences in their hydraulic conductance (Kudoyarova et al., 2007). Thus, it appears there are two ways of regulating water relations under these conditions (increasing K_{root} and/or decreasing gs) and it is important to understand the mechanisms involved.

By measuring component hydraulic conductances within the plant, root hydraulic conductance was identified as the most limiting to whole-plant hydraulic conductance (Fig. 3. 3 B). Similar observations were found in chickpea (Sivasakthi et al., 2020), wheat (Schoppach et al., 2014) and pearl millet (Tharanya et al., 2018) where, lower transpiration under high VPD was associated with limitations in root hydraulic conductance (Sivasakthi et al., 2020), nevertheless limitations in leaf hydraulic conductance restricted transpiration at high VPD in soybean (Sinclair et al., 2008) and peanut (Devi et al., 2012) Thus, different legume species vary in the site of hydraulic limitations.

De-topped plants of both cultivars confirmed the genotypic difference in root hydraulic conductance detected with the evaporative flux method (Fig. 3.4 B & C). Thus, higher whole-plant and root hydraulic conductance in Masterpiece than in Robin Hood allowed plant water status to recover, even though stomatal conductance and transpiration were higher in Masterpiece. These findings also verify the evaporative flux technique as a fast, viable and accurate method for measuring root hydraulic conductance, as in previous studies on soybean, maize sunflower, tomato, kidney bean, green pepper and eggplant (Tsuda and Tyree, 2000).

Differences in plant hydraulics may be explained by root anatomy and/or different populations of water channel proteins (AQPs) since phosphorylation can change their activity (Schäffner, 1998; Tyerman et al., 1999; Guenther et al., 2003). In wheat (Schoppach et al., 2014) root hydraulic limitation was explained by smaller meta-xylem vessels, thinner endodermis and a smaller population of mercury-sensitive aquaporins in the roots, while in pearl millet (Tharanya et al., 2018) and chickpea (Sivasakthi et al., 2020) roots were characterized by low amounts of AQPs. Indeed, future experiments on the anatomy of faba bean roots and the influence of aquaporin inhibitors on plant water relations are essential to explain the greater limitations in root hydraulic conductance at high VPD.

Since root ABA accumulation at high VPD may restrict K_{root} (Markhart et al., 1979; Fiscus, 1981, Davies et al., 1982; Astacio and Iersel, 2011), a possible approach to discriminate among genotypes was to measure their leaf, root and xylem sap ABA concentrations at different VPD levels. As VPD increased from 1.42 to 2.42 kPa, both cultivars increased their leaf and root ABA concentrations. Whereas foliar ABA concentrations decreased at higher VPDs, root ABA concentrations increased by 10 and 22 % in Masterpiece and Robin Hood, respectively (Fig. 3.5 A & B). Decreased leaf and increased root ABA concentrations at high

VPD might result from an inhibition of its pathway from the roots to the leaves. Similarly, air warming increased wheat root ABA concentrations but decreased it in shoots (Kudoyarova et al., 2011).

Since ABA promotes stomatal closure, the decline in leaf ABA concentrations in both cultivars at the highest VPD indicates that stomatal closure is a passive hydraulic ABA-independent process (Brodribb and McAdam, 2011; McAdam and Brodribb, 2015). High VPD can directly promote stomatal closure by decreasing ψ_{leaf} (Brodribb and McAdam, 2011; Buckley, 2019). Indeed, canopy conductance (GC) was negatively and significantly related to VPD (Fig. 3.6), while it negatively correlated with leaf ABA concentration only at the lowest (1.42 kPa) and the intermediate (2.42kPa) VPDs, with no significant relationship between these variables at the highest VPD (Fig. 3.7 A). While these observations are inconsistent with the conventional interpretation that higher leaf ABA concentrations restrict transpiration, ABA accumulation in faba bean leaflets is generalised and guard cells contain only 0.15 % of the bulk leaf ABA in faba bean (Harris et al., 1988). Furthermore, using a guard cell-autonomous reporter system to monitor the distribution of physiologically active pools of ABA indicated accumulation of ABA in shoot vascular tissues after a short period of osmotic stress, with later guard cell accumulation (Christmann et al., 2005). The guard cells have been shown to possess the entire ABA biosynthesis pathway that induces stomatal closure in response to air drying (low relative humidity). This autonomous ABA synthesis in the guard cell allows the plant to change leaf gas exchange according to the environmental conditions (Bauer et at., 2013 a). The presence of ABA in vascular tissues suggests that it may be easily transported not only to the guard cell but also to the roots.

Indeed, root ABA concentration increased in both cultivars as VPD increased, possibly because ABA export from the roots to the shoots is inhibited (Kudoyarova et al., 2011). Indeed, root

xylem ABA concentration decreased by 14-18 % at the highest VPD (Fig. 3.5 C & D). While root and leaf xylem sap ABA concentrations were significantly and negatively associated with GC in Masterpiece (Fig. 3.7 C & D), root ABA concentration was significantly and negatively associated with GC in both cultivars across the entire VPD range (Fig. 3.7 B). This is inconsistent with the previously reported role of root ABA in increasing root hydraulic conductance, thus maintaining higher water status by improving the supply of water to the shoot at high VPD (Thompson et al., 2007). Partially de-rooted maize and wheat plants accumulated ABA in their remaining root axes (Jeschke et al., 1997; Vysotskaya et al., 2004 b). Faster recovery of Ψ_{leaf} and growth upon re-watering was related to larger root hydraulic conductivity and increased ABA concentration in transformed maize lines affected in NCED (9-cis-epoxycarotenoid dioxygenase) gene expression (Parent et al., 2009). However, these responses were transient (Hose et al., 2000) and were positive or negative depending on the ABA concentration (Beaudette et al., 2007). Taken together, elevated VPD causes a progressive ABA accumulation in the roots that maintain higher hydraulic conductance in Masterpiece than Robin Hood at high VPD.

3.5 Conclusions

These results suggest that limited root hydraulic conductance in coordination with active metabolic signals (ABA) restrict transpiration response at high VPD. That is, with increased root ABA export partially closing the stomata to maintain a relatively higher water status in Masterpiece than in Robin Hood.

Chapter 4: Identifying genetic variation in transpiration response to evaporative demand in a faba bean recombinant inbred lines population derived from inbred lines with contrasting drought response strategies

4.1 Introduction

The frequency and intensity of drought incidents are expected to increase due to the rise in the atmospheric temperature, and changes in precipitation as a consequence the climate change (Dai, 2013). 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 drought-adapted crop varieties to farmers 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 vapour pressure deficit (VPD) which works as a water conservation strategy to delay the harmful effects of late-season water deficit. Moreover, various simulation models incorporating this trait in different genotypes of soybean (*Glycine max* (L.) Merr.) (Sinclair et al., 2010), maize (*Zea mays*) (Messina et al., 2015), and lentil (*Lens culinaris* Medik.) (Guiguitant et al., 2017) revealed that limiting TR after 1 to 2 kPa (species dependent) 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, and consequently yield more than genotypes that are not expressing the trait (Sinclair et al., 2016). 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; Sinclair et al., 2016). 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 natural environments 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 influence limited TR at high VPD is urgently needed using environment-independent DNA markers, especially in drought-sensitive crop species such as faba bean (Khazaei et al., 2014). Genomic and transcriptomic approaches now being applied in faba bean open new opportunities for fine mapping and uncovering candidate genes (Khazaei et al., 2021). Considering the strong macro-synteny amongst legumes, a set of expressed sequence tags (ESTs) from *Medicago truncatula*, pea (*Pisum sativum* L.), lentil (*Lens culinaris*) and lupin (*Lupinus luteus* L.), has been included in faba bean maps earlier (Cruz-Izquierdo et al., 2012), and whole-genome sequences of *M. truncatula* (Young et al., 2011) and chickpea (*Cicer arietinum* L.) (Varshney et al., 2013) offered opportunities for translation to faba bean. Furthermore, the way to highly saturated and cost-effective second-generation genetic maps has been facilitated by the recent development of 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., 2021). Furthermore, the University of Reading has recently developed a high-density faba bean genotyping array (as 'Vfaba_v2') which contains 24,929 polymorphic high-resolution SNP markers located in 15,846 different genes (Sullivan et al., 2019). SNP markers provide low genotyping cost per data point, high genomic polymorphism, locus specificity in terms of

accuracy and reproducibility (Yan et al., 2010), simple documentation, co-dominance, a common occurrence amongst elite germplasm, and potential for high-throughput analysis (Cottage et al., 2012 a & b). Thus, DNA markers are considered powerful tools in genetic mapping, association studies, assessing genetic diversity, and positional cloning. In the absence of a faba bean reference genome, several transcriptomes have been reported for faba bean looking for drought adaptation-related traits (see Alghamdi et al., 2018; Wu et al., 2020). Faba bean large genome is currently being assembled (https://projects.au.dk/fabagenome) that will further advance the faba bean genomics and breeding revolution.

Plants can regulate transpiration at high VPD by matching stomatal and hydraulic conductance to maintain a constant water potential (Ψ_{leaf}), thus minimizing the exposure of the leaves to water deficit (Attia et al., 2015). By decreasing stomatal conductance (gs) to water vapour, plants minimize water loss and maintain cellular hydration as VPD increases. The previous chapters with 'Masterpiece' and 'Robin Hood' faba bean cultivars suggested that much larger genetic variation in transpiration (TR) response to vapour pressure deficit could be expected in bigger populations and demonstrated the potential importance of changes in hydraulic conductance for regulating TR at high VPD. Chapter 2 verified the whole-plant gas exchange chamber as a precise technique for measuring TR response to VPD, by tightly controlling atmospheric conditions around the whole plant to achieve a range of VPDs at an almost stable temperature, independently of the time of day or year. Moreover, the evaporative flux method was established as a viable, precise, fast, and easy method to measure hydraulic conductance. Therefore, the high-throughput phenotyping of transpiration response to VPD in faba bean segregation populations is essential for DNA

marker development, particularly for screening drought-adapted faba bean genotypes based on their ability to restrict transpiration at high VPD.

Statement of Research Objectives

This chapter was undertaken using 165 faba bean recombinant inbred lines (RILs) derived from the cross of Mélodie/2 × ILB 938/2 (examined in Khazaei et al., 2014) with the following objectives: 1) To identify genotypic variation in TR to VPD, 2) examine the whole-plant hydraulic conductance and its components as a possible regulatory mechanism for limited TR, and (3) identify genomic regions associated with transpiration response to VPD. It was hypothesized that limited TR response to VPD is associated with restrictions in hydraulic conductance that would not be prominent in genotypes that do not express the limited TR trait.

4.2 Materials and Methods

4.2.1 Plant material

Previous work (2009-2012) at the Department of Agricultural Sciences, University of Helsinki, Finland generated a recombinant inbred lines (RILs) population by single-seed descent in cross Mélodie/2 × ILB 938/2 (IG 13987). The Mélodie/2 is an inbred line selected from the low vicine–convicine cultivar 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 produced to the rate of transpiration) and relatively low productivity (Khan et al., 2007 & 2010; Khazaei et al., 2013 b; Khazaei et al., 2018 a). They also 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 g_s revealed a reverse 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 characters (Table D-1) which confirms the wide genetic variation between them and their suitability for genetic mapping and genomic studies. The wide difference between both parental lines either genetically or geographically makes them quite suitable for building a promising RIL population for successful genetic mapping and QTL detection (Würschum 2012).

4.2.2 Plant culture

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 in the whole-plant gas exchange chamber between 2019-2021. Seeds were chosen randomly and germinated as described earlier (**Section 2.1**). Each RIL was represented by three to four

plants - depending on seed availability- that were planted at different times of the year in a semi-controlled glasshouse to ensure a random distribution of the replicates across varying atmospheric conditions in the glasshouse.

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} photosynthetic photon flux density (PPFD) (mean ± SE, n= 3600, comprising 12 h x 300 days) at the pot surface ~ 2 m below the lamp. Actual air temperature and relative humidity in the centre of the glasshouse were recorded 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 ± 0.6 kPa (mean ± SE, n= 3600), respectively.

4.2.3 Measuring transpiration and hydraulic conductance responses to VPD

After growing the plants for 4 weeks, homogenous plants with 6-7 fully expanded leaves were chosen (leaf area= 299 ± 4 cm^2, means ± SE, n= 560, comprising 100 RILsx 3 replicates & 65 RILs x 4 replicates) for measuring transpiration rate (TR) responses to elevated vapour pressure deficit (VPD) in the whole-plant gas exchange system described earlier (Section 3.1). Whole-plant hydraulic conductance (K_{plant}) and its components, i.e., root hydraulic conductance (K_{root}) and stem hydraulic conductance (K_{stem}) were measured with the

evaporative flux method only at the lowest and the highest VPD as described in Section 3.2. The plants were watered to maximum pot drained capacity and left to drain for about 15 min during which two leaves were covered with aluminium foil to estimate stem water potential (Ψ_{stem}) under the lowest and the highest VPDs. The plants were then sealed into the chamber and left to acclimate for about 30 minutes to the chamber lights. The measurement started by increasing chamber relative humidity to its maximum of 70 % ± 0.6 (means ± SE, n= 560) to generate the lowest VPD while the temperature is stable (25.9 ± 0.09, means ± SE, n= 560). Once CO_2 and H_2O exchange was 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 and one fully expanded transpiring leaf (Ψ_{leaf}) (15-20 % of total leaf area) across both leaves was measured using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) as described in Section 3.2. 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 CO_2 and H_2O values were logged again.

Each plant was exposed to six sequentially decreasing humidity levels achieved by increasing the ratio of dry to humid air (70 %, 58 %, 43 %, 31 %, 18 %, and 11 %), approximately corresponding to VPD values of 1, 1.41, 1.91, 2.32, 2.75 and 3.2 (± 0.02) kPa (means ± SE, n= 560) at stable temperature (25.90 ± 0.09°C, means ± SE, n=3360). At the highest VPD, Ψ_{leaf} and Ψ_{stem} were determined again. The covered leaves were not included in leaf area calculations for transpiration measurements but were included in total leaf area calculations. Leaf area was measured with a leaf area meter (Model LI-3100C, Li-COR, Lincoln, NE, USA).

Whole-plant, root and stem hydraulic conductance were calculated at the lowest and the highest VPDs by dividing transpiration rate (TR) by the $\psi_{gradient}$ as described by (Tsuda and Tyree, 2000), (Section 3.2).

4.2.4 Identifying QTLs associated with transpiration response to VPD

4.2.4.1 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. (2021). Briefly, DNA was isolated from three days 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).

4.2.4.2 Linkage map construction

The linkage map was originally constructed by Gela al. (2021). The linkage map was constructed using ASMap package in R (Taylor et al., 2017) and refined by using MapDisto v. 1.7.7.0.1 (Lorieux, 2012) 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 final linkage groups were assigned to faba bean

chromosomes according to the NV644 ×NV153 genetic map developed at the University of Reading, UK (unpublished data).

4.2.4.3 QTL mapping of transpiration response to VPD

Composite interval mapping (CIM) was used to detect putative QTLs locations of TR-min, TR-max, TR-BP, K_{plant}-min, K_{plant}-max, K_{root}-min, and K_{root}-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 window size of 5 cM. QTL significance thresholds were determined by 1,000 permutations at a significance level of P = 0.05. Only data from 142 RILs which genotyping data was available and used for QTL analysis. To determine candidate genes, the sequences of SNP markers which appeared within the QTL interval were searched using BLASTn (Goodstein et al., 2012) in Phytozome v13 on the reference genome for *Medicago truncatula*.

4.2.5 Statistical analysis

Analysis of TR response to VPD was performed using the segmented linear regression model of GraphPad Prism 9.3.1 (GraphPad Software Inc., San Diego, CA, 2007), which provides a 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 (P< 0.05) genotypic differences in regression parameters (slopes and BPs), TR, Ψ_{leaf}, and hydraulic conductance for the entire population were discriminated against Student's T-test. ANCOVA (for main effects of genotypes, VPD, and their interaction) between the parental lines in their TR, $\Psi_{leaf,}$ and K_{plant} 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 $p < 0.05$.

4.3 Results

Across the entire period of measurements, 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 (25.9 °C ± 0.09, mean ± SE, n= 3360) resulting in VPD ranging from ~ 1 to 3.2 kPa for all genotypes.

