

REVIEW PAPER

Transpiration efficiency: new insights into an old story

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Abstract

Producing more food per unit of water has never been as important as it is at present, and the demand for water by economic sectors other than agriculture will necessarily put a great deal of pressure on a dwindling resource, leading to a call for increases in the productivity of water in agriculture. This topic has been given high priority in the research agenda for the last 30 years, but with the exception of a few specific cases, such as water-use-efficient wheat in Australia, breeding crops for water-use efficiency has yet to be accomplished. Here, we review the efforts to harness transpiration efficiency (TE); that is, the genetic component of water-use efficiency. As TE is difficult to measure, especially in the field, evaluations of TE have relied mostly on surrogate traits, although this has most likely resulted in over-dependence on the surrogates. A new lysimetric method for assessing TE gravimetrically throughout the entire cropping cycle has revealed high genetic variation in different cereals and legumes. Across species, water regimes, and a wide range of genotypes, this method has clearly established an absence of relationships between TE and total water use, which dismisses previous claims that high TE may lead to a lower production potential. More excitingly, a tight link has been found between these large differences in TE in several crops and attributes of plants that make them restrict water losses under high vapour-pressure deficits. This trait provides new insight into the genetics of TE, especially from the perspective of plant hydraulics, probably with close involvement of aquaporins, and opens new possibilities for achieving genetic gains via breeding focused on this trait. Last but not least, small amounts of water used in specific periods of the crop cycle, such as during grain filling, may be critical. We assessed the efficiency of water use at these critical stages.

Key words: Aquaporins, carbon-isotope discrimination, CID, drought, grain filling, hydraulics, post-anthesis water use, vapour-pressure deficit, VPD, water stress.

Introduction

Producing more food per unit of water has become a major concern for humanity because fresh water resources are finite in nature and scarce in areas with large populations and increasing population growth rates. The agriculture sector consumes approximately 75% of the total fresh water, although this proportion falls to approximately 50% in industrial nations where the domestic and industrial use of water is much higher. Therefore, as societies develop there will be

increasing competition for water resources between the agricultural and development sectors, which will undoubtedly increase pressure on the agriculture sector to produce more food with less water. Water productivity can be defined at different levels (e.g. see Condon *et al.*, 2002). At a plot level, it can be defined as water-use efficiency (WUE) = grain yield/water received (in millimetres; through either irrigation or rain) or as WUE = total biomass/evapotranspiration. At a

Abbreviations: CIE, carbon-isotope discrimination; FTSW, fraction of transpirable soil water; SCMR, Soil Plant Analysis Development chlorophyll meter reading; SLA, specific leaf area; TE, transpiration efficiency; VPD, vapour-pressure deficit; WUE, water-use efficiency.

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plant level, the transpiration efficiency (TE), an important component of WUE, is defined as $TE = \text{biomass}/\text{water transpired}$. At the leaf level, TE is defined as the intrinsic WUE; that is, the ratio of instantaneous CO_2 assimilation (A) to transpiration (T) = A/T . These definitions illustrate that water productivity can be approached from different perspectives, broadly in terms of agronomic and genetic aspects. In this review we will focus exclusively on the genetic aspects of TE. The agronomic perspective of WUE has been covered in other reviews (e.g. Hsiao *et al.*, 2007; Kirkegaard and Hunt, 2010; Passioura and Angus, 2010).

The first section of this review will address earlier works on TE, especially concerning the discovery of discrimination against the heavy form of carbon, ^{13}C , by Rubisco and by the stomata and the realization that this discrimination could be used as a screening method for TE analyses (Farquhar *et al.*, 1982). One reason for using surrogate measures of TE is the difficulty of measuring TE gravimetrically, by assessing biomass increases and plant water use on a long-term basis. Because of the cost of carbon-isotope discrimination (CID) and the fact that such measurements are not immediate, other surrogates were subsequently identified, such as specific leaf area (SLA) or Soil Plant Analysis Development chlorophyll meter readings (SCMRs), as proxies of CID (Rao *et al.*, 2001). We review the physiological significance and the use of these different surrogates, especially in relation to whether it the stomata or the carboxylation efficiency that is the main driver of CID variations, and we attempt to draw some conclusions and insights concerning their success. This section will also review new and recent methods that allow robust and accurate gravimetric measurements of TE using a lysimetric approach (Vadez *et al.*, 2008, 2011a, 2011b).

The TE ratio is actually quite complex, as it depends on both genetic and environmental components [$TE = k_d/(e^*_a - e)_d$], where the term in parentheses reflects the vapour-pressure deficit (VPD) and k_d is a coefficient that reflects the CO_2 concentration in the stomatal chamber (Sinclair *et al.*, 1984; Sinclair, 2012). Hence, TE shows an inverse relationship with the VPD (Bierhuizen and Slatyer, 1965; Tanner and Sinclair, 1983). However, much of the research on the genetics of TE over the last three decades has disregarded the aspects of TE that concern this environmental factor, instead concentrating on aspects related to CO_2 in the leaf. Nevertheless, a modelling study showed that setting a maximum transpiration rate during the mid-day period, when VPD is highest, would improve the yield of sorghum under water-limited conditions and increase TE (Sinclair *et al.*, 2005). This genetic trait would confer the capacity to alter the effective VPD value that drives TE. In the past few years, large genotypic variations in the restriction of transpiration under a high VPD have been reported in several legume species, such as soybean (Fletcher *et al.*, 2007), chickpea (Zaman-Allah *et al.*, 2011a), cowpea (Belko *et al.*, 2012), and peanut (Devi *et al.*, 2010), as well as in cereals such as sorghum (Gholipour *et al.*, 2010), pearl millet (Kholova *et al.*, 2010b), and wheat (Schoppach and Sadok, 2012). The discovery of these large genetic differences therefore re-oriented research on TE, providing new and exciting insights into ways to alter the negative influence

of VPD on TE. Therefore, the second section will present the physiological basis for this trait, how it can indeed improve WUE, and how it may explain some of the earlier results that did not fit the CID theory. We will also discuss its mechanistic basis, particularly concerning its relationship with the characteristics of plant hydraulics, and its possible link to the functioning of aquaporins.

