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Understanding of terminal drought tolerance mechanisms in pearl millet (*Pennisetum glaucum* (L.) R. Br.)

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ABSTRACT

The main objective of the thesis work was the detailed characterization of pearl millet (*Pennisetum glaucum* (L.) R. Br.) genotypes contrasting for terminal drought tolerance. For that work, we used a set of near isogenic lines (NIL-QTLs; carrying terminal drought tolerance quantitative trait locus (QTL) from a drougth tolerant donor parent on the genetic background of a sensitive parent) and a recombinant inbred lines population (RIL; developed from a cross between the tolerant and sensitive genotype). In these contrasting genotypes we investigated following physiological traits. Transpiration rate (Tr), transpiration efficiency (TE), transpiration response to increased vapor pressure deficit, threshold in volumetric soil moisture where transpiration begins to decline (FTSW threshold), stomatal density (SD), sensitivity of plants' growth to VPD below and above 2kPa. Regarding biochemical traits, we followed content of chlorophyll (Chl), carotenoids (Car), abscisic acid (ABA), proline (Pro), we conducted isozyme analysis of antioxidative enzymes [superoxid dismutase (SOD), ascorbic peroxidase (APX), catalase (CAT)].

The main leading thread for understanding the drought tolerance mechanisms of pearl millet came from the analysis of traits related to the control of water losses under fully irrigated conditions. We could clearly distinguish drought tolerant genotypes from the sensitive ones based on: i) lower Tr in well-watered conditions measured on full plant basis and on detached leaves ii) higher leaf ABA content in well-watered conditons iii) sensitivity of transpiration to high VPD condition under well-watered conditions. Furthemore, the leaf expansion of tolerant genotypes was sensitive to VPD conditions in which plant development took place and these conditions determined the dynamics of water utilisation during plants development. Based on the biochemical parameters we could rarely distinquish between tolerant and sensitive genotype. Though we documented differences in the activity of APX5 isoenzyme and proline accumulation dynamics under water limiting conditions between tolerant/sensitive genotypes, this variation was probably not directly linked to the yield variation of these genotypes under terminal drought conditions.

It is concluded that the major terminal drought tolerance mechanism of investigated tolerant pearl millet genotypes is linked to their lower Tr. Low Tr of these genotypes probably contribute to saving the water in the soil profile and so leaving a critical amount of water available for grain filling stage (in fact drought avoidance mechanism). It is further discussed that Tr could be influenced by the level of leaf ABA and the hydraulic properties of plant tissues. However, these "water saving" drought tolerance mechanisms seems to be specific to the environmental conditions in which plants` development took place. The importance of these water saving mechanisms is also being validated in RIL population. The biochemical parameters tested under drought conditions appeared to have no major significance for terminal drought tolerance.

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Contents

1. INTRODUCTION

1.1 Pearl millet – genus description

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] (also known under synonyms: *P. americanum* (L.) Leeke or *P. typhoides* (Burm.) Stapf and C.E. Hubb.), an important cereal of traditional farming systems in tropical and subtropical Asia and sub-Saharan Africa, accounts as the sixth most important crop after wheat, rice, maize, barley and sorghum regarding annual global production (FAO 1992). Pearl millet is the staple food grain with a high nutritional value and is also used as a feed, fodder, construction material and even its potential as a source of biofuel is being explored (Wu et al. 2006). It is grown on 29 million ha (FAO 2005) in Africa and Indian sub-continent supporting millions of poor rural families mostly in the drought-prone areas where rainfed agriculture is commonly practiced. Pearl millet is the fourth most important cereal crop in India, after rice, wheat and sorghum, where it is widely grown in the states of Rajasthan, Maharashtra, Gujarat and Haryana where the food security of the poorest population depends vastly on pearl millet production. The average agricultural area sown to pearl millet in India reach to 9.5 M ha with an average annual grain production of 8.3 M tons (FAO 2005). Pearl millet is known to yield up to 6 t/ha, but mainly due to environmental conditions the common average grain yields are lowered to average between 800-600 kg/ha (FAO 2005, 2010).