4.3.1 Responses of the parental lines

4.3.1.1 Genotypic variation in TR response to VPD

The parental lines differed in their TR response to VPD (Fig. 4.1 A & B). While Mélodie/2 exhibited a linear increase in TR for the range of VPD tested, ILB938/2 was well characterized by the two-segmental analysis where its TR steadily increased with increasing VPD to reach 30.45 ± 2.35 mg $H_2O/m^2/min$ at 2.12 ± 0.04 kPa (BP). Thereafter, TR was relatively stable despite increases in VPD, to be 32.53 ± 2.25 mg $H_2O/m^2/min$ at 3.2 ± 0.07 kPa. The genotypes differed in their TR response to VPD, as indicated by significant genotype x VPD interaction

(P= 0.036, Table 4.1). Although both parental lines differed in their minimum TR, they did not significantly differ in their maximum TR (Fig 4.1 C).

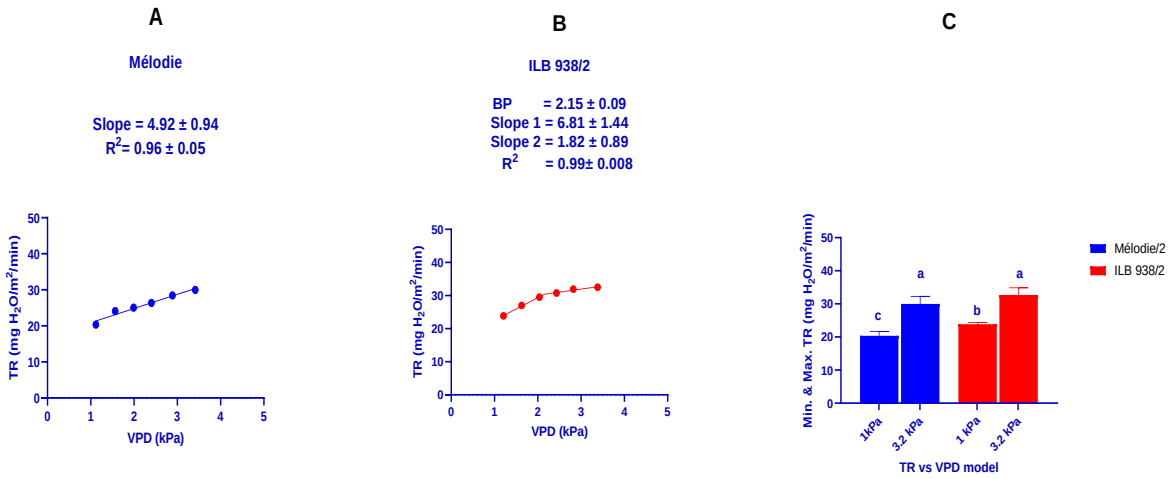


Figure 4.1 TR response to VPD of Mélodie/2 (A) and ILB 938/2 (B) in the whole-plant gas exchange chamber and the difference between the two genotypes in their min (1st column each genotype). and max (2nd column each genotype) TR (C). Each point and column represent 5 minutes of transpiration rate after 15 minutes of steady-state. Symbols are means of four plants for each genotype and error bars were omitted from A and B for clarity, while different letters in C indicate significant (P< 0.001) differences according to T-test. Linear (A) and broken-stick (B) regression lines (P< 0.01) were fitted in Prism. Means ± SE of regression variables i.e., slope 1 and R^2 values of Mélodie/2 and BP, slopes, and R^2 values of ILB 938/2 are represented on the top of panels A & B.

4.3.1.2 Genotypic variation in water potential and hydraulic conductance

Leaf water potential (Ψ_{leaf}) significantly differed between both genotypes at the two tested VPDs with Mélodie/2 having 14 and 9 % higher Ψ_{leaf} than ILB9382/2 at the lowest and the highest VPD, respectively (Fig. 4.2 A). Stem water potential (Ψ_{stem}) was always higher than Ψ_{leaf} by ~ 25 % at the two tested VPDs and decreased by 30 and 26 % at the highest VPD in Mélodie/2 and ILB9382/2, respectively with significant difference between both genotypes (Fig. 4.2 B). Overall, Ψ_{leaf} and Ψ_{stem} responded similarly to the VPD changes in both genotypes (no significant genotype x VPD interaction, Table 4.1). Whole-plant hydraulic conductance (K_{plant}) increased by 10 % and 13 % at the highest VPD in Mélodie/2 and ILB9382/2, respectively, resulting in non-genotypic variation in Kplant as indicated by no significant genotype x VPD interaction (Table 1 & Fig. 2 B).

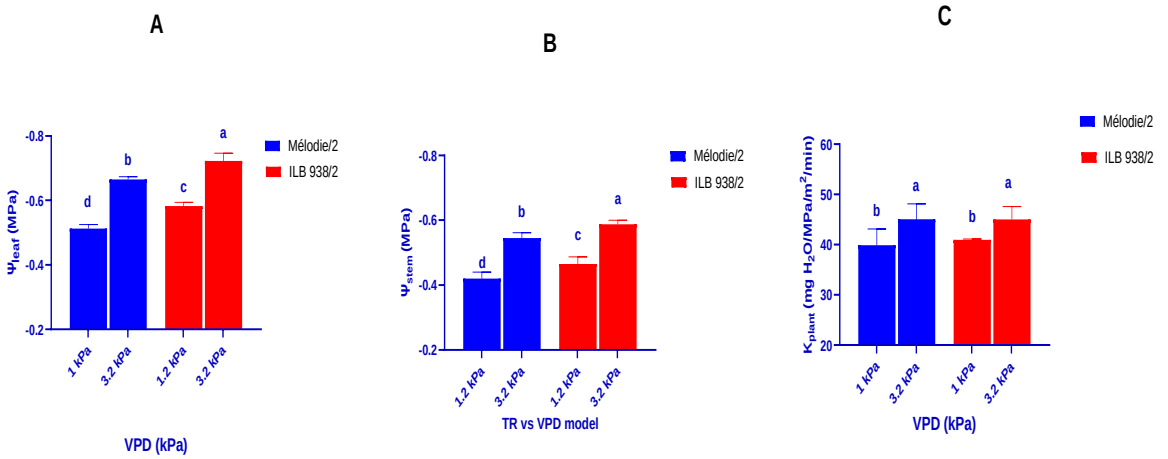


Figure 4.2 Changes in Ψ_{leaf} (A), Ψ_{stem} (B) and K_{plant} (C) in Mélodie/2 and ILB 938/2 from the lowest (1 kPa) to the highest (3.2 kPa) VPD. Data are means ± SE of four plants of each parental

line, with different letters above the bars indicating significant ($P < 0.05$) differences according to the T-test.

To determine where hydraulic conductance is limited, root and stem hydraulic conductance were measured at the lowest and the highest VPDs. Stem hydraulic conductance (K_{stem}) was always higher than K_{root} in both genotypes, by 3 and 3.7-fold at the two tested VPDs (Fig. 4.3 A & B). The genotypes had similar K_{stem} and K_{root} responses to VPD, as indicated by no significant genotype x VPD interactions (Table 4.1). Whereas both genotypes had similar K_{stem} at the lowest VPD, K_{stem} of Mélodie/2 was 8 % higher than that of ILB 938/2 at the highest VPD. In contrast, K_{root} did not significantly differ between both genotypes at any VPD where both genotypes revealed ~ 10 % increase in their K_{root} at the highest VPD (Fig. 4.3 B) While K_{stem} of Mélodie/2 increased by 26 % as VPD increased, ILB 938/2 increased its K_{stem} by only 6 % at the highest VPD. (Fig. 4.3 A). All these results confirm the genotypic variation between both parental lines and show that Mélodie/2 maintains higher water status than ILB 938/2 under high VPD.

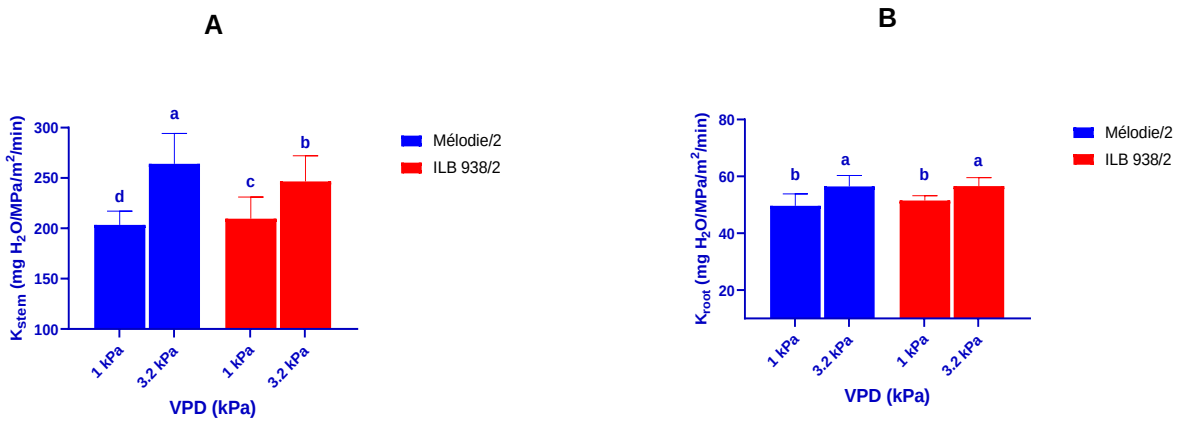


Figure 4.3 Changes in K_{stem} (A) and K_{root} (B) in Mélodie/2 and ILB 938/2 from the lowest to the highest VPD. Data are means ± SE of four plants of each parental line, with different letters above the bars indicating significant (P< 0.05) differences according to T-test.

Table 4.1 ANCOVA variables describing the difference between Mélodie/2 and ILB 938/2 in transpiration (TR), leaf water potential (Ψ_{leaf}), stem water potential (Ψ_{stem}) and hydraulic conductance(K_{plant}) responses to VPD, with significant P values italicised.

Trait	Genotypes	VPD	Genotype x VPD
TR	0.15	*<0.001*	*0.036*
Ψ_{leaf}	*0.03*	*<0.001*	0.94
Ψ_{stem}	*0.002*	*<0.001*	0.41
K_{plant}	0.90	*0.047*	0.96
K_{root}	0.76	0.10	0.79
K_{stem}	0.81	0.06	0.62

4.3.2 Leaf area and potential gradients differences across the RILs

Although leaf area (LA) did not significantly differ among RILs (P= 0.5, Table 4.2), it significantly (P< 0.001) differed across the months the plants were measured in. Plants measured in May had the highest LA (325 ± 7.5 cm^2) while those measured in August had the lowest LA (199 ± 6 cm^2). However, neither genotype nor the genotype x month interaction was significant (Table 4.2 & Fig. D-2) which was expected since the basic criteria for choosing the plants was leaf number (8 leaves). Also across the entire population, leaf water potential (Ψ_{leaf}) was ~ 28 % lower than stem water potential (Ψ_{stem}) at both VPDs (Fig. D-1).

Table 4.2 P-values from ANCOVA for the genotypic differences between the RILs in their leaf water potential (Ψ_{leaf}) and stem water potential (Ψ_{stem}) at the lowest and the highest VPD and P-value for the difference in leaf area (LA) across months the plants were planted, with significant P values, italicised.

Trait	Genotypes	VPD	Genotype x VPD	Month	Genotype x Month
Ψ_{leaf}	*<0.001*	*<0.001*	1		
Ψ_{stem}	*<0.001*	*<0.001*	1		
LA	0.5			*<0.001*	0.7

4.3.2.1 Genotypic variation in TR response to VPD in the RILs

Due to the wide variation between the parental lines, there was considerable variability within the progeny lines. Among the 165 RILs, segmented regression analysis identified a significant break-point (BP) in more than 90 % of the population (150 genotypes) ranging from 1.5<BP<3 kPa, while only 15 genotypes had a linear TR model (Fig. 4.4 A). The RILs that exhibited segmented TR were divided into three sub-groups based on the BP value as follows: 1) 1.5<BP<2 (61 genotypes), 2) 2<BP<2.5 (65 genotypes), and BP>2.5 (24 genotypes). All the RILs (linear & segmented TR) slightly differed in their TR at the lowest VPD, averaging 25.0 ± 0.73 mg H_2O m^{-2} min^{-1} and increasing by 42-51 % as VPD increased to 36.67 ± 1.45 mg H_2O m^{-2} min^{-1}. The highest increase in TR occurred in the 2<BP<2.5 group and the lowest in the linear one, resulting in significant differences between the groups in their maximum TR (Fig 4.4 B).

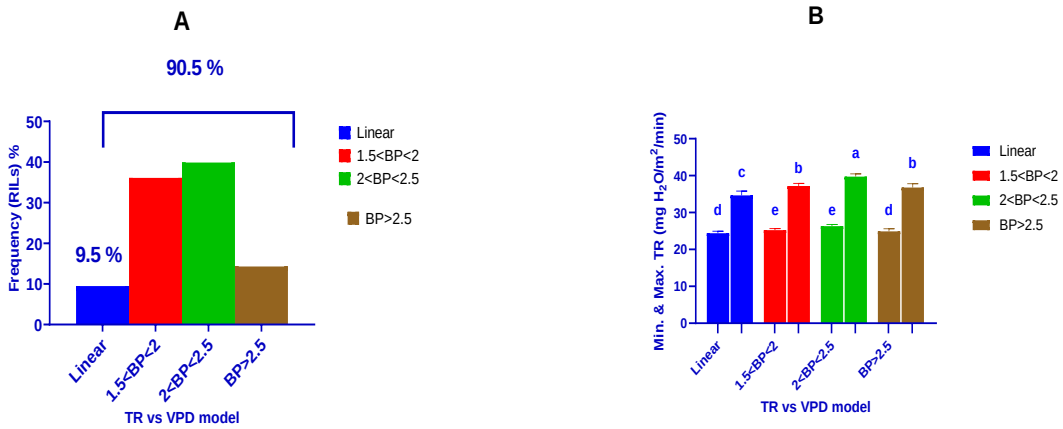


Figure 4.4 Frequency distribution (%) of TR response models to VPD of 165 RILs derived from Mélodie/2 and ILB 938/2 (A) and the difference between the groups in their min. TR (1st column in each group). and max. (2nd column in each group) TR (B). Data are mean ± SE of TR

of the genotypes in each group (n= 1060), with different letters above the bars indicating significant (P< 0.05) differences according to the T-test.

In the group in which a single linear regression represented all the data, the slope averaged 4.86 ± 0.35 mg H_2O m^{-2} min^{-1} kPa^{-1} which is comparable to the parental line Melodie/2 (4.92 ± 0.94 mg H_2O m^{-2} min^{-1} kPa^{-1}) and R^2 averaged 0.95 ± 0.01 (Fig. 4.5 A).

Transpiration of the segmented TR genotypes was relatively stable above the BP (averaging 37.89 ± 0.82 mg H_2O/ m^2/min and with the model highly fitting the data, R^2 averaged 0.976 ± 0.006. Slope 1, BP, and slope 2 differed significantly across the three groups (Fig. 4.6 A & B &C). For the 61 RILs with a low BP (1.5<BP<2), slope 1 averaged 11.93 ± 0.54, mg H_2O m^{-2} min^{-1} kPa^{-1}, which was substantially (28-47 %) greater than the slope 1 in other groups i.e., 9.36 ± 0.36 and 8.14 ± 0.39 mg H_2O m^{-2} min^{-1} kPa^{-1} for 2<BP<2.5, and BP>2.5 kPa, respectively. While slope 2 was 2.5-fold lower and averaged 3.52 ± 0.16 mg H_2O m^{-2} min^{-1} kPa^{-1} which was 0.5-2.5-fold greater than slope 2 for other groups, i.e., 2.93 ± 0.18 and 1.08 ± 0.48 mg H_2O m^{-2} min^{-1} kPa^{-1} for 2<BP<2.5, and BP>2.5 kPa, respectively (Fig. 4.5 B-D). Across the segmented TR genotypes, the two slopes of the segmented TR were significantly (P< 0.001), but poorly (R^2= 0.1) correlated. Similarly, significant (P< 0.001) but poor (R^2= 0.18 & 0.22) correlations were detected between the two slopes and their BP (Fig. 4.7 A & B). Thus, significant genotypic differences in transpiration response to VPD were consistent across the three groups.