In the third section we will refer to the Passioura equation (Passioura, 1977), in which TE is one of the components ($\text{Yield} = \text{WU} \times \text{TE} \times \text{HI}$; where WU is the water used for transpiration and HI is the harvest index), and discuss the strength and limitations of the equation in approaching TE. Part of the weakness lies in assuming that the TE component is constant throughout a plant's life cycle. The previous paragraph and the second section of the paper discuss the greatly varying influence of VPD on TE. Beyond this, it has become increasingly evident that there are certain stages of the crop life cycle that critically require water, such as the grain-filling period. As such, there are now data showing the very high water productivity of the water used during these specific stages (Manschadi *et al.*, 2006; Kirkegaard *et al.*, 2007; Zaman-Allah *et al.*, 2011a; Vadez *et al.*, 2013). Therefore, in this section, we will invert the WUE acronym to examine the efficiency of water use (EWU) and will analyse the importance of securing water for key stages and the genetic means of achieving this. This analysis will be further linked to the second section and traits that, through increasing TE, also contribute to delaying plant water use for later critical stages.

The story since the 1980s, definition, and how to decrease C_i/C_a

Theoretical basis of TE

The definition of the intrinsic TE from equation 4 of (Condon *et al.*, 2002) is that:

$$TE = 0.6C_a * (1 - C_i / C_a) / (W_i - W_a) \quad (1)$$

where C_i and C_a are the stomatal chamber and ambient CO_2 concentrations, respectively, and W_i and W_a are the stomatal chamber and ambient vapour pressures, respectively.

This simple equation lays out the terms that affect TE. First, the C_i/C_a ratio must be kept low to achieve a high intrinsic TE. Because C_a can be considered constant for a given crop, a low ratio comes from a low C_i , which can be achieved in two ways: through (i) a high photosynthetic efficiency or (ii) a low stomatal conductance. As an aside, climate change conditions and the gradual increase in C_a would logically lead to an increase in the TE of crops in the future and partially compensate for the adverse effects of a changing climate (Muchow and Sinclair, 1991). Second, TE shows an inverse relationship with the difference in vapour pressure between the stomatal chamber and the environment (i.e. VPD), whereby low water-vapour gradients would increase TE. This definition is akin to the earlier definition of TE provided by Sinclair *et al.* (1984): $G_d/T = k_d/(e^*_a - e)_d$, where G_d/T is the ratio of daily growth to transpiration, and k_d is a coefficient that reflects the CO_2

concentration in the stomatal chamber. This definition arises from earlier founding work by [de Wit \(1958\)](#).

Equation 1 shows that TE is variable and can be influenced by genetic (the C_i/C_a ratio) and environmental (the $W_i - W_a$ gradient, or VPD) factors. Most, if not all, research on TE over the last few decades has focused on the genetic component of this equation and on finding means of increasing photosynthetic efficiency or decreasing stomatal conductance. In the early 1980s it was discovered that the heavy form of carbon, ^{13}C , is discriminated against by the enzyme Rubisco and by the stomata ([Farquhar et al., 1982](#)). An empirical relationship was later established for wheat ([Farquhar and Richards, 1984](#)) ($\Delta^{13}\text{C} = 4.4 + 22.6 \frac{^{13}\text{CO}_{2\text{int}}}{^{13}\text{CO}_{2\text{ext}}}$), and measuring the CID was then conceived as a way to assess TE, by comparing ^{13}C values in plant tissues and in the atmosphere. According to this theory, $\Delta^{13}\text{C}$ is expected to be negatively related to TE, which has been confirmed by a number of studies ([Hubick et al., 1986](#); [Hubick and Farquhar, 1989](#); [Condon et al., 1990](#); [Ehleringer et al., 1991](#)), although other studies have not found such a relationship (e.g. [Morgan et al. 1993](#) or [Monneveux et al. 2006](#) in wheat; [Ismail and Hall 1992](#) in cowpea; [Comstock and Ehleringer 1993](#) in bean). In the study in cowpea, the conclusion was drawn that the differences in $\Delta^{13}\text{C}$ between genotypes were more consistent than the intrinsic TE measurements, although the physiological basis for the differences in $\Delta^{13}\text{C}$ was not established and CID would then represent a better screening method for breeding purposes.

Carbon isotope discrimination

This spectrometric assessment method has been used successfully to develop wheat cultivars with a lower $\Delta^{13}\text{C}$ and therefore higher TE, leading to higher yields under water-stressed conditions ([Condon et al., 2002](#); [Rebetzke et al., 2006](#); [Richards et al., 2010](#)). The method is less efficient, though, in C_4 plants, where CO_2 leakage occurs between the mesophyll and the bundle sheath, resulting in reduced discrimination ([Henderson et al., 1998](#)). However, the benefits tend to be limited to conditions of severe water stress, whereas under slightly milder water stress conditions a low $\Delta^{13}\text{C}$ would lead to yield penalties ([Condon et al., 2002](#)). Indeed, a low $\Delta^{13}\text{C}$ in wheat has been related to low plant vigour and small plant size, which would reduce light interception and lead to yield penalties under more favourable conditions. The use of CID was expanded as an indirect way to assess TE at the plant level (biomass/water transpired). For instance, a negative relationship was found between TE and CID in four peanut genotypes ([Wright et al., 1994](#)) and ten peanut genotypes ([Rao et al., 1993](#)). Similar results have been obtained in different crops, such as cowpea ([Ismail and Hall, 1992](#)), wheat ([Ehdaie et al., 1991](#)), barley ([Anyia et al., 2007](#)), and sunflower ([Lambrides et al., 2004](#)). In the study in cowpea ([Ismail and Hall, 1992](#)), where the relationship between TE and CID held under both water-stressed and well-watered conditions, the genotype-by-treatment interactions observed for $\Delta^{13}\text{C}$ indicated that $\Delta^{13}\text{C}$ was not only under genetic control but was subject to the complex effects of the water regime. A similar conclusion was drawn from a study in wheat ([Monneveux et al., 2006](#)), where

the relationship between the grain $\Delta^{13}\text{C}$ and yields showed a strong association only under post-anthesis water stress, whereas no or a weak relationship was found under conditions of residual moisture, pre-anthesis water stress, or full irrigation. Fertility levels have also been shown to affect the value of $\Delta^{13}\text{C}$, with an increase in $\Delta^{13}\text{C}$ being observed upon P fertilization in pearl millet ([Bruck et al., 2000](#)) and a decrease in $\Delta^{13}\text{C}$ being found upon N fertilization of N-deficient wheat ([Clay et al., 2001](#)). A number of studies have reported the absence of a relationship between TE and CID ([Devi et al., 2011](#); [Turner et al., 2007](#)), or a weak relationship ([Zacharisen et al., 1999](#); [Krishnamurthy et al., 2007](#)). In summary, while the CID method offers the potential to indirectly assess TE, there are situations where its use has limits, the causes of which are still unclear, and it cannot be employed as a stand-alone tool. To date, CID has only been successfully applied in the case of wheat breeding in relatively dry environments in Australia. We discuss the possible reasons for the inconsistencies in these relationships below.