According to the earlier archeological records, pearl millet originated in Africa and it was domesticated along the Southern margins of the Saharan central highlands 3500-5000 years ago and it was introduced to India about 2000 B.C. (Harlan 1971, Anand Kumar 1989).
Taxonomically, pearl millet belongs to the family of *Panicoidae*, genus *Pennisetum*. The genus *Pennisetum* is divided into five sections: *Gymnothrix*, *Eupennisetum*, *Penicillaria*, *Heterostachya* and *Brevivalvula* (Stapf and Hubbard 1934). Cultivated pearl millet belongs to the section *Penicillaria*. This genus is comprised of over 140 species, with chromosome numbers in multiples of x = 5, 7, 8 and 9 and ploidy ranging from diploid to octaploid levels (Brunken 1977). Sexual, apomictic and facultative apomictic, as well as annual and perennial species are included in this genus. The cultivated crop and its wild progenitors are an annual, sexual diploid (2n = 14), and its chromosomes are designated as the A genome (Jauhar and Hanna 1998). Pearl millet possesses seven pairs of large chromosomes and a haploid DNA content of 2.5 pg (Bennet and Smith 1976). The genome size of pearl millet is about five times larger than that of rice (430 M bp), larger than that of sorghum (750 M bp) and almost equal to that of maize (2400 M bp) (Arumuganathan and Earle 1991). Cultivated pearl millet is a cross-pollinated annual C4 crop with a protogynous flowering habit, and can be intercrossed with a large group of wild relatives (Jauhar 1981, Liu et al. 1994). One of the closest relative is a Napiergrass (*Pennisetum purpureum* Schum.) which is sexual perennial tetraploid (2n = 4x = 28) with chromosomes A` and B. *P. purpureum* readily hybridizes with cultivated crop species and therefore allows continuous gene flow into domesticated genepools (Harlan and de Wet 1971, Harlan 1975).

1.2 Pearl millet – growth conditions and limitation

Pearl millet grows best on well-drained light sandy soils. It can withstand water limited conditions relatively well compared to other crops like sorghum and maize (Burton 1983), therefore is considered as a drought tolerant crop *per se.* Nevertheless, there has been proved variability in the germplasm for drought tolerance indicating the potential for further improvement of drought tolerance for this crop (e.g. Bidinger and Hash 2004, Bidinger et al. 1987, 2007, van Oosterom et al. 1996, Nepolean et al. 2006, Yadav et al. 2002, 2003, 2004, Serraj et al. 2005, Kholová et al. 2010 a, b). Pearl millet also well tolerates high ambient temperature, low soil fertility, soil pH as low as 4 and high concentration of Al, yet responds well to favorable soil conditions (National Research Council 1996). However is increasingly sensitive to flooding and low temperature (FAO 2010). Indeed, in some of the hottest and driest regions of India and Africa, where other crops do not grow well, pearl millet is the only cereal that can be grown reliably and so plays a critical role in food security. Generally, pearl millet is considered more efficient in utilization of soil moisture and has a higher level of heat tolerance than sorghum and maize (FAO 2010). These facts make pearl millet an important food staple in rain-fed regions of sub-Saharan Africa and the Indian sub-continent, especially in the semi-arid regions, where other crops tend to fail because of erratic rainfall, poor soil conditions and inadequate agricultural practices (FAO and ICRISAT 1996). Recently, there is a renewed interest globally in growing pearl millet because of its drought tolerance and high quantity even increased nutritional quality of grain. Pearl millet grains contain 27 – 32% more proteins, higher concentration of essential amino acids, twice the extractable amount of fat and higher releasable energy than maize (Ejeta et al. 1987, Davis et al. 2003). The energy density of pearl millet grains is relatively high, arising from their higher oil content relative to maize, wheat, or sorghum (Hill and Hanna 1990). Also, the amino acid profile is more favorable to human diet than that of common sorghum or maize and is comparable to those of the small grains wheat, barley and rice (Ejeta et al. 1987).

Although pearl millet is one of the most drought tolerant cereals of all domesticated crops (Bidinger and Hash 2004), its grain yield is limited by the poor soil fertility and water-holding capacity of the marginal soils on which the crop is largely grown, combined with traditional management practices (including little use of fertilizers and below optimum levels of tillage) in these stress-prone agricultural production areas. The pest and diseases can also cause considerable yield losses. Rust (*Puccinia substriata* var. Indica), Pyricularia leaf blight (*Pyricularia grisea*) and root knot nematode (*Meloidogyne arenaria*) were shown to reduce the yields considerably (Wilson and Gates 1993, Timper et al. 2002). Similarly grain molds (*Fusarium semitectum* and *F. chlamodosporum*), insect like European corn borer (*Ostrinia nubialis*), corn earn worm (*Helicoverpa zea*) and/or green stink bug (*Nezara viridula*) can also negatively impact yields. Further limitations are imposed by abiotic stresses like salinity and drought stresses. The crop suffers from water deficit at critical growth phases, especially during crop establishment (intermittent drought) and reproductive growth (terminal drought). Therefore, there is a considerable potential for the pearl millet improvement at these research areas.