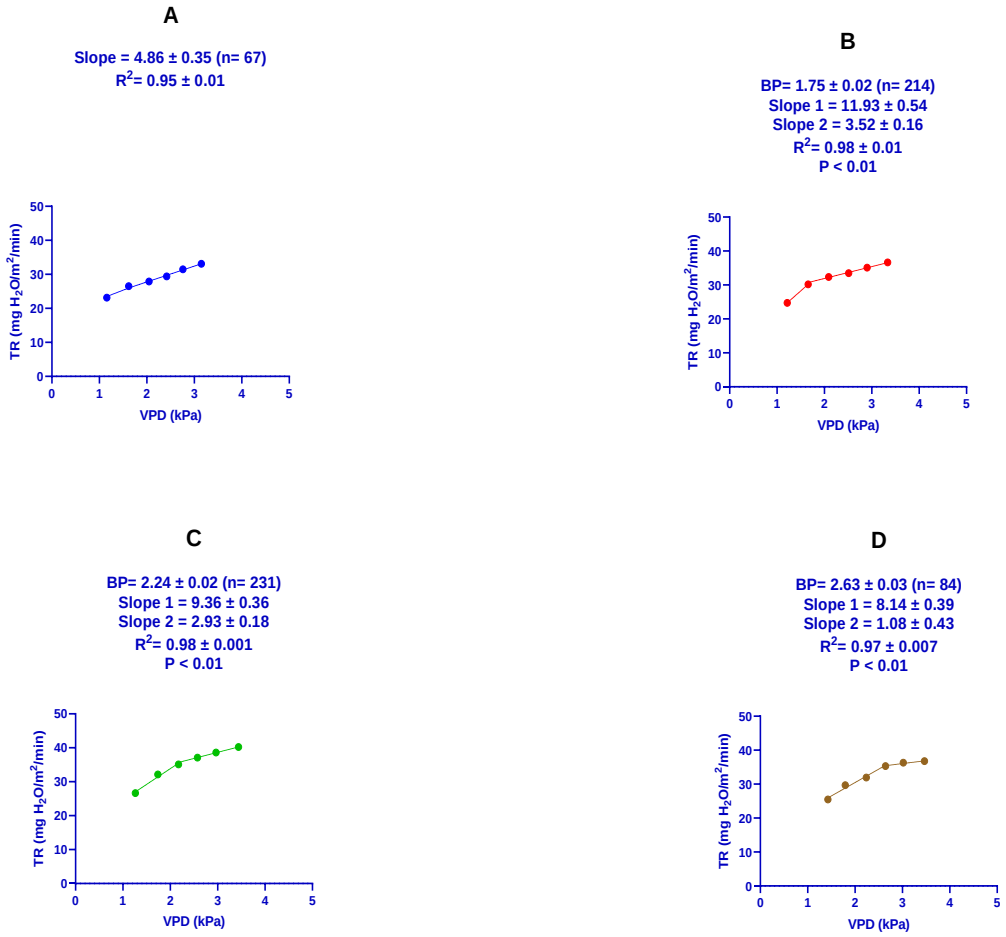


Figure 4.5 TR response to VPD of the linear TR group (A), and the three-segmented TR models i.e. 1.5<BP<2 kPa (B), 2<BP<2.5 kPa (C) and BP>2.5 kPa (D). Data are mean of TR of genotypes in each group. Linear (A) and broken-stick (B, C, D) regression lines (P< 0.01) were fitted in Prism. Means ± SE of regression variables i.e., BP, slopes, $R^{2,}$ and P-value are represented on the top of each panel.

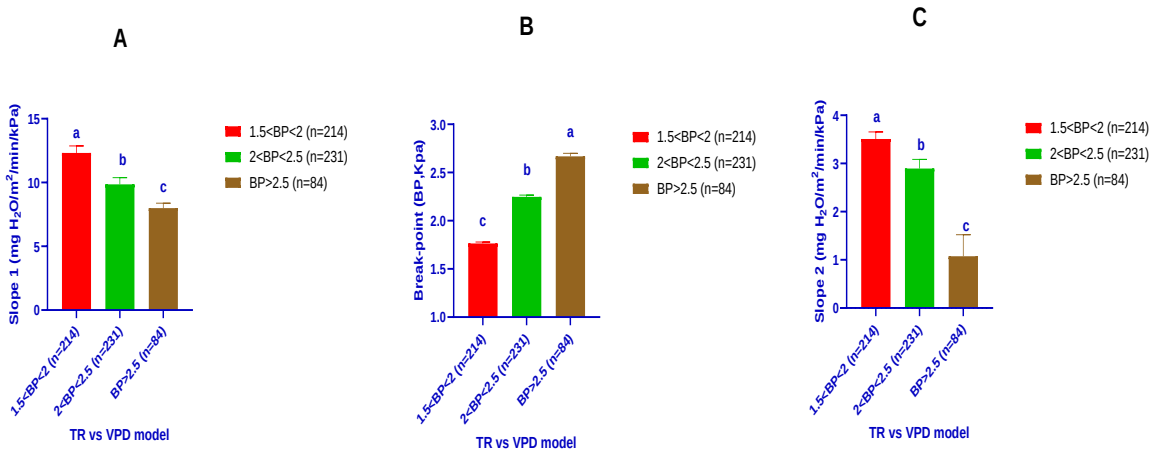


Figure 4.6 Differences between the segmented TR models in their slope 1 (A), BP (B), and slope 2 (C) at *P*< 0.05. Data are means ± SE in each group, with different letters above the bars indicating significant (P< 0.05) differences according to the T-test.

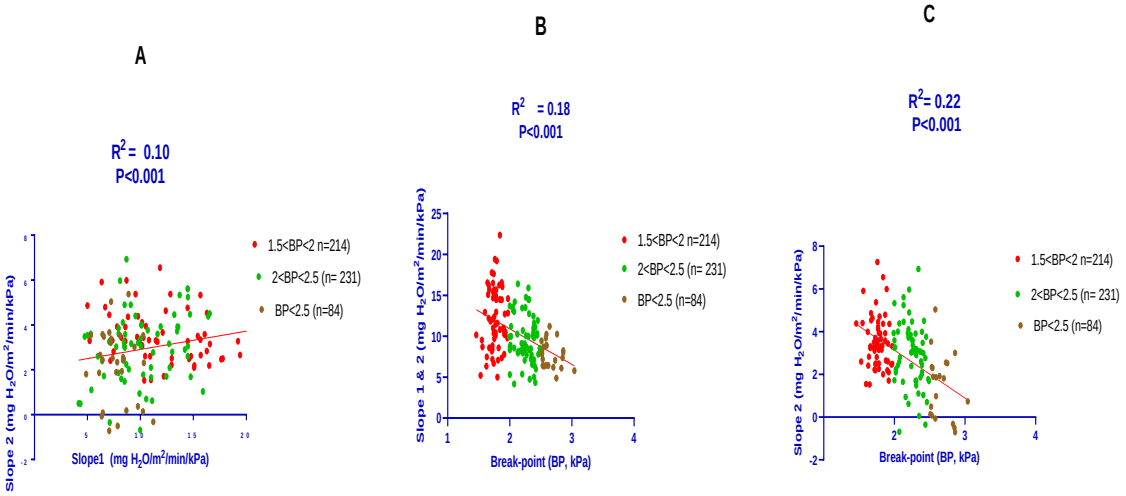


Figure 4.7 Relationships between the two slopes of the segmented TR models (A), slope 1 and the BP (B), and slope and the BP (C). Data are means of 3-4 plants of each RIL. P values and R^2 for regression coefficient analysis are reported on the top of each panel.

Similar to the parental lines, leaf water potential (Ψ_{leaf}) did not differ between the four groups at the two tested VPDs, and averaged -0.567 ± 0.012 MPa at the lowest VPD and 30-35 % lower at the highest VPD to average -0.756 ± 0.016 MPa (Fig. 4.8 A).

Whole-plant hydraulic conductance (K_{plant}) differed significantly across the four groups with greater values in the 2<BP<2.5 kPa group i.e 47.85 ± 0.93 mg H_2O m^{-2} min^{-1} kPa^{-1} and 54.19 ± 1.15 mg H_2O m^{-2} min^{-1} kPa^{-1} at the lowest and the highest VPD, respectively which were 6-12 % higher than K_{plant} in other groups and also 10-24 % higher than the parental lines (Fig. 4.8 B). Tripling the VPD increased K_{plant} by 8.5-13.5 % across the four groups with the highest increase in the 2<BP<2.5 group and the lowest in the linear group resulting in significant differences between the four groups at the two tested VPDs. Within the four groups, K_{plant} was not comparable to either parental line at any VPD where they exhibited 10-20 % higher K_{plant} than the parental lines.

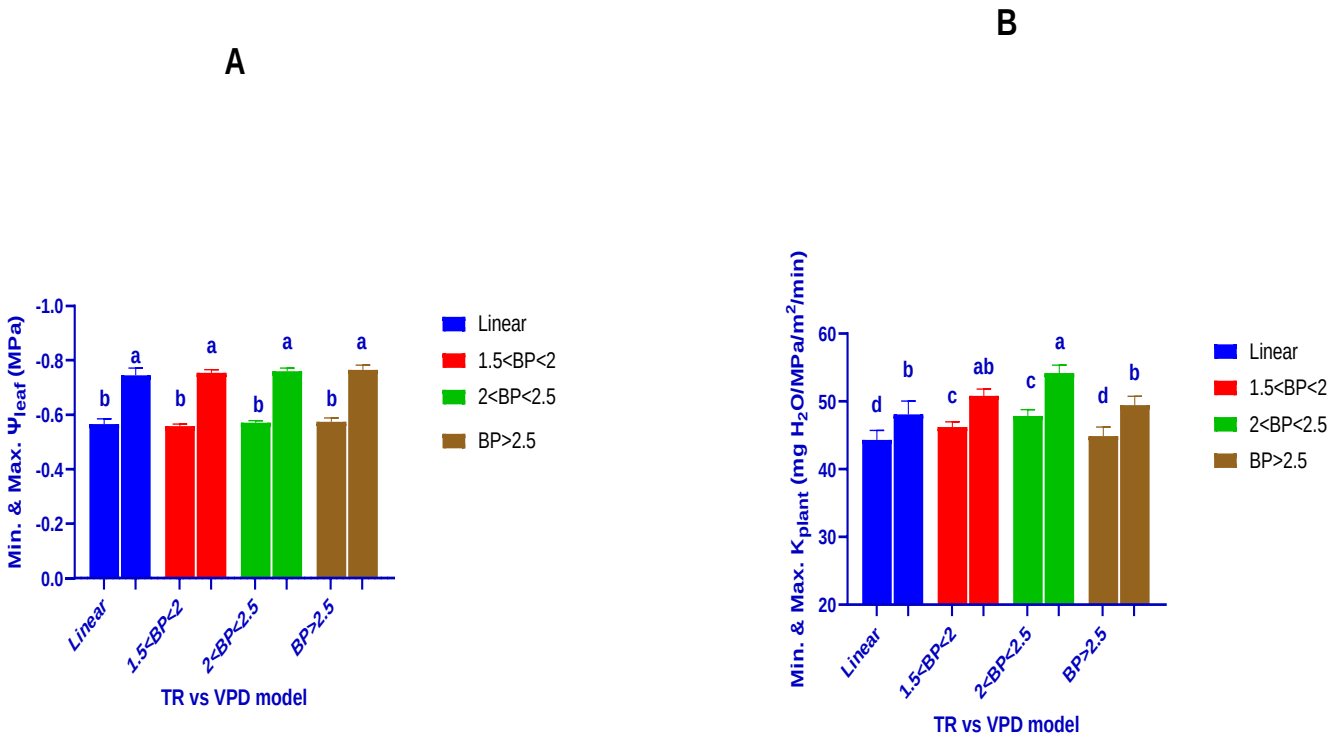
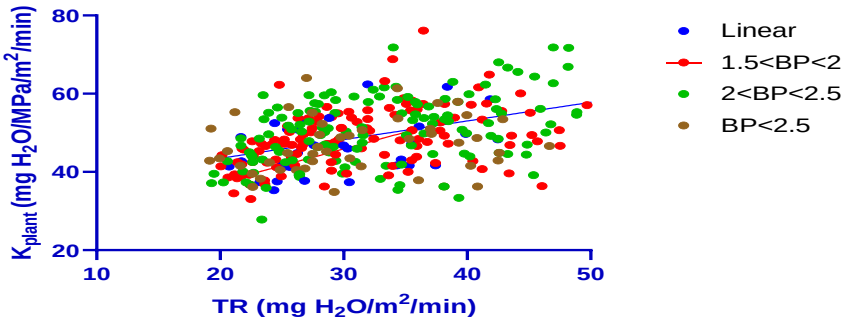


Figure 4.8 Differences between the four TR models of 165 RILs in their Ψ_{leaf} (A) and K_{plant} (B) at the lowest (1st column in each group) and the highest (2nd column in each group) VPD levels. Data are means ± SE of 3-4 plants each RIL with different letters above the bars indicating significant ($P< 0.05$) differences according to the T-test.

To see whether the changes in K_{plant} are attributed to TR and/or Ψ_{leaf}, K_{plant} was regressed against both variables. As TR increased, K_{plant} significantly increased ($P< 0.001$) (Fig. 4.9 A). While Ψ_{leaf} significantly decreased as the transpiration rate increased ($P< 0.001$) (Fig. 4.9 B), there was no significant relationship ($P= 0.53$) between Ψ_{leaf} and K_{plant} (Fig. 4.9 C). Taken together, variation in K_{plant} was better explained by variation in transpiration rate ($P< 0.001$) than variation in Ψ_{leaf}.

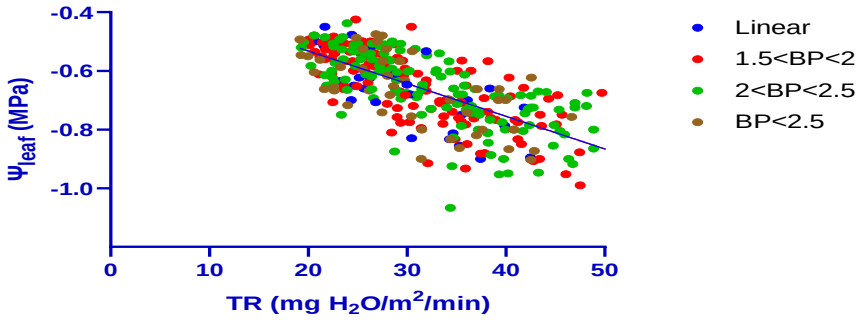
A

R^2= 0.20
P<0.001



B

R^2= 0.47
P< 0.001



C

R^2= 0.001
P= 0.52

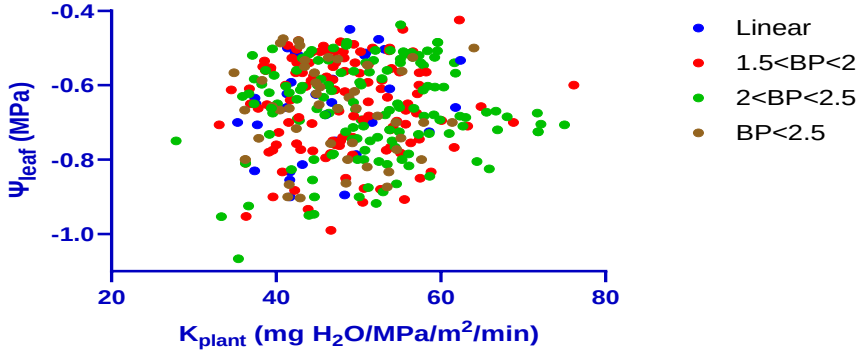


Figure 4.9 Relationships between K_{plant} and TR (A), Ψ_{leaf} and TR (B), and Ψ_{leaf} and K_{plant} (C) of 165 RILs derived from Mélodie/2 and ILB 938/2. Data are means of 3-4 plants of each RIL over the two extreme VPD levels. R^2 and P values for regression coefficient analysis are reported on the top of each panel.

Across the entire population, K_{stem} and K_{root} differed significantly between the groups and the two tested VPDs with ~ 3-fold lower K_{root} values than K_{stem} across the four groups (Fig. 4.10 A & B). K_{stem} and K_{root} increased by 7.5-15 % across the four groups with the highest increase in the 2<BP<2.5 group and the lowest within the linear one (Fig. 4.10 A & B). Thus, the 2<BP<2.5 group sustained higher transpiration rates and better regulated its leaf water status at high VPD, associated with its higher K_{plant} and its components.