SCMR and SLA

Measurements of CID are not immediate, and they are quite expensive, which has triggered a search for alternative surrogates that are cheaper and faster to measure. A study by [Wright et al. \(1994\)](#) showed a strong negative relationship between $\Delta^{13}\text{C}$ and the SLA, indicating that genotypes with thicker leaves would show a higher TE. Subsequently, a significant negative correlation was reported between SLA and SCMR, which can be performed on many leaves relatively quickly, providing a value that serves as a proxy of the chlorophyll content of the leaf ([Rao et al., 2001](#)). Similar associations between TE and SLA were identified in a recombinant F_2 population of *Stylosanthes*, and co-mapped quantitative trait loci for SLA and TE were identified, indicating a causal relationship ([Thumma et al., 1998](#)). SLA and SCMR have been shown to be related to TE in a number of studies ([Comstock and Ehleringer, 1993](#); [Sheshshayee et al., 2006](#); [Thompson et al., 2007](#)). However, other studies have found poor relationships between these surrogates and gravimetric TE measurements ([Krishnamurthy et al., 2007](#); [Devi et al., 2011](#)). Additionally, only minor quantitative trait loci were identified for these surrogates in a population of a recombinant inbred line of peanut ([Varshney et al., 2009](#)). Thus, the common use of these surrogates, applied in breeding programmes, has not improved the rate of genetic gains in peanut ([Nigam et al., 2005](#)).

Limitations on the use of surrogates

Many studies have used these surrogates as factors that are less prone to environmental variation. For instance, $\Delta^{13}\text{C}$ values appear to be more consistent across conditions than TE measurements, from which it was concluded that CID should be a more effective tool than TE measurements for breeding purposes (e.g. [Hall et al., 1992](#); [Ismail and Hall, 1992](#)). However, this conclusion overlooks the large genotype-by-treatment interaction reported for the leaf gas exchange

efficiency. In another case, in peanut, SCMR and SLA were used to screen for 'drought tolerance' (Upadhyaya, 2005), although these experiments were carried out under fully irrigated conditions, and no TE measurements were performed in even a subset of germplasm. These are cases where the simplicity of a surrogate trait overshadows its ecophysiological meaning. A study in sorghum found large differences in TE in the investigated germplasm (Hammer *et al.*, 1997) but also detected a weak association between TE and CID and suggested that screening for TE should not be based on a single screen but, rather, on establishing the physiological basis of the differences in TE. CID measurements are indeed known to be very closely dependent on the environment, which reflects different responses of specific physiological traits associated with CID to environmental conditions (e.g. Misra *et al.*, 2010; Tambussi *et al.*, 2007; Condon and Richards, 1992).

Another limiting factor in the use of surrogates is the time required. While measurements of intrinsic TE are instantaneous, CID involves integration over time, which depends on the sampled plant parts, which are either flag leaves or grain in many studies. Therefore, unless the conditions under which the intrinsic TE is measured are environmentally stable, there is a chance of genotype-by-environment interactions occurring. For instance in cowpea, gravimetric and intrinsic TE were not found to be correlated (Anyia and Herzog, 2004). In fact, while $\Delta^{13}\text{C}$ does not always correlate with TE measurements under a given water regime, $\Delta^{13}\text{C}$ always decreases under water stress, indicating the tight dependence of CID on leaf conductance. The stomatal aperture is regulated by a number of environmental factors in addition to soil moisture, including light, temperature, nutrition, and relative humidity (Comstock *et al.*, 2005), and any of these factors would therefore transiently alter CID. Hence, it is interesting that a good correlation between $\Delta^{13}\text{C}$ and TE has been identified under environmentally stable conditions (e.g. Ismail and Hall, 1992).

Stomata or photosynthesis: what drives differences in CID?

Both SLA and SCMR are surrogates that serve as indirect proxies for the carboxylation efficiency of the mesophyll, where higher chlorophyll content from more packed mesophyll cells (lower SLA) leads to more active removal of C_i from the stomatal chamber, thus reducing the C_i/C_a ratio and increasing TE. These two surrogates are therefore not useful in situations where a low leaf conductance is driving differences in TE. The CID assay does not distinguish between whether differences in CID are driven by mesophyll efficiency or leaf conductance. The lack of a relationship between TE and CID could be derived from these two mechanisms operating in opposition. This might have been the case in a study in bean, where CID was indeed found to be positively related to both photosynthetic activity and leaf conductance but was not correlated with TE (Comstock and Ehleringer, 1993). The differences in the degree of discrimination against ^{13}C between Rubisco and the stomata are another source of confusion in interpreting these results. The discrimination against

^{13}C by Rubisco is to a factor of 2.7% (Farquhar *et al.*, 1989), whereas the discrimination by stomata is to a factor of 0.4%, which is approximately seven times less. Therefore, stomatal conductance-driven differences in $\Delta^{13}\text{C}$ would lead to much weaker CID signatures than those driven by differences in carboxylation efficiency. In cowpea, the differences in the gas exchange efficiency were found to be more strongly driven by differences in the assimilation capacity (A) than by stomatal conductance (Gs) (Hall *et al.*, 1992). A pathway analysis also showed that the main factor explaining the differences in TE evolved over the life of the plant (Hui *et al.*, 2008). In peanut, many authors have argued that differences in TE were driven by the mesophyll efficiency (Hubick *et al.*, 1986; Rao *et al.*, 1993; Wright *et al.*, 1994; Udayakumar *et al.*, 1998). However, more recent results reveal a close relationship between stomatal conductance and TE (Bhatnagar-Mathur *et al.*, 2007). An additional dimension is provided by the sensitivity of stomatal conductance to VPD, which varies across genotype (details in the next section), and the fact that genetic differences have been found in peanut (Devi *et al.*, 2010). Interestingly, the study in bean cited above (Comstock and Ehleringer, 1993) also found high variation among genotypes in the response to decreasing air humidity, and the authors attributed the lack of a relationship between TE and CID to differences in leaf temperature.

In summary, while there is no question about the validity of the theory regarding the differences in discrimination against ^{13}C and its drivers, whether differences in CID are consequences of differences in the photosynthesis or the leaf conductance is still unclear. From a breeding point of view, it would be more effective to decipher the physiology and genetics of both components of discrimination, rather than attempting to harness the genetics of CID as a whole. Until there is a better understanding of what role environmental conditions play in CID, especially for VPD, it will be difficult to use this method on a routine basis in breeding programmes. More research is therefore needed on the ecophysiological meaning and significance of this discrimination phenomenon.