Drought research on pearl millet conducted at CGIAR (Consultative Group on International Agricultural Research) centers over the past decades can be grouped into 3 broad areas: screening and evaluation of genotypes for yield drought tolerance;

strategic research of different morphological, anatomical, physiological, biochemical and genetic parameters/mechanisms; and applied research on drought management. Future genotypic selection based on desirable drought tolerant traits contributing directly or indirectly to superior yield performance under drought conditions might be possible.

Table 1. Pearl millet physiological traits investigated in relation to drought resistance improvement

Traits investigated	references
Grain and stover yield & quality	Ibrahim et al. (1985), Kumari S (1988), Bidinger et al. (1987, 2007), Singh and Singh (1995), van Oosterom et a.l (1996), Nepolean et al. (2006), Yadav et al. (1999a,b, 2002, 2003, 2004) Serraj et al. (2005)
ABA accumulation	Henson et al. (1981), Henson (1983), Henson et al. (1983), Henson (1984)
Water potential	Henson (1982)
Osmotic potential	Henson (1982)
Osmolytes	Patil et al. (2005), Kholová et al. (2008)
Antioxidative enzymes	Patil et al. (2005), Kholová et al. (2008)
Photosynthetic pigments	Ibrahim et al. (1985), Ashraf et al. (2001)
Transpiration related traits	Ibrahim et al. (1985), Squire (1979), Black and Squire (1979), Henson et al. (1981), Henson (1984), Kholová et al. (2008, 2010 a, b, c)
Canopy temperature	Singh and Kanemasu (1983)

Current research is focused particularly on the understanding of mechanisms responsible for drought resistance and on the evaluation of their easily scalable marker parameters such as transpiration rate and/or root characteristics.

1.3 Drought stress

Drought stress, the major constrain for crop productivity, is affecting 1/3 of arable land world-wide and will probably increase in the on-going climate changes. Therefore, future sustaining the productivity of land will be, at least partially, dependent on production of crops with increased drought tolerance.

Till date, much has been done in research for drought resistance/tolerance, however the outcome of these efforts has not met the demand for crops production. This could be explained with the extreme variability and complexity of drought stress effects. Firstly, drought can affect plants in differential stages of their growth; either early during plant establishment, in vegetative developmental stage (intermittent drought) or at the end of growing season in reproductive stage (terminal drought). Out of these, terminal drought is shown to contribute to the most severe yield losses as it affect spikelet establishment and reduces its fertility (Bernier et al. 2007). Secondly, drought has different intensities and effects and plants have developed several strategies to deal with them on different levels of their phenological, morphological and anatomical structure as well as on the levels of various physiological and biochemical processes. Therefore, since there exists diverse drought patterns and various plant adjustments to counteract the drought effects, it is very important to define which type of drought stress is targeted by a breeding program.

There are basically several levels of plant ***drought tolerance/resistance***.
Drought escape: The success of plant in water deficient environment can be dependent only on its phenology; e.g. plant will complete its reproduction during the wet season before drought occurs (early flowering).
Drought avoidance and drought adaptation: both strategies relate to the morphological, anatomical and/or biochemical plant's adjustment to avoid water deficit in plants` tissues (e.g. development of succulence of leaves and roots, reducing of transpiring surfaces, sunken stomata, presence of specialized photosynthetic pathways, thick cuticule, extensive root growth, efficient water use). These mechanisms can be either induced by drought (avoidance) or could have constitutive character e.i. are also present in non-stressed conditions (adaptation).
Drought acclimation (sometimes referred as drought tolerance): Is commonly understood as series of biochemical adjustments induced by water deficit (e.g. osmoprotection, anti-oxidative enzymes induction, chlorophyll degradation).
All of above mentioned strategies can by potentially used in drought resistance breeding depending on target environmental conditions.