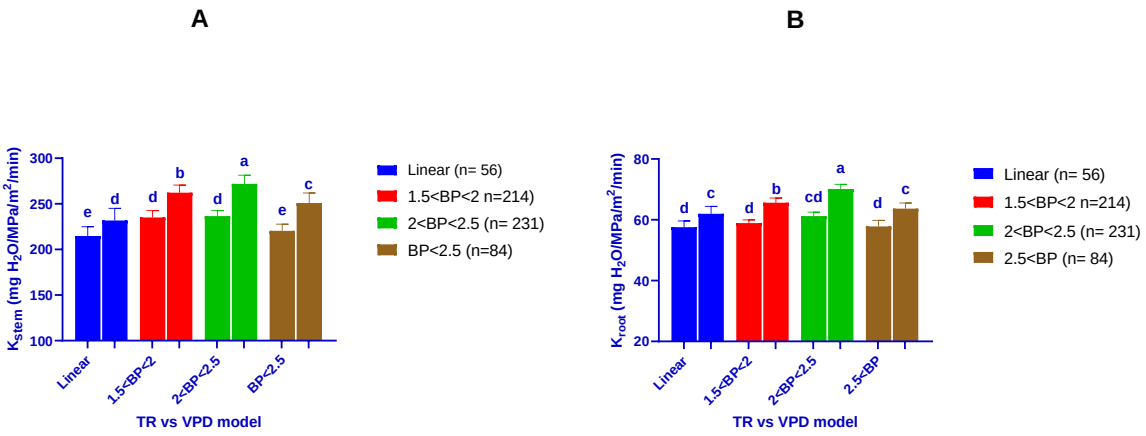


Figure 4.10 Differences between the four TR models of 165 RILs in their K_{stem} (A) and K_{root} (B) at the lowest (1st column in each group) and the highest (2nd column in each group) VPD levels. Data are means ± SE with different letters above the bars indicating significant (P< 0.05) differences according to T-test.

4.3.3 Linkage map construction

A total of 35,367 SNP markers were filtered for polymorphism between the parents, significant segregation distortion, and missing data. The final genetic map was composed of 4,089 SNP markers, which mapped to six linkage groups (LGs) representing the six chromosomes of faba bean (Table D-2). The linkage was 1253.13 cM long with 0.3 cM marker intervals. The LGs varied in their genetic distance with the shortest distance (122.3 cM) in LG3 (fewest SNPs) and the longest in LG1 (417.86 cM) where it contained the most SNPs (Table D-2).

Quantitative trait locus analysis was performed for minimum, maximum and break-point transpiration, and whole-plant and root hydraulic conductances at the minimum and maximum VPDs. Thirteen QTLs were identified in total, three QTLs for the minimum transpiration rate (TR_{min}) at chromosomes 1 and 3, one QTL for maximum transpiration rate at chromosome 3 (TR_{max}), one for transpiration at the BP (TR_{BP}) at chromosome 5, two QTLs for minimum hydraulic conductance at chromosome 1 ($Kplant_{min}$), three QTLs for maximum hydraulic conductance at chromosomes 1 and 3 ($Kplant_{max}$), two QTLs for minimum root hydraulic conductance at chromosome 1 ($Kroot_{min}$) and one QTL for maximum root hydraulic conductance at chromosome 3 ($Kroot_{max}$) (Fig. 4.11 & Table 4.3). The QTLs $qTR_{min}1.1$ and $qTR_{min}2.1$ accounted for 9.1 and 9.4 % of PVE (phenotypic variance explained), respectively. The QTLs $qKplant_{min}1.1$, $qKplant_{min}2.1$, $qKplant_{max}1.1$ and $qKplant_{max}2.1$ accounted for 11.5 %, 12.5 %, 9.9 % and 11.8 % of PVE, respectively. These QTLs were derived from ILB 938/2 and Mélodie/2 respectively, QTL qTRBP5.1 explained 10.8 % of the PVE (Table 4.3).

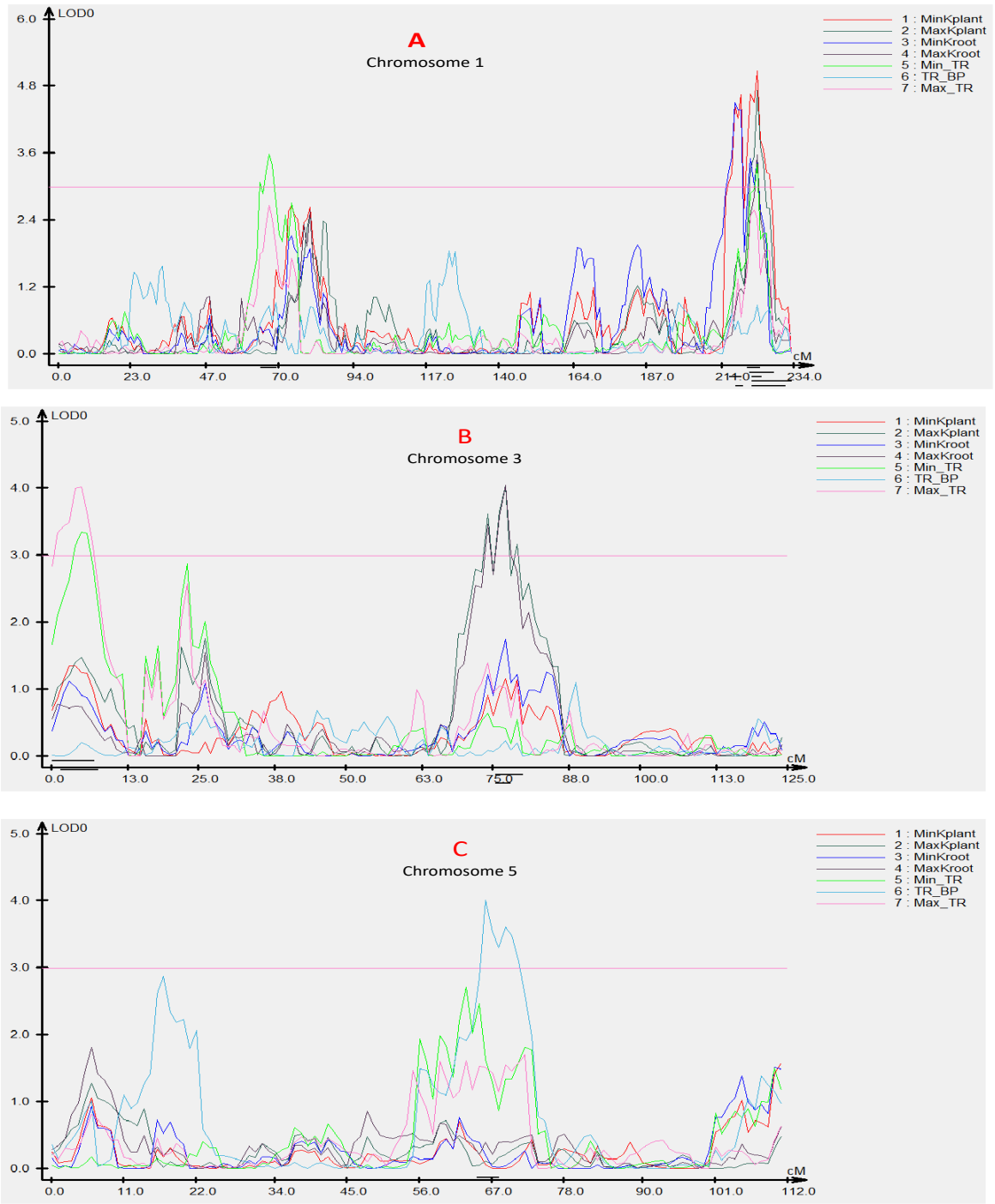


Figure 4.11 Composite interval mapping results for minimum, maximum and break-point transpiration (TR), whole-plant (K_{plant}) and root hydraulic conductance (K_{root}) and for break-point transpiration (TR-BP). The vertical axis is logarithm of odds (LOD) score, while the horizontal axis is the genetic distance along chromosomes 1 (A), 3 (B) and 5 (C), ascending in genetic position from left to right.

Table 4.3 Quantitative trait loci (QTL) for minimum and maximum transpiration, whole-plant and root hydraulic conductance and break-point transpiration traits in 142 RIL population derived from Mélodie/2 x ILB 938/2 at F8.

QTL	Chromosome	Peak (cM)	QTL interval	LOD	R^2 (%)[a]	Add[b]
$qTR_{min}1.1$	1	67.01	65.0-69.0	3.59	9.10	-1.02
$qTR_{min}2.1$	1	222.01	217.0-219.0	3.41	9.40	1.18
$qKplant_{min}1.1$	1	217.01	217.0-218.0	4.64	11.50	2.34
$qKplant_{min}2.1$	1	222.01	221.0-226.0	5.07	12.50	2.40
$qKplant_{max}1.1$	1	222.01	221.0-229.90	4.73	11.80	2.90
$qKplant_{max}2.1$	1	222.01	220.30-224.80	3.57	9.90	3.50
$qKroot_{min}1.1$	1	215.0	214.0-217.0	4.51	11.10	3.11
$qKroot_{min}1.1$	1	222.01	221.0 -225.30	3.51	8.70	2.80
$qTR_{min}1.3$	3	5.01	1.90-9.50	3.35	9.30	-1.12
$qTR_{max}1.3$	3	5.01	0.40-8.60	4.02	10.80	2.03
$qKplant_{max}1.3$	3	77.01	75.70 - 78.0	4.05	10.60	2.79
$qKroot_{max}1.3$	3	77.01	75.8 - 79	4.04	10.88	3.73
$qTR_{BP}1.5$	5	66.0	63-68	4.01	10.80	1.55

[a]R^2 - Percentage of phenotypic variance explained by QTL, [b]Additive genetic effect

BY using the SNP markers identified between two stable QTLS as BLASTn queries of the *M. truncatula* genome, several candidate genes were identified in the corresponding regions that may play a role in plant response to water deficit (Table 4.4).

Table 4.4 Candidate genes associated with QTLs for minimum and maximum transpiration, whole-plant and root hydraulic conductance and break-point transpiration traits based on BLASTn sequence similarity searches in the *Medicago truncatula (*Mt4.0v1) genome. The genes were queried using sequences of the SNP markers identified in the interval between the two stable QTLs.

QTL	Gene ID#	Position#	e -value	Descriptions
qTR_{min}1.1	Medtr2g025120	chr2:8925944..8928216	1.234e-19	OXIDOREDUCTASE, 2OG-FE
	Medtr4g132765	chr4:55522668..55527302	4.01e-13	/OXYGENASE FAMILY PROTEIN
qTR_{min}1.3	Medtr1g022365	chr1:7086220..7088202	3.76e-7	PTHR33472:SF3 - OXIDOREDUCTASE. Co-expressed with genes in roots-
qTR_{min}1.1	Medtr2g025540	chr2:9128940..9133614	1.82e-17	PTHR12956//PTHR12956:SF17 &
qTR_{min}1.3	Medtr4g133000	chr4:55643714..55648105	7.25e-10	SF28 ALKALINE CERAMIDASE-
	Medtr1g021670	chr1:6571053..6574863	4.89e-12	RELATED.Co-expressed with genes in leafspecific co-expression subnetwork.
qTR_{min}1.1	Medtr2g025180	chr2:8956289..8961013	1.71e-11	PTHR11216//PTHR11216:SF76 - EH DOMAIN. Co-expressed with genes in roots-specific co-expression subnetwork.
qTR_{min}1.1	Medtr2g025710	chr2:9201966..9203846	4.89e-12	Long-chain-alcohol O-fatty-acyltransferase / Wax synthase. Co-expressed with genes in roots.

$qTR_{min}1.1$	Medtr3g083370	chr3:37628014..37630303	7.25e-10	PTHR10209:SF193 –
	Medtr5g085330	chr5:36870741..36872929	1.71e-11	1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 3-RELATED. Co-expressed with genes in leaf-specific co-expression subnetwork.
$qTR_{min}2.1$	Medtr3g115220	chr3:53861604..53869489	2.53e-9	PTHR31818:SF1 – O
$qKplant_{min}1.1$				FUCOSYLTRANSFERASE-LIKE
$qKroot_{min}1.1$				PROTEIN. Co-expressed with genes in leaf-specific co-expression subnetwork.
$qKplant_{min}2.1$	Medtr5g097010	chr5:42463028..42466972	9.43e-15	PTHR22572:SF101 - GLUCOSE-1-
$qKplant_{max}1.1$				PHOSPHATE ADENYL TRANSFERASE
$qKroot_{min}1.1$				SMALL SUBUNIT, CHLOROPLASTIC
$qKplant_{max}2.1$				Co-expressed with genes in leaf-specific co-expression subnetwork.
$qKplant_{min}2.1$	Medtr5g096890	chr5:42397183..42404142	3.52e-20	PTHR12670:SF4 - NEUTRAL
$qKplant_{max}1.1$	Medtr3g061720	chr3:24621565..24627200	5.95e-11	CERAMIDASE-RELATED.
$qKroot_{min}1.1$				Co-expressed with genes in leaf
$qKplant_{max}2.1$				specific co-expression subnetwork.
$qKplant_{min}2.1$	Medtr5g096660	chr5:42271151..42278841	1.31e-25	PF00485//PF14681 –Phosphoribulo-
$qKplant_{max}1.1$				kinase / Uridine kinase family (PRK)
$qKroot_{min}1.1$				// Uracil phosphoribosyltransferase
$qKplant_{max}2.1$				(UPRTase). Co-expressed with genes in leaf specific co-expression.

qKplant$_{min}$2.1 **qKplant$_{max}$1.1** **qKroot$_{min}$1.1** **qKplant$_{max}$2.1**	Medtr7g029450	chr7:10536308..10543432	4.89-12	PTHR10285:SF71 - URIDINE KINASE-LIKE PROTEIN 1, CHLOROPLASTIC-RELATED. Co-expressed with genes in leaf-specific co-expression subnetwork.
qKplant$_{min}$2.1 **qKplant$_{max}$1.1** **qKroot$_{min}$1.1** **qKplant$_{max}$2.1**	Medtr3g061030	chr3:24230200..24237140	5.95e-11	2.4.2.9//2.7.1.48 - Uracil phosphoribosyl-transferase / UMP pyrophosphorylase // Uridine kinase / Uridine monophosphokinase. Co-expressed with genes in nodules-specific co-expression
qKplant$_{min}$2.1 **qKplant$_{max}$1.1** **qKroot$_{min}$1.1** **qKplant$_{max}$2.1**	Medtr5g096670	chr5:42278942..42281440	5.27e-24	(PTHR11627:SF19 -FRUCTOSE-BISPHOSPHATE ALDOLASE-RELATED Exhibits leaf specific expression.
qKplant$_{min}$2.1 **qKplant$_{max}$1.1** **qKroot$_{min}$1.1** **qKplant$_{max}$2.1**	Medtr5g096830 Medtr3g095790	chr5:42354421..42358871 chr3:43769207..43772320	2.08e-10 3.76e-7	PTHR23289 - CYTOCHROME C OXIDASE ASSEMBLY PROTEIN COX15. Co-expressed with genes in nodules-specific co-expression subnetwork. Co-expressed with genes in leaf specific co-expression subnetwork.
qKplant$_{min}$2.1 **qKplant$_{max}$1.1** **qKroot$_{min}$1.1**	Medtr5g096970	chr5:42438617..42447850	6.79e-23	PTHR11902:SF13 - CYTOSOLIC ENOLASE 3. Co-expressed with genes in leaf-specific co-expression

qKplant$_{max}$1.1	Medtr5g094770	chr5:41420060..41421993	1.5e-18	PF01535//PF13041//PF13812 - PPR repeat (PPR) // PPR repeat family (PPR_2) // Pentatricopeptide repeat domain (PPR_3).
qKroot$_{min}$1.1	Medtr1g018480	chr1:5334172..5343203	3.08e-8	PTHR11216:SF72 - CALCIUM-BINDING EF HAND-CONTAINING PROTEIN.
qKroot$_{min}$1.1	Medtr5g099240	chr5:43501965..43509933	1.5e-18	PTHR24349:SF83 - CALCIUM-DEPENDENT PROTEIN KINASE 6. Co-expressed with genes in roots-specific co-expression subnetwork.
qKplant$_{max}$1.1	Medtr5g095470	chr5:41729018..41729984	1.4e-12	PTHR23416:SF56 - SERINE ACETYLTRANSFERASE 1, CHLOROPLASTIC-RELATED.
qTR$_{min}$2.1 **qKplant$_{min}$1.1**	Medtr4g100810	chr4:41599236..41604242	2.37e-3	PF10250 - GDP-fucose protein O-fucosyl-transferase (O-FucT).
qTR$_{min}$2.1 **qKplant$_{min}$1.1** **qTR$_{min}$2.1**	Medtr5g098940 Medtr5g087820	chr5:43313469..43316302 chr5:38090611..38094429	1.31e-25 7.74e-16	PTHR11753//PTHR11753:SF16 - CLATHRIN COAT ASSEMBLY PROTEIN. Co-expressed with genes in leaf specific co-expression subnetwork.
qTR$_{min}$2.1 **qKplant$_{min}$1.1**	Medtr5g099010	chr5:43363456..43367269	9.43e-15	PTHR31818:SF0 - PROTEIN ROOT HAIR SPECIFIC 17.
qTR$_{max}$1.3	Medtr1g026540	chr1:8655619..8658952	2.89e-21	PTHR10366//PTHR10366:SF355 - NAD DEPENDENT EPIMERASE/DEHYDRATASE