A new method for evaluating TE

The use of indirect methods arose due to the difficulty of precisely measuring transpiration under field conditions or measuring plant transpiration over the long term in pot experiments. A lysimetric method, involving long, large PVC tubes installed outdoors, has been developed (Vadez *et al.*, 2008). The tube size and spacing are designed to provide the plants with a similar space and soil volume to be explored to those found in field conditions (Fig. 1). This approach allows the monitoring of plant water use and biomass accumulation (both vegetative and grain) from very early plant stages until maturity, and it allows extremely robust TE assessments to be conducted with very low experimental error (Ratnakumar *et al.*, 2009; Vadez *et al.*, 2011a, 2011b). Using this system, transpiration is measured over almost the entire crop cycle, avoiding possible artefacts found in short-term experiments when there is high T variation. This experimental setup has



Fig. 1. Description of the lysimetric system. Panel (a) shows the layer of beads applied on top of the tubes (in addition to a plastic sheet below the beads in other crops than peanut) to prevent soil evaporation. Tubes are lifted for weighing with an extension load cell (b). The lysimetric system is versatile and usable for many crops; e.g. cowpea (c), sorghum (d), and peanut (e). Panels (d) and (e) show that the plants are cultivated individually but are part of a crop canopy, and cultivated at a density that reflects the plant population in the field.

been shown to be suited to a wide range of crops and has been designed to cater for breeding programmes due to its several-thousand-tube capacity. Additionally, this system allows plant water use to be monitored at different times during the crop cycle. Last but not least, it is the first system that allows measurements of all terms of the Passioura equation ($\text{Yield} = \text{WU} \times \text{TE} \times \text{HI}$) to be conducted on the same plants and then allows the weight of each term on yield to be assessed. Both of these last factors are discussed at length in the third section of the paper.

How to decrease $(W_i - W_a)$, the transpiration response to high VPD

Theoretical considerations underlying the VPD response

Here, we come back to the equation of Sinclair *et al.* (1984), where $G_d/T = k_d/(e_a^* - e)_d$, with G_d representing the daily increase in biomass and TE showing an inverse relationship with the VPD faced by the plants. The term $(e_a^* - e)_d$ represents the difference between the saturated vapour pressure (e_a^*) and the ambient one (e), calculated as the mean over 1 day. To measure TE over a long period, this term needs to be integrated over a season and then to be weighted by the transpiration rate of the plant over the course of the measurement period (Tanner and Sinclair, 1983). Applying the same logic to a single day, the weighting of this component based on the transpiration rate throughout the day leads to the possibility of obtaining altered values if there are genotypic variations in the transpiration profile throughout the

day (Sinclair, 2012). The sensitivity of the stomatal aperture to the VPD has long been established (Turner *et al.*, 1984; Grantz, 1990), but it was not until fairly recently that genotypic variation was demonstrated in various species (e.g. Fletcher *et al.*, 2007; Kholova *et al.*, 2010b). This search was triggered by evidence from simulation modelling using weather data from a sorghum production area, showing that restricting the maximum transpiration would increase the TE and yield of sorghum (Sinclair *et al.*, 2005). Therefore, the significance of this trait is that if a given genotype possesses the attributes to restrict transpiration at the time when VPD is highest, the proportion of transpiration taking place under a high VPD is lower, which decreases the integrated value of the VPD [the $(e_a^* - e)_d$ component] used to calculate TE and therefore increases TE. Of course, restriction of transpiration under high VPD conditions would also partially increase the leaf temperature and, hence, the e_a^* term, which would partially offset the benefit of a reduced effective VPD. However, simulations have shown a large beneficial effect of that trait on yields in soybean (Sinclair *et al.*, 2010), and more similar evidence has come from peanut (Vadez *et al.*, unpublished data) and sorghum (Kholova *et al.*, unpublished data).

In summary, the restriction of transpiration under conditions of high VPD in certain genotypes across different species decreases the $(e_a^* - e)_d$ term of the TE equation, thereby presenting the opportunity of including a genetic component in a term that had long been seen as purely dependent on the environment and therefore 'out of genetic reach'. There is clearly a possibility of using the genetic component of that term of the equation to enhance TE, specifically for crops that are most likely to face high-VPD conditions.

Genetic differences in the VPD response and its hydraulic basis

Large genetic variation in the capacity to restrict transpiration under a high VPD has been identified in the past 5 years in soybean (Fletcher et al., 2007), chickpea (Zaman-Allah et al., 2011a), peanut (Devi et al., 2010), cowpea (Belko et al., 2012), pearl millet (Kholova et al., 2010b), maize (Yang et al., 2012), sorghum (Gholipour et al., 2010), and wheat (Schoppach and Sadok, 2012). Figure 2 shows an example of the VPD response from a recent study (Kholova et al., 2010b). The fact that rapid changes in transpiration occur once certain VPD thresholds are crossed implies that hydraulic signals are needed to induce fairly rapid stomatal closure and avoid loss of turgor. In soybean, the sensitivity of transpiration to a high VPD of genotype PI416937 was found to be related to a lower leaf hydraulic conductance (Sinclair et al., 2008), and there is preliminary evidence of a similar process in peanut (Devi et al., 2012). In other crops, the source of hydraulic limitation is instead the roots, for instance in wheat (Comstock, 2000; Schoppach et al., 2014; Tharanya et al., unpublished data). In a study in which 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme activity was enhanced, the concentration of abscisic acid increased in the shoots while stomatal conductance decreased, and TE and root hydraulic conductivity were increased (Thompson et al., 2007). Additionally, in a study where root hydraulic conductivity was decreased through aquaporin inhibitor treatments, the stomatal conductance was not affected under a low VPD, but the stomata closed under a high VPD (Ehlert et al., 2009).