1.4 Drought resistance improvement

1.4.1 Breeding for drought escape strategies

Drought escape strategy of the crops (in terms of early flowering) has been recognized as a major factor determining relative cultivar performance in individual stress environments (Bidinger et al. 1987) and is often a major cause of Genotype × Environment interaction especially in harsh environmental conditions (van Oosterom et al. 1996). The breeding for altered life cycle is extremely useful in the environments where the drought periods are highly predictable (Bernier et al. 2007). The basic principle of the successful implementation of this strategy into breeding is that shortening of crops life cycle ultimately translates into the relatively extended duration of grain filling stage prior to drought occurs (e.g. Bort et al. 1998, Richards 2000). Anyhow, the crops cultivars possessing the shortened life cycle might not be necessarily considered truly drought tolerant.

1.4.2 Breeding for drought acclimation strategies

In past, a lot of attention was paid to crop drought acclimation improvement. The breeding for drought acclimation improvement could be potentially useful in highly unpredictable environments. The literature is filled with proposed traits dealing with the lower level of plants organization (i.e. molecular, biochemical), which frequently show only poor and inconsistent relationship with crop yield (e.g. Richards, 1996, Araus et al. 2001, Chowdury and Choudhuri 1985; Irigoyen et al. 1992; Űnyayar et al. 2005, Bhatnagar-Mathur et al. 2007, Conti et al. 1994, Mugo 1999, Cellier et al. 1998, 2000). Yield is a very complex trait; throughout the plant cycle it takes many levels of plant organization – from the molecular level to the canopy development. Therefore, any simpler candidate trait related to yield should also integrate the processes in time and should be highly environmental specific (Slafer and Araus, 1998, Araus 2002).

1.4.2.1 Potential of osmolytes in breeding for drought acclimation strategies

As a candidate traits in this category can stand traits related to osmotic adjustment (e.g. accumulation sugars, nitrates, simple aminoacids). The primary idea of osmolytes benefit under water deficit conditions was that accumulation of these compounds could decrease the cell osmotic potential and thus maintain water absorption and cell turgor pressure, which might contribute to sustaining physiological processes, such as stomatal opening, photosynthesis and growth (Ludlow and Muchow 1990, Blum 1996). Osmolytes have been emphasized as a selection criterion for yield improvement in dry environments (Ludlow and Muchow 1990, Zhang et al. 1999). Soon after this idea reflected in identification of QTLs linked to osmolyte accumulation capacity (e.g.Van Deynze et al. 1995, Price and Courtis 1999, Teulat et al. 1998) and development of crops (through transgenosis or marker assisted breeding (MAB - e.g. Zhang et al. 1999, Nguyen et al. 1997) with increased levels of osmolytes. Several stable transgenic events resulting in plants over-expressing osmolytes were documented for *A. thaliana* (manitol – Thomas et al. 1995, glycine betaine – Waditee et al. 2003), tobacco (sorbitol – Sheveleva et al. 1998, inositol – Sheveleva et al. 1997, Majee et al. 2004, trehalose – Pillion-Smits et al. 1998, proline – Zhang et al. 1995, LaRosa et al. 1991), *Diospyros kaki* (sorbitol – Gao et al. 2001), rice (proline - Zhu et al. 1998) or soybean (proline - DeRonde et al. 2000). Despite producing higher amounts of osmolytes, these plants showed only marginal improvement of drought tolerance. But at least, developed transgenic plants contributed to progress understanding of the osmolytes function in plant tissues.

Overall, according to current opinion, traits enhancing osmotic adjustment are of little benefit for yield in the field conditions. In fact these traits are more likely to cause early exhaustion of soil moisture (Sinclair and Serraj 2002, Kholová et al. 2010a, b) and rapid transition of plant to the survival mode where even putative benefits are of little use for growers (for review see Sinclair and Serraj 2002, Blum 2005, 2009).