$qTR_{max}1.3$	Medtr2g087640	chr2:36868869..36873363	1.5e-18	PTHR19877 - WD40 REPEAT PROTEIN.
$qTR_{max}1.3$	Medtr1g024005	chr1:7759631..7761462	2.53e-9	PTHR10108//PTHR10108:SF837 – METHYLTRANSFERASE. Co-expressed with genes in leaf specific co-expression subnetwork.
$qTR_{max}1.3$	Medtr1g023690	chr1:7618000..7621776	3.52e-20	PTHR13690:SF76 - BASIC-LEUCINE ZIPPER (BZIP) TRANSCRIPTION FACTOR FAMILY PROTEIN.
$qTR_{max}1.3$	Medtr4g019450	chr4:6083889..6090270	1.71e-11	PTHR11850:SF82 - BEL1-LIKE HOMEODOMAIN PROTEIN 8-RELATED. Co-expressed with genes in leaf-specific co-expression subnetwork.
$qTR_{max}1.3$	Medtr1g019870	chr1:6062058..6066368	4.89e-12	KOG2659 - LisH motif-containing protein. Co-expressed with genes in roots-specific co-expression subnetwork.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g022360	chr1:7083958..7084997	6.79e-23	PF01535 - PPR repeat (PPR) Co-expressed with genes in leaf specific co-expression subnetwork.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr3g112020	chr3:52412285..52417563	2.08e-10	PTHR13061//PTHR13061:SF10 - DYNACTIN SUBUNIT P25. Co-expressed with genes in roots-specific co-expression subnetwork.

$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g022225	chr1:6955941..6957574	1.5e-18	PTHR13930 - RSAFD1-RELATED. Co-expressed with genes in roots specific co-expression subnetwork.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g022190	chr1:6934778..6938822	4.01e-18	PTHR10996:SF123 - ERYTHRONATE-4-PHOSPHATE DEHYDROGENASE FAMILY PROTEIN.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g022160	chr1:6892246..6896988	2.08e-10	PTHR32227:SF16 - GLUCAN ENDO-1,3-BETA-GLUCOSIDASE 7-RELATED. Co-expressed with genes in roots specific co-expression subnetwork.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g021925	chr1:6671114..6675091	1.82e-17	PTHR10983:SF25 - 1-ACYL-SN-GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE 4-RELATED. Co-expressed with genes in leaf-specific co-expression subnetwork
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g021895	chr1:6658323..6660432	1.82e-17	PTHR12313//PTHR12313:SF10 - RNF5. Co-expressed with genes in roots specific co-expression subnetwork.
$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr1g021950	chr1:6680254..6688043	9.43e-15	Leishmanolysin / Promastigote surface endopeptidase. Co-expressed with genes in roots-specific co-expression subnetwork.

$qTR_{min}1.3$ **$qTR_{max}1.3$**	Medtr3g111530	chr3:52111957..52113811	2.89e-2	4.2.1.92 – Hydroperoxide dehydratase / Hydroperoxide isomerase Co-expressed with genes in leaf.
$qKplant_{max}1.3$ **$qKroot_{max}1.3$**	Medtr1g032520	chr1:11570552..11577948	4.58e-6	PF07058 - Microtubule-associated protein 70 (MAP70). Co-expressed with genes in nodules specific co-expression subnetwork
$qKroot_{max}1.3$	Medtr8g105260	chr8:44396772..44400394	6.79e-4	KOG0508//KOG4412 - Ankyrin repeat protein // 26S proteasome regulatory complex, subunit PSMD10. Co-expressed with genes in leaf specific co-expression subnetwork.
$qTR_{BP}5.1$	Medtr1g059700 Medtr7g105050	chr1:25950770..25955589 chr7:42588771..42592563	7.74e-16 7.74e-16	3.2.1.26 - Beta-fructofuranosidase / Saccharase
$qTR_{BP}5.1$	Medtr4g099100	chr4:41027737..41033129	2.89e-2	PTHR12439 - PLACENTAL PROTEIN 11-RELATED
$qTR_{BP}5.1$	Medtr4g052940	chr4:19205590..19210680	1.31e-6	KOG0110//KOG0131//KOG0148 - RNA-binding protein (RRM superfamily) // Splicing factor 3b, subunit 4 // Apoptosis-promoting RNA-binding protein TIA-1/TIAR (RRM superfamily).

qTR$_{BP}$5.1	Medtr7g105790	chr7:42945241..42946436	5.95e-11	PTHR33057:SF17 - GB Co-expressed with genes in leaf specific co-expression subnetwork.
qTR$_{BP}$5.1	Medtr7g105800	chr7:42948167..42955987	7.25e-10	PTHR13390 – LIPASE co-expressed with genes in nodules-specific co-expression subnetwork.
qTR$_{BP}$5.1	Medtr7g105870 Medtr7g106010 Medtr7g106000	chr7:42989208..42990385 chr7:43054682..43055402 chr7:43048977..43049967	2.53e-9 5.58e-5 2.37e-3	PTHR31415:SF4 - HARPIN-INDUCED PROTEIN-LIKE-RELATED. Co-expressed with genes in roots specific co-expression subnetwork
qTR$_{BP}$5.1	Medtr7g105100	chr7:42613545..42617300	4.01e-13	PTHR10992//PTHR10992:SF691 - ALPHA/BETA HYDROLASE FOLD-CONTAINING PROTEIN Co-expressed with genes in leaf specific co-expression subnetwork.
qTR$_{BP}$5.1	Medtr7g105170	chr7:42639030..42640463	1.08e-7	PF02365 - No apical meristem (NAM) protein (NAM).
qTR$_{BP}$5.1	Medtr7g105030	chr7:42575595..42582054	2.39e-11	PTHR24115:SF536 - 125 KDA KINESIN-RELATED PROTEIN-RELATED. Co-expressed with genes in roots-specific co-expression subnetwork.

$qTR_{BP}5.1$	Medtr7g104890	chr7:42517178..42524740	1.5e-18	PTHR11564//PTHR11564:SF19 - GTPASE CONTAINING FAMILY OF SIGNAL RECOGNITION PARTICLE PROTEINS. Co-expressed with genes in leaf-specific co-expression subnetwork.
$qTR_{BP}5.1$	Medtr7g104800	chr7:42483009..42489150	2.89e-21	KOG0117 - Heterogeneous nuclear ribonucleoprotein R (RRM superfamily). Co-expressed with genes in roots-specific co-expression subnetwork.
$qTR_{BP}5.1$	Medtr7g010360	chr7:2533492..2542856	2.37e-3	KOG0123 - Polyadenylate-binding protein (RRM superfamily). Co-expressed with genes in leaf-specific co-expression subnetwork.
$qTR_{max}1.1$	Medtr2g011480	chr2:2789349..2793998	3.52e-20	PTHR13063 - ENOS INTERACTING PROTEIN. Co-expressed with genes in nodules-specific co-expression subnetwork.
$qTR_{max}1.1$	Medtr2g009980 Medtr7g092250 Medtr3g111350	chr2:2169922..2176627 (PAC:31067297) chr7:36534179..36538356 chr3:52030513..52035160	6.79e-23 5.58e-5 2.53e-9	(PTHR22601: SF8, SF11, SF23 - OLIGOPEPTIDE TRANSPORTER 1,2,4. Co-expressed with genes in leaf-specific co-expression subnetwork.

$qTR_{max}1.1$	Medtr3g080870	chr3:36602875..36608789	1.31e-6	PTHR22601//PTHR22601:SF12 - ISP4 LIKE PROTEIN Component of root urea treatment-specific co-expression subnetwork

4.4 Discussion

This chapter focused on identifying genetic variation in transpiration response to evaporative demand, studying hydraulic conductance as a regulatory mechanism, and identifying genomic regions for the response in a population of 165 faba bean RILs and their parental lines.

Though both parental lines were considered drought-adapted (Abdelmula et al., 1999; Khan et al., 2007), they significantly differed in their TR response to VPD whereas Mélodie/2 had a linear TR model and ILB 938/2 revealed a segmented one. Consequently, the RILs showed a wide range of responses in all studied traits at the applied VPDs. Although ILB 938/2 (segmented TR model) always had a lower Ψ_{leaf} than Mélodie/2 (linear TR) at the two tested VPDs, Ψ_{leaf} of both genotypes responded similarly to VPD as indicated by no significant genotype x VPD interaction (Fig. 4.2 A & Table 4.1). Consequently, K_{plant} (TR/ Ψ_{leaf}) significantly differed between genotypes at both VPDs (Fig. 4.2 B & Table 4.1).

Since the parental lines had different responses to VPD, it seemed possible that RILs selected from this population would exhibit different TR responses to VPD such as linear comparable to Mélodie/2 and a segmented TR response with BPs within the range of ILB 938/2. Only 15 (9.5 %) were represented by a single linear regression over the entire range of VPD that matched Mélodie/2, and 65 genotypes (40 %) exhibited segment TR response with a 2<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 kPa, 61 RILs) and higher (BP> 2.5 kPa, 24 RILs) than the BP of ILB 938/2. The low BP of 61 RILs seemingly indicates a limitation on hydraulic conductance that may be more severe 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. Such a conclusion is consistent with the study of Sadok and Sinclair (2009 b), where none of the soybean genotypes that had a segmented TR genotype in their pedigree expressed the segmented TR trait. These results indicate that the trait responsible for this response is either recessive or dependent on a combination of alleles. Such a possibility may allow a better understanding of the genetic basis of this variability through QTL analysis on TR response to VPD. The observed stability or even the slightly higher TR after the BP (Fig. 4.5 B-D) is beneficial for improving crop performance under mild abiotic stress, as an alternative to stomatal closure under severe stress conditions (Collins et al., 2008).

In this population, a 3-fold increase in VPD decreased ψ_{leaf} by 30-35 %, possibly due to the feed-forward response of the stomata to high evaporative demand (Bunce, 1997; Buckley, 2005). Modelling trials revealed that changes in leaf hydraulic conductance could result in the feedforward response (Dewar, 2002; Buckley, 2005). Sadok and Sinclair (2010 b) suggested that 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 stomatal conductance and preventing a decline in TR in response to high VPD. The positive relationship between K_{plant} and TR (Fig. 4.9 A) is consistent with earlier observations (Weatherley, 1982; Else et al., 1995; Steudle and Peterson, 1998). Thus, the increase in hydraulic conductance observed from the lowest to the highest VPD minimized the decrease in Ψ_{leaf}, while

transpiration was raised. The dependence of Ψ_{leaf} on TR in well-watered plants (Fig. 4.9 B) is described by the equation $\Psi_{leaf} = \Psi_{soil} – K.TR$, where Ψ_{soil} is soil water potential, K is the hydraulic resistance on the way of water from the soil to leaf and E transpiration rate (Jones, 1983). So, a decline in Ψ_{leaf} is an inevitable result of an increase in transpiration since K (TR/ Ψ_{leaf}) is less affected by changes in Ψ_{leaf} (Fig. 4.9 C).

Based on measuring component hydraulic conductance within the plant, stem hydraulic conductance was always higher than root 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. This is comparable to the response of Masterpiece and Robin Hood (Chapter 3) and also consistent with the observations of Sivasakthi et al. (2020) in chickpea where limited root hydraulic conductance restricted TR at high VPD.

The high genetic variation between the RILs in transpiration response to VPD and other related traits promises the use of the limited transpiration trait at high VPD in breeding and genetics programs. Quantitative trait loci analysis for the RILs population was done to associate the potential QTLs with the minimum, maximum and BP transpiration and minimum and maximum whole-plant and root hydraulic conductance traits. Twelve QTLs were identified for minimum and maximum transpiration, whole-plant and root hydraulic conductance traits, while only one locus was detected on chromosome 5 associated with TR at the BP (Table 4.4 & Fig. 4.11). Interestingly, chromosomes 1 and 5 were previously reported to accommodate some QTLs associated with yield and yield distribution characters (Ávila et al., 2005). It is essential in MAS to validate these identified QTLs in improving faba bean for dry/water-limited conditions. Validating QTL involves testing whether the same QTL appears when growing the genetic material in other geographical locations or years and if it is still

detected when introduced into a different genetic background (Landi et al., 2005). This will help in unravelling the molecular basis of transpiration response to VPD, which will provide novel opportunities for more applications such as identifying genes responsible for transpiration traits. Most of the identified candidate genes were previously reported to regulate plant response to several abiotic stresses. Of these, OXIDOREDUCTASE, 2OG-FE /OXYGENASE FAMILY PROTEIN (Karati, et al., 2010), 1-CARBOXYLATE OXIDASE 3-RELATED (Kim et al., 1998), ALKALINE CERAMIDASE-RELATED genes (Zheng et al., 2018) and 1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 3-RELATED (synthesise ethylene, Houben and Poel (2019), Neutral Ceramides (Jasmonates & salicylicacid-related, Zienkiewicz et al. (2020), OLIGOPEPTIDE TRANSPORTERs, Protein kinases (Zhu, 2016; Qi et al., 2018; Gong et al., 2020) that are known to play crucial roles in plant responses to several abiotic stresses e.g., water deficit and reactive oxygen species (ROS). Interestingly, whole-plant and root hydraulic conductance accommodated a CYTOCHROME C OXIDASE that its deficiency in Arabidopsis thaliana resulted in less sensitivity to abscisic acid (Garcia et al., 2016), (Table 4.4). Similarly, in soybean, limited transpiration with associated with two QTLs that harbour several candidate genes, including a gene involved in abiotic stress tolerance (Sarkar et al., 2022). In wheat, six QTLs associated with transpiration response to VPD were identified in 143 RILs, of which, one major QTL accommodates genes involved in root hydraulic conductance and ABA signalling (Schoppach et al., 2016). It will be an interesting approach to associate these QTLs with other agronomic traits related to water conservation strategies to increase faba bean yield under dry conditions. These findings confirm that advanced mapping populations can reveal QTLs for water conservation traits under complicated genetic control to enhance limited TR at elevated VPD in a population of faba bean RILs population derived

from Mélodie/2 × ILB 938/2. The identified QTLs will be useful in molecular breeding for sustaining under water limited conditions.

4.5 Conclusion

More than 90 % of the faba bean RIL population restricted TR at high VPD, with considerable variation in the BP at which this occurred. Since maintaining low maximum TR at high VPD may be well suited for water-deficit environments, variation in the BP could allow cultivars to be developed with specific BP for differing water-deficit environments. A genotype with a low BP would likely be more suited for a dry environment than one with a high BP. These findings also confirm the role of plant hydraulic conductance in regulating TR at high VPD. The substantial decrease in root hydraulic conductance relative to stem hydraulic conductance restricted the water flow to the shoot and hence maintained transpiration at high VPD. This study provides the first QTL identification for transpiration response to VPD using high-resolution SNP markers. These identified QTLs can be used as potential targets for further genetic studies, and the linked DNA markers can enable the possibility of using marker-assisted selection in breeding faba bean for limited transpiration rates after validation in appropriate germplasm.