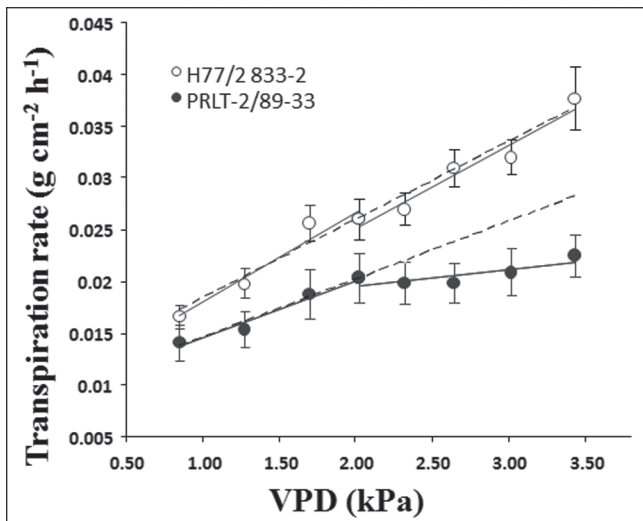


Fig. 2. Transpiration response to a ladder of increasing VPD conditions, in a terminal-drought-sensitive (H77/833-2, open circles) and a terminal-drought-tolerant (PRLT-2/89-33, closed circles) pearl millet genotype. Transpiration was measured gravimetrically at each VPD level by regularly weighing potted plants (wrapped in a plastic bag around the stem to avoid soil evaporation). Leaf area was destructively measured at the end of the VPD response experiment and the transpiration rate computed as the ratio of transpiration values divided by the leaf area. Redrawn from Kholova J, Hash CT, Kumar PL, Yadav RS, Kocova M, Vadez V. 2010b. Terminal drought-tolerant pearl millet *Pennisetum glaucum* (L.) R. Br. have high leaf ABA and limit transpiration at high vapour pressure deficit. *Journal of Experimental Botany* **61**, 1431–1440.

Therefore, there is a hydraulic basis for the differences in the transpiration response to a high VPD. In soybean, genotypes showing contrasting responses to the VPD also display a contrasting sensitivity to silver nitrate in a population of aquaporins present in the leaves (Sadok and Sinclair, 2010). Similarly, peanut genotypes exhibiting differing transpiration responses to a high VPD also differed in their transpiration response to silver nitrate in de-rooted plants (Devi et al., 2012). In both studies, the genotypes of soybean or peanut in which transpiration was constrained by a high VPD did not show inhibition by the aquaporin inhibitor, suggesting that transpiration constraint due to plant hydraulics was related to the absence of some forms of aquaporins. We have also found that differences in the response of transpiration to high VPD in sorghum was related to differences in the gene expression of several PIP isoforms (our unpublished data). Similar evidence was recently shown for an increase in aquaporin gene expression under higher evaporative demand (Laur et al., 2013) or osmotic stress (Hachez et al., 2012). This fits well with the role of aquaporins, which have been described as facilitators of water movement in different organs (Maurel, 1997; Tyerman et al., 1999; Chrispeels et al., 2001; Javot and Maurel, 2002; Li et al., 2013). Aquaporins are also well known in their response to water stress (e.g. Aroca et al., 2012; Henry et al., 2012). Of course, there are many aquaporin isoforms in the different crop species, which are expressed in different plant compartments, with expression varying with age and time of the day. Therefore the challenge still remains to explain which of these, if any, has any direct functional effect on the VPD response phenotype.

In summary, genetic differences in the response of transpiration to a high VPD have been identified in different crop species and this response appears to have a hydraulic basis, in which aquaporin might play a role. However, the conditions under which altered hydraulic properties lead to stomatal closure and increases in TE are still not clear, and more work is required to elucidate them. Additionally, soil hydraulic properties may play a critical role that needs to be better understood, especially concerning how they are translated through root signalling to the shoots, in a hydraulic or biochemical manner, to alter plant development and/or stomatal opening. However, the possibility of harnessing genetic components of these mechanisms in a way that improves water productivity opens an exciting arena of research.

Transpiration response to soil drying

A strong water stress effect on CID has generally been reported (see citations above), and plants being exposed to intermittent water stress would therefore experience alternating periods of sufficient and insufficient water supplies. The plant response to progressive soil drying follows a fairly common pattern across crop species, displaying an initial phase in which the water supply to transpiring leaves is insufficient, and stomatal closure begins to avoid loss of turgor and leads to a decline in transpiration (Fig. 3). Plants under water stress (in phase 2) would indeed likely transpire in the early hours

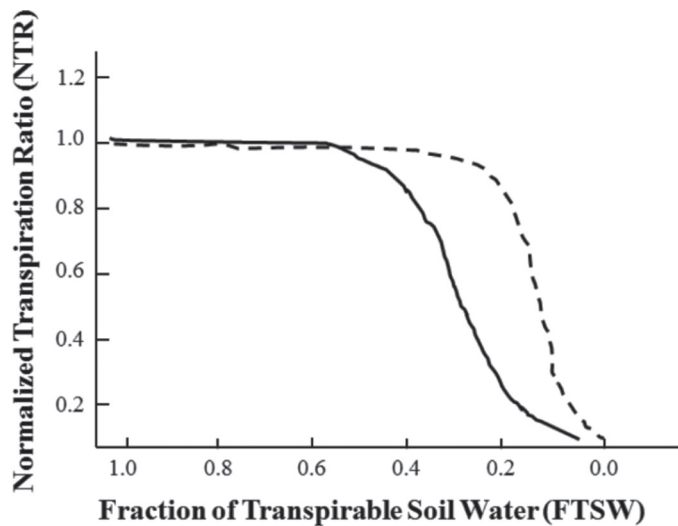


Fig. 3. Typical response of transpiration to progressive soil drying in two hypothetical genotypes: a genotype with an early decline in transpiration at a fraction of transpirable soil water (FTSW) of approximately 0.5 (solid line) and a genotype with a late decline in transpiration at FTSW of approximately 0.2 (dashed line). The y axis depicts the normalized transpiration ratio (NTR), which derives from the normalization of transpiration data against a fully irrigated control (see Kholova *et al.* 2010a or Zaman-Allah *et al.* 2011a for details about the normalization). Where NTR is constant at a value of 1.0, water-stressed plants transpire, and therefore grow, as much as fully irrigated controls. NTR drops below 1.0 when roots cannot fully supply transpiration demand. At an NTR value below 0.1 it is considered that stomata are fully closed and there are no more transpiration-related water losses. At this stage, all the water available for transpiration has been depleted and the FTSW reaches a value of zero.

of the morning and close their stomata when approaching higher-VPD conditions later in the day. What is quite critical here is the genetic variation in the soil moisture thresholds (measured based on the fraction of transpirable soil water; FTSW) where water stress starts affecting the plants (Sadras and Milroy, 1996; Devi *et al.*, 2009; Kholova *et al.*, 2010a; Zaman-Allah *et al.*, 2011a; Belko *et al.*, 2012; Gholipour *et al.*, 2012) (a representative example is shown in Fig. 3). This plant attribute would offer another means of altering the $(e_a^* - e)_d$ term of the TE equation, as transpiration would be more heavily weighted toward lower VPD periods of the day. Genetic differences in the FTSW threshold at which the decrease in transpiration is initiated are therefore another major factor that might alter this term by genetic means, and there is a possibility of exploring genetic variation for such traits at a larger scale. Although there are now available data documenting the genetic variation in the transpiration response to a high VPD, there is most likely a need to screen a large germplasm for putative variation in the transpiration response to soil drying. It should be mentioned, however, that this is not an easy phenotype to measure. Assessing it on a large scale is possible and would require a phenotyping platform allowing frequent weighing and automatic re-watering.