1.4.2.2 Potential of photosynthetic pigments and anti-oxidative enzymes in breeding for drought acclimation strategies

Drought stress often causes the changes in photosynthetic pigment content and ratio; (e.g. Anjum et al. 2003, Farooq et al. 2008, Messacci et al. 2008). The magnitude of these changes could in turn negatively influence photosynthesis and so contribute to yield losses. The potential of engineering plants to maintain the pigments content under drought was heavily discussed especially when some experiments revealed positive correlation with the grain yield (e.g. in maize, wheat and groundnut; Pastori and Trippi 1992, Kraus et al. 1995, Arunyanark et al. 2008). Nevertheless, the physiological constrain of pigment maintenance (especially chlorophylls) in plants facing drought simultaneously give rise to elevated production of reactive oxygen species (ROS) and can in turn destroy other molecules in photosystems (Schmid 2008). Therefore, unbalanced photosynthetic pigments content could in fact accelerate the damage of photosystems if ROS production is not regulated further (Maslova and Popova 1993, Keiper et al. 1998, Tardy et al. 1998, This et al. 2003, Farrant et al. 2003). There are basically two detoxification mechanisms plants have developed to avoid excessive ROS production (as in Scandalios 1997, Shalata and Tal 1998, Gomez et al. 1999); (i) Non-enzymatic radical scavengers, e.g. carotenoids, glutathione, mannitol, ascorbate, tocopherol, flavonoids and some alkaloids; (ii) Enzymatic anti-oxidants of the Hallivel-

Asada cycle (Asada 1994), which involves ROS reactions with superoxide dismutase (SOD), ascorbic peroxidase (APX) and catalase (CAT) and regeneration of substrate for APX assisted by glutathionreductase (GR). There also appeared evidence in some plant species (wheat, mangrove, sesame) tolerance to abiotic stresses could be related to enhanced capacity to scavenge ROS (Sairam and Srivastava 2001, Parida et al. 2004, Fazeli et al. 2007). From the other hand, studies on jute, alfalfa and tomato haven't confirmed these results (Chowdury and Choudhuri 1985, Irigoyen et al. 1992, Űnyayar et al. 2005, Bhatnagar-Mathur et al. 2009). There appears to be limited effort to transform plants for specific modulation of chlorophylls or antioxidative enzymes level in order to improve their drought tolerance. However, increased chlorophyll levels were observed as a secondary effect in plants transformed with eg. nicotianamine synthase gene enhancing iron uptake in *Lolium perenne* (Zhang and Zheng 2008), these plants simultaneously showed improved drought tolerance. Similarly, rice carrying additional *„Triticum aestivum* salt tolerance-related gene (*TaSTRG*) " with unknown function had enhanced salt and drought tolerance accompanied by chlorophyll content maintenance (Zhou et al. 2009). In tobacco transformed with isopentenyltransferase, which induces cytokinins synthesis and so delay senescence, there were observed enhanced levels of several antioxidative enzymes along with enhanced drought tolerance (Rivero et al. 2007). Contrarily to chlorophylls and antioxidative enzymes, there has been effort to produce plants with increased levels of carotenoids but more likely in order to meet the nutritional demand in human/livestock diet (eg. "golden rice" over-expressing ß-carotene (Ye et al 2000, Bayer et al. 2002, Paine et al. 2005)) than to increase plant drought tolerance.

After all, the contribution of photosynthetic pigment contents maintenance, its relation to ROS scavenging systems and finally the link to the yield stability under drought is still not clear and seems to be highly variable depending on species, developmental and metabolic state of plant, and the duration of stress (Smirnoff 1993, Castillo 1996).

1.4.2.3 Potential of plant hormone regulators in breeding for drought acclimation strategies

The discussion about use of plant hormone regulators in breeding programs still persist. The debate focuses mainly on potential of abscisic acid (ABA), which has been shown to play the role in stomata functioning (e.g. Schulze 1986, Davies and Zhang 1991, Sharp 1996, Bray 2002). However, other hormones like cytokinins are also likely to be involved in the regulation of stomatal aperture, either in isolation or acting in conjunction with ABA (Wilkinson and Davies 1999, 2002). The regulation through ABA is far from being simple and involves both long-distance transport and modulation of ABA concentration at the guard cells to a given dose of the hormone (Wilkinson and Davies 2002). Among the factors implicated in the ABA action are xylem sap and leaf tissue pH, which may increase in condition of high evaporative demand such as high vapor pressure deficit (VPD), high light intensity and high leaf temperature. Large inter/intra-specific variation in ABA levels has been reported (Conti et al. 1994, Mugo 1999, Chandrasekar et al. 2000, Li and Wang 2003, Yin et al. 2005, Zhang et al. 2005). In wheat and some woody plants, higher ABA level was correlated with drought tolerance (Chandrasekar et al. 2000, Li and Wang 2003, Yin et al. 2005, Zhang et al. 2005), although no such correlation was reported in maize and sunflower (Conti et al. 1994, Mugo 1999, Cellier et al. 1998, 2000) and in phaseolus no ABA increment was detected during drought exposure (Trejo and Davies 1991). So, the