Chapter 5 General Discussion

Recently, plants have experienced progressive increases in vapour pressure deficit (VPD) due to the rise in global land surface temperature (0.2°C per decade) as a consequence of climate change (IPCC, 2019). Thus, identifying drought-tolerant traits and incorporating them into high-yielding cultivars is essential to sustain agriculture's viability under current and future climates. Although numerous physiological traits involved in plant responses to drought have been identified, only a few plant traits have been considered to improve drought tolerance. Plants respond to changes in VPD by regulating stomatal aperture, which in turn affects transpiration. Limiting maximum TR at high VPD has been reported to ameliorate the effects of late-season water deficit on plant performance and yield (Richards and Passioura, 1989, Sinclair et al., 2005, Zaman- Allah 2011 a & b). Genetic variation in TR response to VPD has been reported in many crop species including legumes (Fletcher et al., 2007; Sadok and Sinclair 2009 a & b; Devi et al., 2010; Zaman-Allah et al., 2011 a & b; Belko et al., 2012). Additionally, a few simulation studies have investigated the benefits of limited TR on crop yields (Sinclair et al., 2005 & 2010 & 2014; Messina et al., 2015; Sadok et al., 2019). However, a review of the available literature identified no information about such response and its physiological controlling mechanisms in faba bean (Chapter 1). Thus, this thesis aimed to identify genetic variation in TR response to VPD in faba bean and determine possible physiological and genetic (QTL analysis) mechanisms that regulate the limited TR trait.

Initially, TR response to VPD was studied in two British faba bean cultivars (Masterpiece and Robin Hood) by measuring whole-plant and single leaf transpiration using three different approaches i.e., gravimetric measurements using digital balances, single leaf TR using infra-red gas analysis and whole-plant TR in the whole-plant gas exchange chamber (Chapter 2).

To understand why both faba bean cultivars limited their TR at different VPD thresholds (2.3-3.0 kPa), whole-plant, root and stem hydraulic conductance along with leaf, root, and their xylem sap ABA concentrations were studied at different VPD levels (Chapter 3). The information obtained from the preceding chapters lead to a large-scale investigation of TR response to VPD to locate putative genes associated with the trait. Thus, Chapter 4 aimed to identify genetic variation in TR and hydraulic conductance response to VPD in 165 faba bean RILs generated by crossing Mélodie/2 and ILB 938/2 (Khan et al., 2007 & 2010; Khazaei et al., 2013 b), as well as undertaking QTLs to associate potential QTLs with transpiration response to VPD.

5.1 Identifying genetic variation in TR response to VPD in faba bean

At the whole-plant and single leaf levels under controlled and semi-controlled environments, regression analysis identified a segmented TR response model to VPD with a BP of 3.05 and 2.33 kPa in Masterpiece and Robin Hood, respectively (Fig. 2.4). These values are within the range reported in other legume species in which limited TR at high VPD has been identified (Sadok and Sinclair, 2009 a & b; Devi et al., 2010; Zaman-Allah et al., 2011 a; Belko et al., 2012). This could help develop genotypes with specific BP for differing water-deficit environments. The BPs and the slopes before (Slope 1) and after the BP (Slope 2) significantly differed between cultivars resulting in genotypic variation as indicated by significant genotype x VPD interaction (Table 2.1). The response was also confirmed across different times of the year/day, indicating the stability of the trait and the possibility to select for it directly.

The genotypic variation between Masterpiece and Robin Hood in their TR response to VPD suggests that much greater variability could be expected in a more diverse range of cultivars (Schoppach and Sadok, 2012), as well as complex inheritance of the trait that may be

regulated by more than one controlling mechanism. Indeed, investigating TR response to VPD in 165 RILs displayed diverse responses where almost 90 % (150 genotypes) showed a distinct response by limiting their TR when VPD reached about 1.7 to ~ 2.9 kPa while only 15 genotypes had consistently increased TR with increasing VPD. This decline in TR at high VPD may conserve soil water for use later in the growing season (Sinclair et al., 2017). However, in this study, the genotypes with linear TR response to VPD had similar maximum TR as those expressing the limited TR trait. Nevertheless, the maximum TR in genotypes expressing the limited TR trait occurred at lower VPDs (1.7-2.9 kPa) than genotypes without TR limitation (3.2 kPa). Thus, under late-season water deficit, the limited TR genotypes could utilise conserved soil water which would enhance yield compared to the genotypes without the limited TR trait. Indeed, simulation analysis of soybean production in the United States and sorghum in Australia revealed a yield increase in about 75 % of the seasons, as a result of limiting transpiration at high VPD (Sinclair et al., 2005 & 2010). However, under wet conditions, the trait resulted in yield loss, indicating it was beneficial only under mild and severe drought conditions (Sinclair et al., 2010).

Of course, future simulations in faba bean genotypes that express the limited TR would help in assessing the utility of this trait for yield improvement in environments where mild or severe stress occurs. Most of the existing models in faba bean are focused on relating yield with phenology stages and irrigation regimes. For instance, mid-flowering in faba bean was simulated under different sowing dates and watering regimes in south-eastern Australia (Zeleke and Nendel, 2019). Under rainfed and semi-arid environments, early flowering cultivars complete their reproductive cycle relatively early and consequently might not be significantly influenced by late water deficit and heat stress incidents. Hence, a relatively stable production can be expected. The fully irrigated plants had only a slightly higher yield

than those irrigated only during the reproductive stage (late October) since grain yield is more determined by changes in the environmental conditions (drought or rainfall/irrigation) at the starting- and during grain filling than pre-anthesis periods (De Costa et al., 1997). Therefore, the environmental conditions and growth after flowering can have significant effects on the final grain yield.

5.2 Low hydraulic limits transpiration under high VPD in faba bean

Limited TR response to VPD has been widely confirmed in several crop species, however, the mechanism of stomatal response to VPD remains poorly understood (McAdam and Brodribb, 2015). Some studies have suggested limited TR under high VPD occurred passively due to limitation in plant hydraulic conductance (Sinclair et al., 2017), while others proposed that stomatal regulation occurred actively via the plant hormone ABA in co-ordination with the passive hydraulic process (McAdam and Brodribb, 2014). These metabolic processes are prompted by low Ψ_{leaf} (McAdam et al., 2016) which might limit TR. Indeed, both Masterpiece and Robin Hood significantly decreased their K_{Plant} (Fig. 3.1 D) at the highest VPD (3.57 kPa) with K_{Plant} highly positively associated with TR (Fig. 3.2 B), but not with Ψ_{leaf} (Fig. 3.2 C). These restrictions in K_{Plant} were mainly located in the roots since measuring root and stem hydraulic (K_{root} & K_{stem}) conductance revealed lower conductance in the roots than in stems (Fig. 3.3 A & B). Similar responses were observed in the RILs as well, where K_{Plant} was significantly relattoith TR (Fig. 4.9 A) and K_{root} was always lower than K_{stem} (Fig. 4.10 A & B) across the entire population. This is also consistent with the observations in chickpea (Sivasakthi et al.,

2020), wheat (Schoppach et al., 2014) and pearl millet (Tharanya et al., 2018) where lower root hydraulic conductance restricted transpiration at high VPD.

5.3 ABA accumulation in coordination with passive hydraulic signals limit transpiration under high VPD in faba bean

Xylem sap ABA concentrations (and leaf ABA levels) increased as VPD increased until 2.4 kPa, then declined significantly at the highest VPD consistently in Masterpiece and Robin Hood (Fig. 3.5 A & C & D). This is inconsistent with the common model where ABA levels increase continuously with VPD. On the other hand, root ABA concentrations increased in both genotypes as VPD increased with a greater increase in Robin Hood than in Masterpiece (Fig. 3.5 B). The decline in leaf ABA at high VPD indicates that stomatal closure is a passive hydraulic ABA-independent process (Brodribb and McAdam, 2011; McAdam and Brodribb, 2015). One possible explanation for decreased ABA levels at high VPD is that the decrease in Ψ_{leaf} was not sufficient to trigger leaf ABA accumulation at the highest VPD. In pea (*Pisum sativum* L.), ABA accumulation was not observed unless the leaves were pressurized to more than -1 MPa, sufficient to induce turgor loss and hence ABA accumulation (McAdam and Brodribb, 2014). In this investigation, at the highest VPD (> 3.5 kPa) Masterpiece and Robin Hood lowered their Ψ_{leaf} to levels (<-1 MPa) that were insufficient to induce foliar ABA accumulation (according to McAdam and Brodribb, 2014).

One possibility for the continuous increase in root ABA as VPD increases is inhibition of the ABA export from the roots to the shoots (Kudoyarova et al., 2011). Indeed, a 14-18 % decline in ABA concentration was detected in the xylem sap at the highest VPD (Fig. 3.5 D). The strong negative relationship between root ABA and canopy conductance (Fig. 3.7 B) supports the

hypothesis that stomatal closure occurs passively in coordination with the metabolic active signals (ABA). However, this is inconsistent with the conventional role of root ABA in increasing root hydraulic conductance, thus maintaining higher water status by improving the supply of water to the shoot at high VPD (Thompson et al.,2007). In transformed maize lines with higher NCED (9-cis-epoxycarotenoid dioxygenase) gene expression, higher root hydraulic conductance and ABA concentrations were associated with faster recovery of Ψleaf and growth upon re-watering (Parent et al., 2009). However, these responses were transient (Hose et al., 2000) and ABA dose-dependent (Beaudette et al., 2007).

The role of exogenous ABA application on influencing root hydraulic conductance is still controversial with a positive effect (Ludewig et al., 1988; Zhang et al., 1995; Hose et al., 2000; Mahdieh and Mostajeran, 2009), no effect (Aroca et al., 2003), and variable effect depending on the applied concentration or a negative effect in the shoot (Pantin et al., 2013). These varied results are possibly due to species differences or the experimental approaches to modify ABA, e.g., the exogenous ABA concentration or the exposure period. This variable effect of ABA on plant hydraulics may be reconciled by a unified dose-response curve to exogenous ABA application (Dodd, 2013). Taken together, elevated VPD induces root ABA accumulation with varied effects on plant hydraulics based on its concentrations, such that relatively high concentrations can sustain water status, but even higher concentrations restrict hydraulic conductance and consequently decreases TR.

The important role of hydraulic signals in regulating stomatal conductance has been largely investigated, while the role of ABA in regulating hydraulic conductance is still equivocal. Therefore, a detailed understanding of the mechanism(s) by which endogenous ABA regulates hydraulic conductance under prolonged exposure to VPD awaits further investigation. This may include investigating its effect on inducing aquaporins (AQP) genes (Maurel et al., 2008).

In the short-term (45 min) experiments conducted here, it is doubtful that any changes in AQP expression are likely to affect corresponding protein levels. Alternatively, ABA is likely to determine post-transcriptional level of AQP (Sharipova et al., 2016). Since ABA can regulate AQPs activity via their phosphorylation (Chaumont and Tyerman, 2014), the high association between leaf and root xylem saps ABA and hydraulic conductance (Table 3.1) could be explained through this mechanism.

5.4 Identifying QTLs and candidate genes associated with plant transpiration and hydraulic conductance response to VPD

Molecular marker technologies could supplement traditional faba bean breeding procedures to help generate cultivars appropriate for addressing the expanding global demand for faba bean. To associate transpiration response to VPD of the RILs (Chapter 4) with putative QTLs, quantitative trait loci analysis was undertaken for three transpiration traits e.g., minimum, maximum transpiration and break-point transpiration rates, whole-plant and root hydraulic conductance traits. One locus was identified for break-point transpiration on chromosome 5 while, twelve QTLs were associated with other traits distributed between chromosomes 1 and 3. (Fig. 4.11 & Table 4.3). Most of the identified QTLs harboured candidate genes related with regulation plant response to several abiotic stresses (Table 4.4). Oxidoreductase and oxygenase family proteins (Karati, et al., 2010) are well known to regulate plant response to ROS. Downregulation of CYTOCHROME C OXIDASE in *Arabidopsis thaliana* decreased the sensitivity to abscisic acid (Garcia et al., 2016). Also, AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 3-RELATED, the final step in ethylene synthesizing pathway (Houben and Poel, 2019) suggested an association between plant transpiration and ethylene accumulation.

5.5 Concluding remarks

Taken together, this research has shown:

Phenotyping transpiration response to VPD is independent of the measurement approach and could be done whenever the targeted VPD is achievable.

Evaporative flux method is a viable technique for measuring whole-plant hydraulic conductance and its components.

Limiting maximum transpiration is the most common response of faba bean to high VPD with considerable genetic variation in the break-point at which this occurred.

Limited transpiration is a stable trait and independent of time of day/year when measured.

Lower break-point is always associated with higher slope 1 values indicating maximizing gas exchange at low VPD and shifting to water conservation strategy as VPD increased.

Genetic variation in whole-plant hydraulic conductance is better explained by variation in transpiration than leaf water potential.

Limited transpiration response under high VPD is regulated by limitation in root hydraulic conductance in coordination with root ABA accumulation.

Minimum transpiration rates are associated with three QTLs on chromosomes 1 (2 QTLs) and 3 (one QTL), while maximum and break-point transpiration rates are associated with one locus each on chromosome 3 and 5, respectively.

Minimum whole-plant hydraulic and root hydraulic condutances are associated with four QTLs on chromosome 1 (two each), while maximum whole-plant and root hydraulic conductances are associated with four QTLs (two each) on chromosomes 1 and 3. These QTLs accommodate some candidate genes that regulate plant response to multiple abiotic stresses, e.g., water deficit and reactive oxygen species.

Refereces

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Appendices

Appendix A

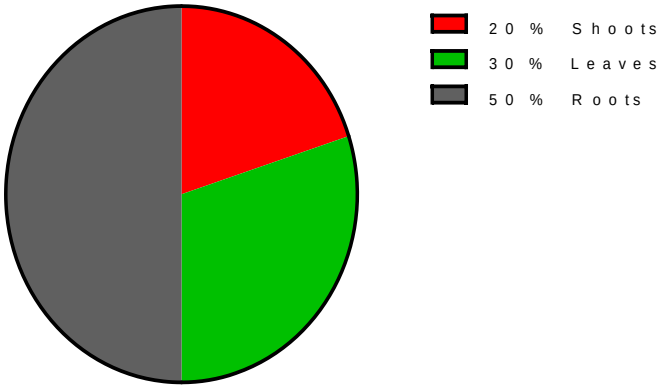

Figure A-1 Plant organ's contribution in the whole-plant hydraulic resistance in dicotyledons (A) where half of the resistance is located in the roots and the other half is attributed to the aerial parts of which, 30 % of the whole-plant resistance is located in the leaves (Sack et al., 2003; Sack and Halbrook, 2006).

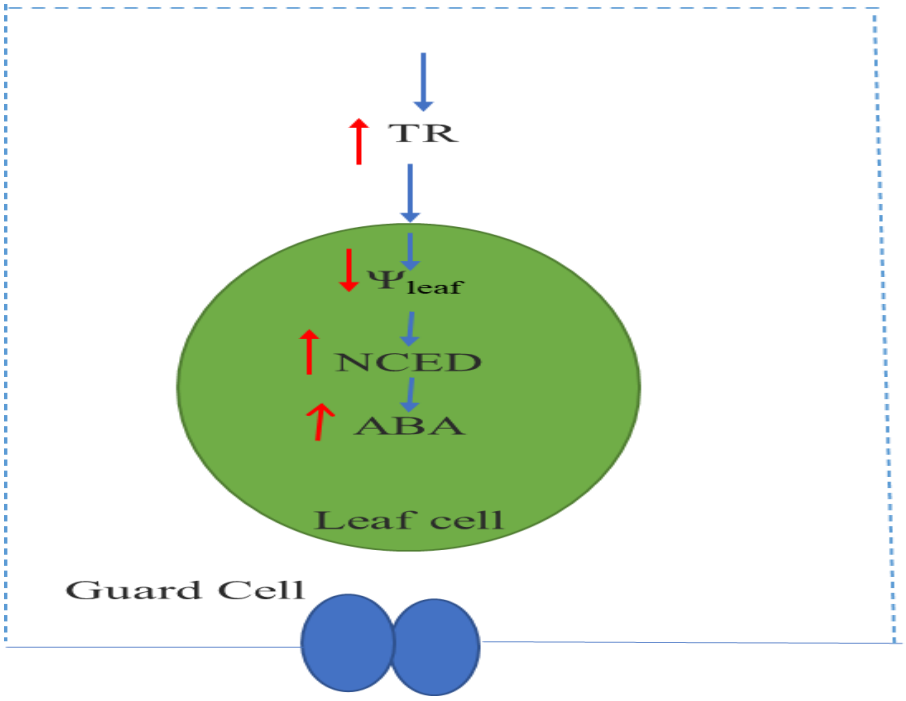

Figure A-2 A schematic of the proposed mechanistic model for ABA-mediated stomatal closure in response to increased VPD in angiosperms. At high VPD, transpiration (TR) increases, decreasing leaf water potential (Ψ_{leaf}) and leaf turgor pressure (Sussmilch et al., 2017).