A consequence of the genetic variation in FTSW thresholds at which transpiration declines is that genotypes with high thresholds are water-stressed at FTSW levels where low-threshold genotypes are not water stressed and function like fully irrigated plants. From a CID perspective, high-threshold

genotypes would display a lower $\Delta^{13}\text{C}$. From a VPD perspective, higher-threshold genotypes would be conferred a higher TE because of a lower integrated daily VPD value in the calculation of TE. Experimental evidence of this hypothesis was recently reported (Devi *et al.*, 2009), although the obtained polynomial relationship was relatively weak ($R^2 = 0.39$) and was conditioned by one extreme genotype. Nevertheless, more convincing, but contradictory results were also reported recently. A strong negative correlation ($R^2 = 0.88$) was found between the TE and the FTSW thresholds of a set of transgenic (*rd29::DREB1A*) peanut plants grown under a fairly high average VPD in a glasshouse. In cowpea, a strong negative linear relationship ($R^2 = 0.62$) was also found between TE and the FTSW threshold for transpiration declines under conditions of a high VPD, although no relationship was found under low-VPD conditions (Belko *et al.*, 2012). In the last study, the genotypes exhibiting unrestricted transpiration at a high VPD also presented higher FTSW thresholds, and these results are in agreement with those found in pearl millet, where high-threshold genotypes also show unrestricted transpiration at a high VPD (Kholova *et al.*, 2010a). In these two studies we argue that the FTSW threshold calculation, which implies normalization to the transpiration of well-watered plants, would be altered if any factor affected the transpiration of well-watered plants, which was the case for the transpiration response to high VPD. Therefore, a high FTSW threshold may not always lead to increases in TE, especially in genotypes that exhibit transpiration restriction at a high VPD.

Stomatal patchiness

One quite intriguing finding is that differences in the transpiration response to a high VPD in a set of pearl millet genotypes did not lead to differences in TE (Kholova *et al.*, 2010b). In this paper it was argued that the experiment had been carried out in a glasshouse and that the plants may not have been exposed to the VPD threshold at which differences in transpiration occur. However, even when TE was measured over the long term in lysimeters and under high-VPD conditions this parameter did not differ between these genotypes (Vadez *et al.*, 2013). Part of the reason for this result could lie in the opposing effects of transpirational sensitivity to VPD and a low FTSW threshold for transpiration declines (e.g. genotype *PRLT/89-33*). Another possible interpretation is that stomatal closure may not take place uniformly at the leaf level. It is indeed assumed that plants showing transpirational sensitivity to a high VPD partially close all of their stomata upon increases in VPD. It is this uniform response that would lead to an effect on TE, from its effect on stomatal conductance (see TE definition in the first section). A similar effect on transpiration under high VPD could be achieved if only part of the stomata were completely closed, while others remained fully open. In such a case there would be no decrease in stomatal conductance in some parts of the leaf, and full closure in others, and then overall no effect on TE. Evidence of stomatal patchiness has long been reported (Pospisilova and Santrucek, 1994), and the stomatal responses to environmental stimuli

are known to be extremely heterogeneous, which appears to be related to hydraulic interactions (e.g. Mott and Buckley, 2000; Peak *et al.*, 2004). Stomata patchiness might create spots in the leaf having different temperature, and how this would affect the VPD of the micro-environment above these spots is unknown. We might speculate that air circulation at the leaf surface and the small size of these spots would make the VPD homogenous across the leaf surface. Although the interpretation of leaf gas exchange data becomes complex if stomata close in patches, it might partly explain the observation that while transpiration under a high VPD could be restricted (because of the complete closure of some stomata), the intrinsic TE at the level of stomata remaining open would be unchanged.

In summary, while the genetic variability of the transpirational sensitivity to VPD and to soil drying may lead to substantial increases in TE and therefore make it possible to include a genetic component in the $(e_a^* - e_d)$ term of the TE equation, the relationship between this mechanism and TE is not straightforward because (i) the sensitivity to VPD and to soil drying can work in opposition (sensitivity to VPD and insensitivity to soil drying) and (ii) stomatal patchiness would make the transpiration response to VPD or to soil drying quite heterogeneous at the leaf canopy level.

How these findings could explain earlier controversial results

In the paragraphs above there are a number of examples that might explain why the earlier CID theory, when put into practice, has not always 'worked'. CID being driven by CO₂ assimilation or intrinsic transpiration would not result in the same CID signature, although both would alter the C_i/C_a ratio. Hence, unless there is a clear predominant driver of the differences in CID, experimental situations that induce variations in stomatal stimuli are bound to have extremely complex consequences for the CID signature. Hence, we have observed the criticality of the $(e_a^* - e_d)$ term of the TE equation (Sinclair *et al.*, 1984) and that there are several genetic components capable of altering that term. Among these components, we have described the sensitivity of transpiration to VPD and to soil drying, and overall the processes that influence stomata opening, from plant hydraulic differences, soil hydraulic conditions and stomatal heterogeneity. We have seen that some of these processes can work in opposition (for instance, the sensitivity to VPD and to soil drying), which would obscure the resulting effect on TE. Therefore, progress in improving the TE of plants can be made by deciphering the individual components that contribute to increasing the intrinsic TE. This starts by clearly distinguishing the role of the photosynthetic capacity in explaining differences in CID. Many authors have argued that CID is driven by A (e.g. Rao and Wright, 1994; Condon *et al.*, 2002; Arunyanark *et al.*, 2008). Although the role of the stomata in altering CID has been reported (Ehleringer *et al.*, 1991; Udayakumar *et al.*, 1998), it has largely been overlooked, as has the fact that stomatal opening is extremely sensitive to environmental stimuli. Thus, in cases where the stomata serve as the major driver of

differences in CID, which have been reported to be more complex to interpret (Condon *et al.*, 2002), a number of potential factors that modulate stomatal movements need to be better understood. Only when these components are identified can their genetics be deciphered and put to use to benefit crop-improvement programmes. Positive correlations between grain yields and CID are usually found in situations of no or mild water stress, whereas under terminal stress conditions negative relationships prevail between grain yields and CID (Tambussi *et al.*, 2007).