ABA-tolerance link is, as expected, highly crop and environment specific. There has been also progress in identification of QTLs affecting ABA concentration under drought conditions especially in maize leaves and xylem sap (Lebreton et al. 1995, Landi et al. 2005, Tuberosa and Salvi 2007). Some of these results suggest that ABA concentration in plant tissues might be tightly associated with rooting characteristics, especially root internal architecture and relative water content in maize plants. However, the putative effect of these QTLs on the yield under water limited conditions persist questionable (Tuberosa and Salvi 2007). To study ABA effect to further extend, there have been developed transgenic plants over-expressing ABA constitutively or inducibly under drought conditions in tobacco and *A. thaliana* (Borel et al. 2001, Thompson et al. 2007, Iuchi et al. 2001). Some of these transgenic events lead, indeed, to the enhanced plants drought tolerance (Borel et al. 2001, Iuchi et al. 2001), however these plants also showed delayed germination and their water management considerably altered. Logically, limitation in water usage in ABA transgenics may in turn restrict plant growth and so can be contra-productive for use in agricultural systems.

Furthermore, importance of ABA independent mechanisms coordinating plants' water use is being emphasized (Cellier et al. 1998, 2000, Davies et al. 1994, Yamaguchi and Yamaguci-Shizonaki 1997). After all, the complexity of plant response to ABA is apparent and the selection for high capacity ABA accumulation has yet to provide conclusive data that could help shape crop breeding for drought conditions (Pekic et al. 1995).

1.4.3 Breeding for drought avoidance improvement

Breeding for drought avoidance improvement holds also putative potential for crops grown under variable environmental drought patterns. The success in drought avoidance crops improvement mostly depends on understanding the complex physiological processes of plants under drought. As a starting point for identification of the crucial mechanisms of drought avoidance in crops there has been often used the simple concept where Yield = T x TE x HI (T- amount of water transpired, TE – transpiration efficiency, HI – harvesting index; Passioura 1977). According to the component analysis proposed by Passioura (1977, 1996) the traits convenient for breeding selection should be those increasing i) the capacity to capture more water, ii) the efficiency for producing dry matter per unit of absorbed water and iii) the ability to allocate an increase proportion of the biomass into grain. All these mechanism were thought of as the breeding targets in various crops (e.g. groundnut, sorghum). However, it is also important to take into consideration that this formula overlooks possible interactions between the parameters mentioned in the equation. In particular, it overlooks the fact that there may be stages where water utilization (T) might be critical for some other component of the equation (e.g. HI). Therefore, it appears clearer that, at least for certain crops and conditions, the timing of water utilization throughout plants development might be a principal component of drought adaptation even more important to consider than the components of the Passioura's equation (Sinclair et al. 2005, Blum 2009, Kholová et al. 2010a, b). Based on recent understanding the breeders efforts should be rather focused to improvement of plants' use in well-watered conditions which can result in a soil water conservation and further in availability of water in soil profile during the prolonged drought (e.g. Mortlock and Hammer 2001, Condon et al. 2002, Serraj et al. 2004, Kholová et al. 2010 a, b, Sinclair 2010). This

was practically demonstrated in pearl millet genotypes tolerant to terminal drought stress which were able to restrict transpiration rate (Tr) before stress conditions occurred and to save water in the soil profile for period of grain filling (Kholová et al. 2010a, b). Similar results were obtained for wheat (Richards 2000), soybean (Sinclair 2005), groundnut (Bharat-Matur et al. 2009) or recently chickpea (Zaman et al. unpublished).