Appendix B

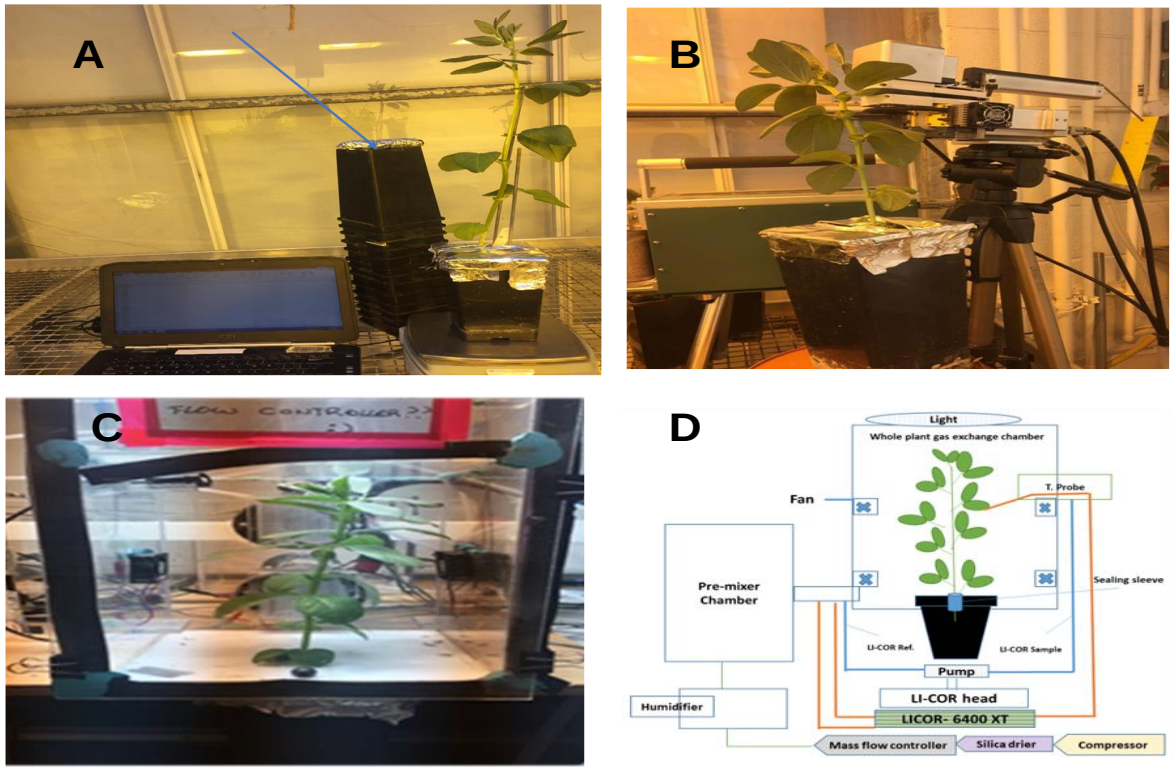

Figure B-1 Measuring whole-plant transpiration response to VPD gravimetrically in the glasshouse (A), where environmental conditions were recorded with the sensor probe (blue arrow) which was wrapped in aluminum foil to shield it from radiative heating, measuring leaf transpiration response to VPD using infra-red gas analysis in the glasshouse (B), measuring whole-plant transpiration in the whole-plant gas exchange chamber (C) and a schematic of whole-plant gas exchange chamber system (D) adapted from Jauregui et al., 2018.

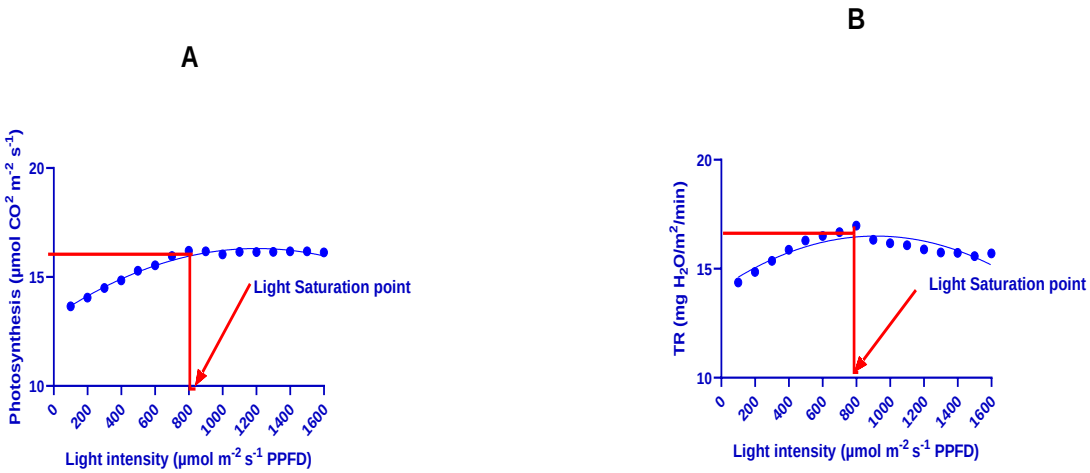

Figure B-2 Single leaf photosynthesis (A) and transpiration (B) light response curve of an individual Robin Hood plant in the glasshouse under stable atmospheric temperature (28 ^{o}C). A range of 100-1600 µmol m^{-2} s^{-1} was imposed in the IRGA cuvette and an average of 5 minutes of photosynthesis and transpiration rates were recorded after about 5 minutes of steady-state. Maximum values were achieved at 800 µmol m^{-2} s^{-1} (saturation point) thereafter photosynthesis stabilized while transpiration declined at the higher light intensities.

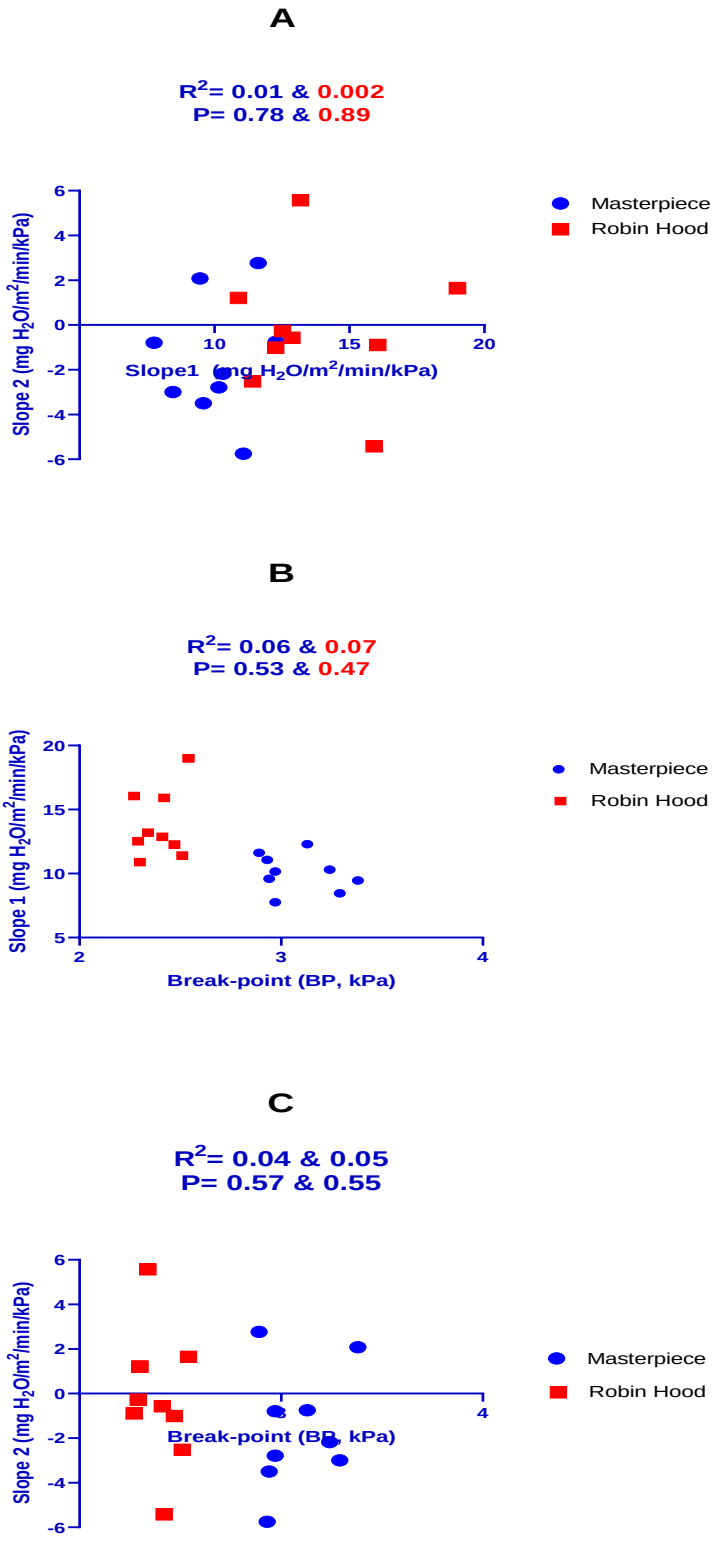

Figure B-3 The relationship between the two slopes (A), slope 1 and the BP, and slope and the BP (C) of Masterpiece and Robin Hood on the three systems. Data are individual plants of

each cultivar, P values, and R^2 for regression coefficient analysis are reported on the top of each panel.

Table B-1 Measurement sequence of Experiment 1 which used different measurement approaches with the same plants over consecutive days.

Date	Plant	System	Time of the day	VPD range in the glasshouse (kPa)
10/06/2018	RH 1	Balance		0.55 – 3.47
11/06/2018	RH 2	Balance		0.49 – 2.74
12/06/2018	RH 3	Balance	24 h	0.39 - 3.07
13/06/2018	MP 1	Balance		0.49 - 4.40
14/06/2018	MP 2	Balance		0.54 - 4.04
15/06/2018	RH 1	Infra-red gas analyser	Morning	0.80 - 4.40
	RH 2		Afternoon	
	RH 3		Late afternoon	
	MP 3	Balance	24 h	
16/06/2018	RH 1	Whole-plant gas exchange	Morning	0.78 - 3
	MP1	chamber	Afternoon	
	RH 2		Late afternoon	
	MP 1	Infra-red gas analyser	Morning	
	MP 2		Afternoon	
	MP 3		Late afternoon	
17/06/2018	RH 3	Whole-plant gas exchange	Morning	0.50 - 2.91
	MP 2	chamber	Afternoon	
	MP 3		Late afternoon	

Table B-2 Measurement sequence of Experiment 2 which used gravimetric measurements over 24 hours at two different times of the year.

Date	Plant	Min. Temp.	Max.Temp.	VPD range in the glasshouse (kPa)
17/07/2018	MP 1	19.1	39.9	0.66 - 4.50
18/07/2018	RH 1	18.3	38.9	0.75 - 4.76
19/07/2018	MP 2	18.7	39.9	0.65 - 4.70
20/07/2018	RH 2	17.8	37.2	0.50 – 4.50
21/07/2018	MP 3	19.9	39.8	0.59 – 4.97
22/07/2018	RH 3	17.1	37.3	0.50 – 4.00
09/10/2018	RH 4	15.6	31.3	0.67 - 3.70
10/10/2018	MP 4	15.2	33.8	0.69 - 4.15
11/10/2018	RH 5	18.3	29.6	0.66 - 3.47
12/10/2018	MP 5	15.5	30.3	0.64 – 4.40
13/10/2018	RH 6	17.7	28.6	0.56 – 3.01
14/10/2018	MP 6	16.7	30.3	0.73 - 4.42

Table B-3 Measurement sequence of Experiment 3 within the whole-plant gas exchange chamber at three different times of the day.

Date	Plant	Time of the day	VPD range in the glasshouse (kPa)	VPD range in the chamber (kPa)
19/11/2019	MP 1	Morning	0.48 - 2.36	1.53 – 3.58
	RH 1	Afternoon		1.33 - 3.73
	RH 2	Late afternoon		1.38 – 3.26
20/11/2019	RH 3	Morning	0.64 - 3.35	1.41 – 3.53
	MP 2	Afternoon		1.54 – 3.64
	RH 4	Late afternoon		1.10 – 3.31
21/11/2019	MP 3	Morning	0.60 - 2.49	1.55 – 3.51
	RH 5	Afternoon		1.28 – 3.76
	MP 4	Late afternoon		1.53 – 3.63
22/11/2019	RH 6	Morning	1.09 -1.97	1.56 – 3.18
	MP 5	Afternoon		1.44 – 3.64
	RH 7	Late afternoon		1.00 – 3.54
23/11/2019	MP 6	Morning	0.97 - 2.37	1.30 – 3.33
	RH 8	Afternoon		1.34 – 3.83
	MP 7	Late afternoon		1.49 – 3.60
24/11/2019	RH 9	Morning	0.78 - 2.01	1.35 – 3.00
	MP 8	Afternoon		1.50 – 3.54
	MP 9	Late afternoon		1.42 – 3.51

Appendix C

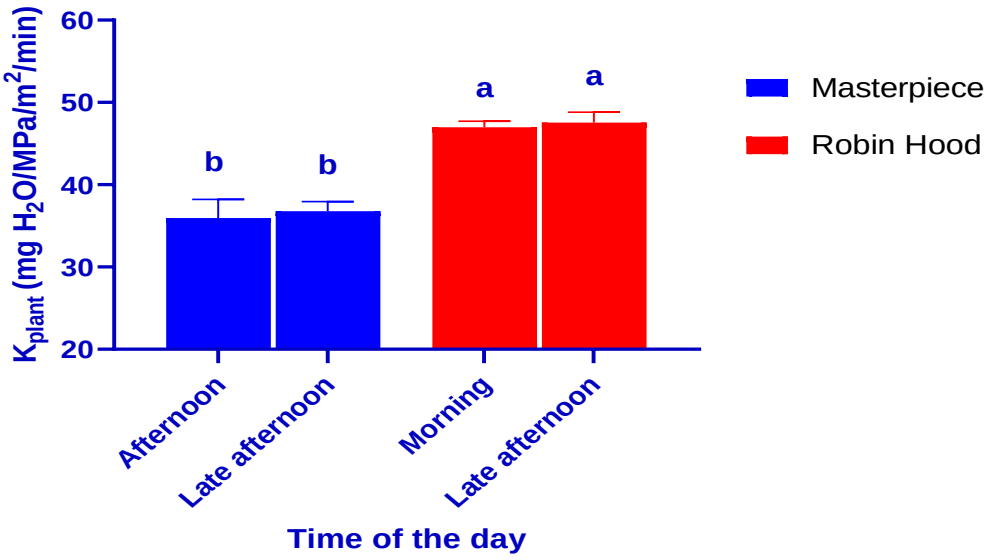

Figure C-1 K_{plant} of Masterpiece and Robin Hood at (1.39 ± 0.01) kPa achieved at different times of the day. Data are means ± SE of 2-3 plants at each time of day, with identical letters above the bars indicating non-significant (P> 0.05) differences according to T test.