Timing of plant water use

In this section, we wish to take a different stand with regard to water by exploring possible interactions between the terms of the Passioura equation (Yield = WU × TE × HI) (Passioura, 1977). For instance, it has been reported that selection for high TE might also select plant types with low vigour and productivity and, hence, a low T term (Condon *et al.*, 2002; Blum, 2009). In the terms of the Passioura equation (Passioura, 1977), the variation in water use by the plant over time, the T term is taken as a linear term showing a similar influence on yields across the entire cropping cycle, but recent work demonstrates the importance of having available water for critical stages in crops, such as the reproduction and grain-filling periods. Therefore, we also want to go beyond the discussion on the means of improving TE and explore the means of achieving efficient timing of water use.

Are the terms of the Passioura equation mutually exclusive?

Recent reviews take the stand that a high WUE is certain to be related to low productivity (Blum, 2005, 2009). This notion is supported by a number of studies in which a high yield was achieved by genotypes with high stomatal conductance to maximize plant water use (e.g. Reynolds *et al.*, 1994; Araus *et al.*, 2002). This indeed appears to be the case under Mediterranean environments, where the grain or leaf CID appears to be positively related to grain yields; that is, higher grain yields are achieved in crops with a high $\Delta^{13}C$ and, hence, a low intrinsic TE (e.g. Monneveux *et al.*, 2006). Low $\Delta^{13}C$ wheat lines in Australia were also found to show low vigour and to present a lower yield in higher rainfall environments (Condon *et al.*, 2002), putatively because their lower stomatal conductance led to less biomass accumulation, smaller leaf area, and less light interception. These are cases in which lower stomatal conductance would indeed reduce the potential to maximize soil water use. However, as noted in the previous section, transient alterations of stomatal conductance during high-VPD conditions can dramatically increase TE. In recent studies, a lysimetric system has been used to assess TE, along with the other terms of the Passioura equation, in a large range of genetic materials (germplasm, breeding populations) (Vadez *et al.*, 2008). In a study in sorghum, no relationship was found between the TE

and T components of the equation (Fig. 4a) (derived from Vadez *et al.*, 2011b). Similar data were obtained in other studies in sorghum (Vadez *et al.*, 2011a) and pearl millet (Vadez *et al.*, 2013). Figure 4b and c provide similar information for 268 entries of the reference collection of pearl millet and 280 entries of the peanut reference collection (Vadez *et al.*, unpublished data). These studies were conducted under conditions of medium-to-high-VPD conditions, and sorghum genotypes achieving a high TE also displayed transpirational sensitivity to a high VPD (Vadez *et al.*, unpublished data). Additionally, this work in sorghum showed that among the terms of the Passioura equation, besides the obvious importance of the harvest index, yields were closely related to TE, but not to the water-use term (Vadez *et al.*, 2011a, 2011b), which contradicts earlier claims (Blum, 2009). In other recent studies testing soybean genotypes with contrasting sensitivities to a high VPD, a higher photosynthetic rate was able to compensate for the transiently lower stomatal conductance of VPD-sensitive genotypes (Gilbert *et al.*, 2011a, 2011b). This is related to the logarithmic shape of the relationship between the photosynthetic rate and stomatal conductance (Wong *et al.*, 1979) and the marginal increases in the photosynthetic rate above a certain stomatal conductance. These examples provide clear contradictory evidence that a high TE is not necessarily related to low productivity and water use.

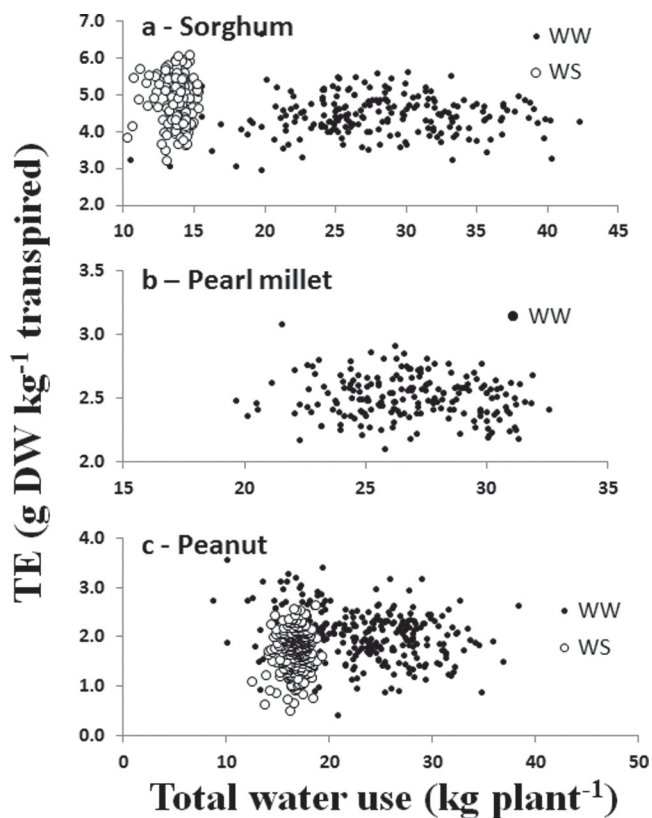


Fig. 4. Relationships between TE in g biomass·kg⁻¹ water transpired) and total plant water use (in kg·plant⁻¹) in a set of 152 sorghum germplasm (a), 210 pearl millet germplasm (b), and 280 peanut germplasm (c) under either fully irrigated (well-watered, WW; closed circles) or water-stress (WS; open circles) conditions. Transpiration was monitored throughout most of the cropping cycle, using lysimeters of 2.0 m length and 25 cm diameter (sorghum and pearl millet) or 1.2 m length and 20 cm diameter (peanut).

Again, this situation stresses the need to properly decipher the mechanistic components of TE.

Importance of water availability at key critical stages

It is widely recognized that the reproductive stages are particularly sensitive to water deficits and that water availability during and after anthesis is critical. For instance, higher durum wheat grain yields were found to be closely related to increasing the water input during the post-anthesis period (Araus *et al.*, 2003). Similar results were obtained across 11 grain legume species (Siddique *et al.*, 2001), where increasing water use during the post-anthesis period led to a higher harvest index and grain yield. A finer demonstration of these facts was made in the lysimetric system described at the end of the first section, where water extraction was monitored from the vegetative stage until maturity in pearl millet (Vadez *et al.*, 2013) and chickpea (Zaman-Allah *et al.*, 2011b) exposed to terminal water stress (Fig. 5). In these studies, higher grain yields were achieved by genotypes that had lower plant water use prior to anthesis, which made water available for extraction during the reproductive and grain-filling period. A similar conclusion was drawn from a set of peanut genotypes exposed to intermittent water stress, where higher pod yields were related to higher water extraction during the grain-filling period (Ratnakumar *et al.*, 2009).