As mentioned above, basic mechanisms of drought avoidance strategies mostly relates to the plant's control of the water usage. Allover, plant's use of water principally depends on the balance between the water absorption by the root system, and water, that is released through the leaves by transpiration (which is in the simplistic way the function of plant water conductivity and ambient environment). Existing variability in root system characteristics could be well utilized in breeding programs. A deeper root system has been shown to allow crops to extract more water from the soil, resulting in higher yield potential under drought (e.g. Johansen et al. 1997, Kashiwagi et al. 2006, Bernier et al. 2007). Therefore, efficient regulation of root/shoot growth could be an important characteristic of drought tolerant crops genotypes. However, if too much carbon is invested in root growth, yield may be affected negatively. Contrarily, if no enough growth is invested in roots, plants can suffer from drought and reduced yields as well. Therefore, the contribution of root depth to drought avoidance is considered highly site specific (Bernier et al. 2007). Other root characteristic which can influence plants water use is the root conductivity. There has been demonstrated variability in the ability to extract water from the soil under water limited conditions in maize and some legumes species (Ray and Sinclair 1997, Bhatnagar-Mathur et al. 2007, Hufstetler et al. 2007). But even internal root structure (constitutive or inducible by water deficit) could influence the axial water flow and so might be considered to influence drought tolerance. In this regard, differences in xylem vessels hydraulic properties (xylem diameter and chemical composition) were shown the potential to influence drought tolerance in e.g. agave (Pena-Valdivia and Sanchez-Urdaneta 2009), rice (Umayal et al. 2001) or wheat (Richards and Passioura 1989). Furthermore, radial transport through plant tissues can also play an important role in the drought resistance. In this regard, the role of root aquaporines in the restriction/enhancement of water absorption during the crucial periods of drought is intensively studied. Aquaporines are transmembrane proteins triggering symplastic (cell-to-cell) movement of water molecules. Substantial inter- and intra-specific variability in aquaporin numbers and types has been shown (e.g. Tyerman et al. 2002, Javot and Maurel 2002, Bramley et al. 2007, 2009). However, exact role of aquaporines in drought resistance is yet to be explored. Another basic mechanism how plants can tune the water usage is through stomata parameters like are stomata density, stomata conductivity and also sensitivity of stomata conductance to soil drying (Muchow and Sinclair 1989, Henson et al. 1983, Masle et al. 2005). Reduced stomata numbers, smaller stomata size and their early response to declining soil moisture decrease the gas exchange and so reduce water loss what could be potentially advantageous traits improving plants drought resistance. On the other hand, reduced gas exchange could result in serious yield loss in environments with short, frequent and mild droughts. Separate category with similar impact on plant's water usage is the regulation of stomata aperture by plants phytohormones. In this matter, the negative stomata conductance regulator – ABA is widely discussed (see above). One of other mechanisms which may be beneficial for plants suffering drought involves the plants' stomata closure reaction to increased evaporative demand. This response may not necessarily involve direct action of ABA, but may be the result of

long term ABA effect on plants morphogenesis (Aasamaa et al. 2001, 2002), or the mediation of stomata closure by hydraulic signals. Some plant species (typically C4 plants from arid and semi-arid regions) have been found sensitive to high VPD levels, usually above 3-4 kPa where the stomata close to avoid wasteful water loss during the harsh midday conditions (Squire 1979, Sinclair et al. 2007, Kholová et al. 2010b). Not much attention was paid to possible genetic variations in this strategy, although recent modeling data show that a restriction of daily transpiration rate would indeed contribute to saving water in the soil profile and would increase transpiration efficiency TE (Sinclair et al. 2005, 2010, Oosterom et al. 2010).

1.4.4. Recent progress in understanding the pearl millet drought avoidance strategies

In pearl millet, stomata play an important role in minimizing crop water use during pre-anthesis water deficit (Winkel et al. 2001). However, controlling leaf water losses when water is non-limiting for plant development was also considered as a suitable adaptation strategy (Kholová et al. 2008, 2010a, b). It was shown that pearl millet genotypes carrying a terminal drought tolerance QTL are characterized by a lower rate of water loss per unit leaf area under well-watered conditions (Tr, in g cm^{-2} day^{-1}) (Kholová et al. 2008, 2010a, b). This water saving mechanism operating under non-stressed conditions was proposed to leave water available in the soil profile for grain filling and could be beneficial for terminal stress conditions. Though, how certain pearl millet genotypes achieve low Tr is still unclear. The daily Tr actually "integrates" the regulation of stomata over substantial length of time, but may not exactly determine transient genotypic differences in stomata regulation occurring during the course of the day. As such, the Tr assessment does not indicate whether Tr differences between genotypes are constant during the day or whether transient changes in environmental conditions lead to transient larger Tr differences between genotypes. It was determined, that the probable mechanism of low Tr maintenance relates to the fact, that tolerant pearl millet genotypes tend to restrict their Tr when exposed to high VPD to the greater extend than drought sensitive genotypes. However, the VPD response may not be the only source of Tr variability between tolerant and sensitive genotypes. The water saving mechanism including lower leaf conductance could relate to high leaf ABA differences as well, since tolerant genotypes were shown to maintain considerably higher level of ABA in leaves in well watered conditions (Kholová et al. 2010 b). Ongoing work is also aimed at the study of the role of aquaporines in tolerance to drought stress. It was determined the existence of a huge variability in types and numbers of root aquaporines. Tolerant genotypes have less number of different types of aquaporines compared to sensitive genotypes tested (Vadez et al. personal communication). Simultaneously, we have recently explored variability in anatomy of root endodermis. It was found that tolerant genotypes possessed smaller cells of root endodermis close to the apical root zone, where majority of water is absorbed. Both of mentioned traits, aquaporines and endodermis variation, may considerably influence the symplastic radial water transport and eventually cause the hydraulic limitations as discussed above (Vadez et al. personal communication).