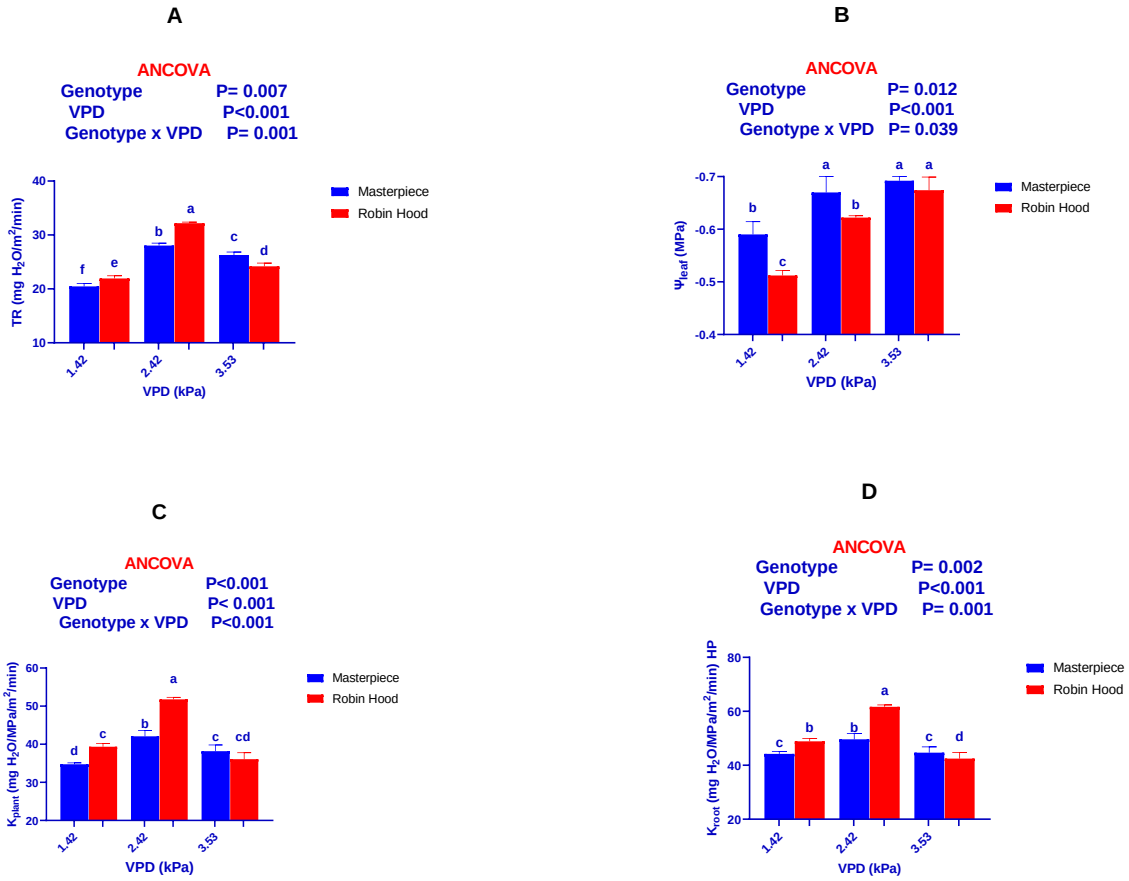

Figure C-2 TR (A), Ψ_{leaf} (B), K_{plant} (C) and K_{root} (D) of Masterpiece and Robin Hood cultivars at different VPD levels in Experiment 3. Data are means ± SE of five replicates, with different letters above the bars indicating significant (P< 0.05) differences according to T test. P values from ANCOVA are indicated above each panel.

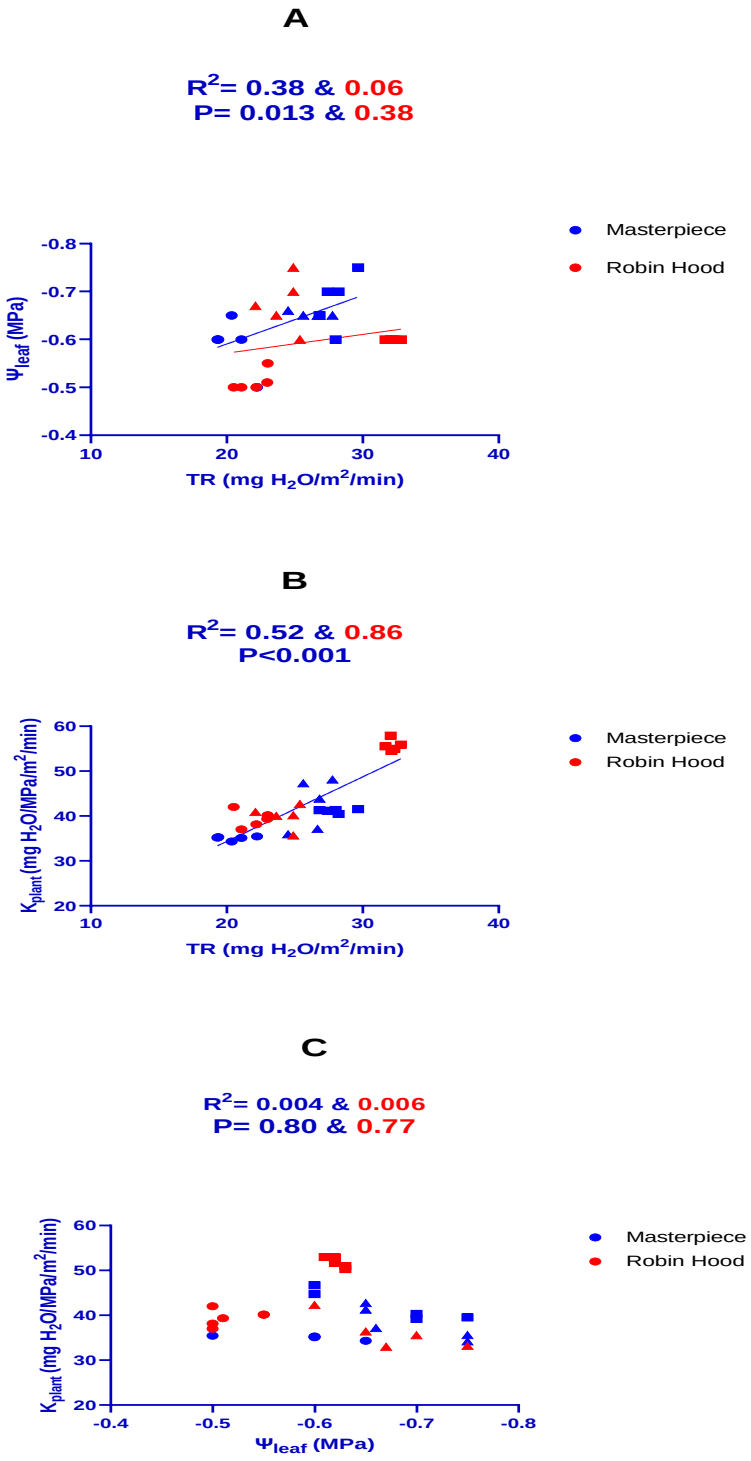

Figure C-3 The relationship between Ψ_{leaf} & TR (A), K_{plant} & TR (B), and K_{plant} & Ψ_{leaf} (C) of Masterpiece and Robin Hood in Experiment 3. Points are individual plants at the lowest (●), the intermediate (■) and the highest VPD (▲). $R^2$and P values are indicated above each panel.

Table C-1 Experimental design of the measurement sequence of TR response to VPD of Masterpiece (MP) and Robin Hood (RH) in the Whole-plant gas exchange chamber.

Exp.	Measurements	Pot type	Date	Time	Genotype / Plant No.	Applied VPD (kPa)
1. Measuring whole-plant hydraulic conductance and it components with the evaporative flux method (EF).	TR, Ψ_{leaf}, Ψ_{stem}, K_{plant}, K_{root} and K_{stem}	Rectangular 2-L pots (12.5 top × 10.5 base × 21 cm height)	28/01/2019	10:30	RH 1	1.42
				12:45	MP 1	1.43
				15:00	RH 2	1.34
				17:15	RH 3	1.40
				19:30	MP 2	1.44
			29/01/2019	10:45	RH 4	1.40
				12:45	MP 3	1.42
				14:30	RH 5	1.38
				16:15	MP 4	1.33
				18:30	MP 5	1.34
			30/01/2019	10:50	RH 6	2.42
				12:40	RH 7	2.42
				14:30	MP 6	2.40
				16:45	RH 8	2.47
				19:00	MP 7	2.45
			31/01/2019	10:20	RH 9	2.40
				12:30	MP 8	2.42
				14:30	RH 10	2.40
				16:40	MP 9	2.42
				18:55	MP 10	2.43
			01/02/2019	10:15	RH 11	3.55
				12:30	RH 12	3.51
				14:15	MP 11	3.45
				16:25	RH 13	3.70
				18:30	MP 12	3.46

			02/02/2019	10:40	RH 14	3.70
				12:45	MP 13	3.55
				14:25	RH 15	3.70
				16:15	MP 14	3.61
				18:45	MP 15	3.49
2. Root hydraulic conductance with pressure chamber technique	K_{root} (EF) and K_{root} (RP)	One-litre cylindrical pots (20 cm high×9 cm diameter)	12/03/2019	10:22	MP 1	3.65
				12:23	RH 1	3.50
				14:52	MP 2	3.60
				17:05	RH 2	3.58
			13/03/2019	10:28	MP 3	3.60
				12:32	RH 3	3.62
				14:23	MP 4	3.66
				16:56	RH 4	3.63
3. **Measuring ABA responses to different VPD levels.**	TR, Ψ_{leaf}, Ψ_{stem}, K_{plant}, K_{root}, K_{stem} and leaf, root, leaf xylem sap and root xylem sap ABA concentrations	One-litre cylindrical pots (20 cm high×9 cm diameter)	25/11/2020	9:30	RH1	1.44
				11:35	MP1	1.40
				14:01	RH 2	1.44
				15:55	MP 2	1.47
				17:23	RH 3	1.40
			26/11/2020	10:05	MP 3	1.42
				11:40	RH 4	1.44
				13:18	MP 4	1.44
				14:56	RH 5	1.41
				17:23	MP 5	1.38
			27/11/2020	09:33	RH 6	2.44
				11:23	MP 6	2.38
				13:30	RH 7	2.42
				15:20	MP 7	2.43
				17:30	RH 8	2.40

28/11/202	09:48	MP 8	2.40
	12:44	RH 9	2.39
	14:39	MP 9	2.46
	16:15	RH 10	2.38
	18:05	MP 10	2.50
29/11/2020	09:00	RH 11	3.45
	11:30	MP 11	3.46
	13:14	RH 12	3.44
	14:48	MP 12	3.48
	16:32	RH 13	3.55
30/11/2020	10:17	MP 13	3.53
	11:56	RH 14	3.65
	13:48	MP 14	3.50
	15:11	RH 15	3.60
	17:09	MP 15	3.58

Appendix D

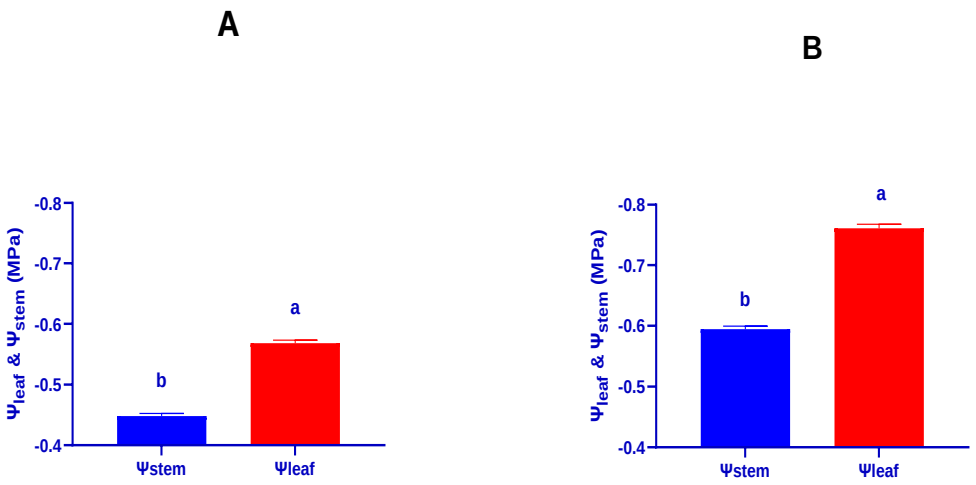

Figure D-1 Differences between Ψ_{leaf} and Ψ_{stem} of the RILs population derived from Mélodie/2 and ILB 938/2 at the lowest (A) and the highest (B) VPD. Data are means ± SE of four plants of each RIL across 165 RILs, with different letters above the bars indicating significant ($P< 0.05$) differences according to the T test.

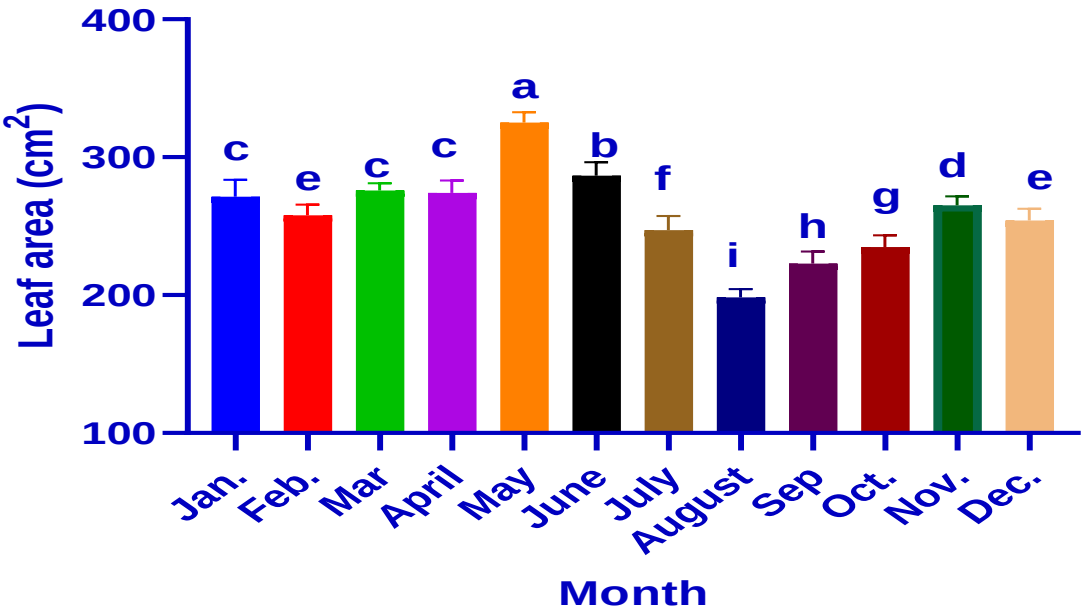

Figure D-2 differences in leaf area of RILs derived from Mélodie/2 and ILB 938/2 across the months the plants were harvested. Data are means ± SE of four plants of each RIL, with different letters above the bars indicating significant (P< 0.05) differences according to the T test.

Table D-1 Mean (± SD) of some agronomic and morphological characters in Mélodie/2 and ILB938/2 measured under glasshouse conditions in 2013 at the Department of Agricultural Sciences, University of Helsinki, Finland.

Parental line	Dry weight/ plant	No. of seeds/ plant	No. of pods/ plant	No. of seeds/ pod	Vicine-convicine (% of dry seed)	Seed coat colour	Hilum colour	Stipule spot pigmentation colour	Funicle colour
Mélodie/2	26.40	32.29	10.2	4.57	0.02	beige	colourless	coloured	brown
SD	2.77	4.2	4.0	0.65	-	-	-	-	-
ILB 938/2	14.35	8.09	4.0	2.23	1.17	green	coloured	colourless	yellow
SD	1.46	1.39	1.07	0.27	-	-	-	-	-

† Khamassi et al. (2013)

Khamassi K, Jeddi FB, Hobbs D, Irigoyen J, Stoddard FL, O'Sullivan DM, Jones H (2013) A baseline study of vicine–convicine levels in faba bean (*Vicia faba* L.) germplasm. Plant Genet Resources 11:250–257

Table D-2 Characteristics of the genetic linkage map for the 142 faba bean RIL population derived from Mélodie/2 x ILB 938/2 at F8. (**Source Gela et al., 2021**)

Chromosomes (Linkage groups)	**Number of markers Map length (cM)**	**Map length**	**Average marker interval (cM)**
1	1262	417.86	0.33
2	656	220.99	0.34
3	488	122.30	0.25
4	668	183.73	0.28
5	488	140.40	0.28
6	527	167.58	0.32
Total	4089	1252.86	