The WUE for grain yield production (WUE_{yield}) of 11 grain legumes under a Mediterranean climate ranged from 13 to 16 kg·ha⁻¹·mm⁻¹ (Siddique *et al.*, 2001), which is within the range observed for canola under similar conditions (11–15 kg·ha⁻¹·mm⁻¹) (Robertson and Kirkegaard, 2005). These data were computed from the water used during the

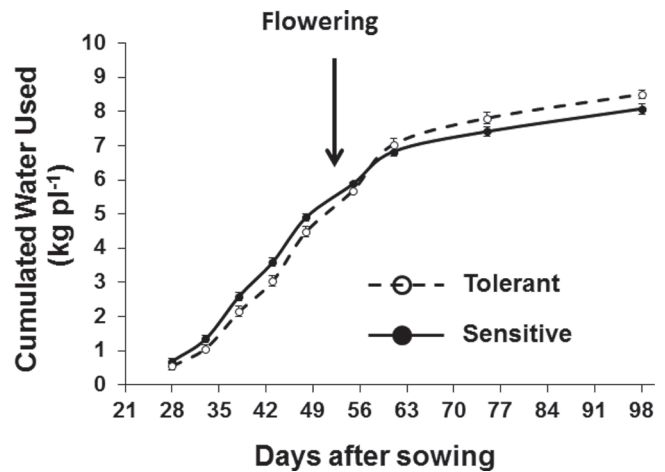


Fig. 5. Cumulated water use (in kg plant⁻¹) as a function of time after sowing and under terminal water stress conditions in chickpea. The plants were grown in lysimeters of 1.2 m length and 20 cm diameter and the last irrigation was applied at 21 days after sowing. The data are the average of the mean values (five replicated plants per line) of 12 tolerant lines (open circles and dashed line) and eight sensitive lines (closed circles and solid line), which were selected from several field experiments. Redrawn from data of Fig. 1 in Zaman-Allah M, Jenkinson DM, Vadez V. 2011b. A conservative pattern of water use, rather than deep or profuse rooting, is critical for the terminal drought tolerance of chickpea. *Journal of Experimental Botany* **62**, 4239–4252.

course of the entire crop life cycle, and the WUE for grain yields could be twice as much for water used during the grain-filling period (Wasson *et al.*, 2012). For instance, the slope of the regression between grain yields and the water extracted during the third week after emergence of the flag leaf were observed under two levels of terminal water stress and could be extrapolated to WUE_{yield} values of 37 and 45 $kg \cdot ha^{-1} \cdot mm^{-1}$ in pearl millet (Vadez *et al.*, 2013) or 40 $kg \cdot ha^{-1} \cdot mm^{-1}$ in chickpea (Zaman-Allah *et al.*, 2011b). Values in a similar range were reported in wheat, for which every millimetre extracted during the grain-filling period showed a WUE_{yield} of 55 (Manschadi *et al.*, 2006) and 59 $kg \cdot ha^{-1} \cdot mm^{-1}$ (Kirkegaard *et al.*, 2007).

In summary, while crops of course require water throughout the cropping cycle and must maximize water use, there are stages in which the water supply is particularly critical. Therefore, the application of the Passioura equation must take into account the interactions between its terms and their different weightings based on yields at different times. Here, the interaction between water use at key times and the harvest index is of particular importance.

Means of securing water at key times

There are several means of securing water availability during the grain-filling period, including those presented in the second section of this paper. In a study in soybean, large yield improvements arose from a higher FTSW threshold for a transpiration decline (Sinclair *et al.*, 2010). The transpirational sensitivity to a high VPD was the factor that made more water available during grain filling in pearl millet genotypes (Kholova *et al.*, 2010b; Vadez *et al.*, 2013). Higher-yield chickpea genotypes show lower leaf conductance during the vegetative stage and present a smaller leaf canopy in the vegetative stage (Zaman-Allah *et al.*, 2011a). Therefore, beyond aspects related to leaf conductance and the control of the stomatal aperture, the control of leaf water losses by adjusting the leaf area based on water availability is another critical factor. For instance, genotypes with higher leaf appearance rate would show reduced tillering and therefore a reduced leaf area at anthesis, making more water available after anthesis in sorghum (van Oosterom *et al.*, 2011). Of course, limiting the leaf area would also lead to yield penalties under conditions where there is only mild water stress. Therefore, the key message here is that securing water availability for the reproductive and grain-filling stages relies on the following simple, but fundamental factors: (i) there should be no more water available in the soil profile at maturity and (ii) plant water requirements need to match the water supply. All of these aspects are the topic of a recent review (Vadez *et al.*, 2014). Additionally, we need to consider the case of grain legumes relying on symbiotic nitrogen fixation. Symbiotic activity usually declines during the post-anthesis period because of competition for carbon sources between grain filling and nodule activity. Therefore, high levels of growth and N accumulation prior to anthesis might be needed as well (Sinclair and Vadez 2012).

Conclusions

Improving the productivity of water in agriculture is a necessity. This issue began to be addressed in the early 1980s, and much research on this topic has been undertaken since that time. While the theory concerning the intrinsic WUE in crops has helped investigators to focus on critical issues to achieve a higher TE, successful breeding applications have been limited and are restricted to fairly stable and severe terminal stress situations in wheat in Australia. Here, we argue that a part of this lack of applications is first due to overlooking the high level of interaction between mechanistic traits underlying TE and the environment. There are indeed many factors that can alter CID, and unless their ecophysiological significance is understood there is little possibility of making practical use of this method. The second reason is the subject of this review and involves the terms of equations for determining TE, particularly the VPD term, which has been thought to be purely dependent on the environment. Here, we have reviewed possible ways to 'genetically' alter the effective VPD that is used in the determination of TE, which hold great promise regarding yield increases. The sensitivity of transpiration to soil drying and to VPDs shows a wide range of genotypic variation in a number of crops, and simulation studies predict large possible yield increases. Of course, the use of these traits requires that they be thoroughly understood, especially in relation to hydraulic issues in plants, as should their interactions, with the aim of harnessing the genetics of these traits.

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