Therefore, many questions regarding the water conserving mechanisms persist unanswered. Especially, recent demonstration that the variation in pearl millet water utilization strategy is conditioned by the environmental characteristics in which plants' development take place (Kholová et al. 2010 c).

1.5 Marker assisted breeding approach in pearl millet

Till date, cereal breeding (including pearl millet) has been based principally on empirical selection for yield *per se* (e.g. Loss and Siddique 1994, ArvindKumar et al. 2008). However, this approach is far from being optimal, since yield is characterized by low heritability and a high genotype × environment (G×E) interaction (Jackson et al. 1996). However, following the above mentioned context it is more clear that an indirect (or analytical) approach, based on the understanding the crop physiology in connection to its molecular background, can help to target the key traits which can directly translate into yield benefits both under optimal and stress conditions. This approach can complement conventional (empirical) breeding programs and hasten yield improvement (Araus 1996, Slafer and Araus 1998). In this approach, the molecular biology tools appear extremely powerful. One of the useful molecular tools for breeding acceleration are the molecular assisted breeding (MAB) techniques enabling location of key DNA regions (e.i. quantitative trait loci, QTLs) and their rapid introgression into the desired genetic background. QTL is a chromosomal region where one or more genes affect phenotypic values of a quantitatively inherited trait such as grain yield. QTL is detected by correlating phenotypic values of lines with different marker genotyped at a given chromosomal location. There are three steps to QTL analysis: i) phenotypic evaluation of relatively large population segregating for polymorphic genetic markers, ii) genotyping of the population, iii) statistical analysis to identify the loci that are affecting the trait of interest. Such mapping studies are performed to detect possible linkage of a molecular marker to a phenotype of interest. It then becomes possible to select for desirable genes based on the presence of the marker genotype, which is faster and easier than the field phenotyping. This technique, known as marker-assisted selection, is theoretically more reliable than selection based on phenotype, but there are persisting problems such as QTL × environment interactions, QTL × genetic background interactions and also understanding of yield-determining physiological processes in the varying environment (Yadav et al. 2002, Tuberosa et al. 2007).

In 1990 the pearl millet breeding unit of the Cereal Program at the ICRISAT began to create segregating populations of pearl millet (*Pennisetum glaucum* (L.) R. Br.) suitable for mapping of the genome. Due to its highly out-crossing breeding behavior, its apparent origin from several independent domestication events (Poncet et al. 1998) and the wide range of stressful environments in which it has traditionally been cultivated, pearl millet exhibits a tremendous amount of polymorphisms at phenotypic level (Liu et al. 1992, 1994). However, the limited availability of marker polymorphisms makes genetic diversity studies in this species more complicated than in other cereals. Despite, the breeding behavior of pearl millet and the existing phenotypic diversity within this species, have strong implications for the use of molecular markers in its diversity assessment. In all crop species, phenotypic estimates of genetic diversity are biased by the environment(s) in which evaluation occurs. Further, in pearl millet and other cross-pollinated seed-propagated species, these estimates can also be considerably influenced by inbreeding depression that occurs as a result of a closed population structure during the obtaining of desired recombinant populations. The impact of regeneration procedures on diversity in accessions maintained in gene bank are unknown, therefore, for genetic diversity assessment in cross-pollinated and highly genetically polymorphic pearl millet, molecular markers offer considerable advantages over methods based on phenotypic evaluation. The genetic analysis requires preferably co-dominant markers

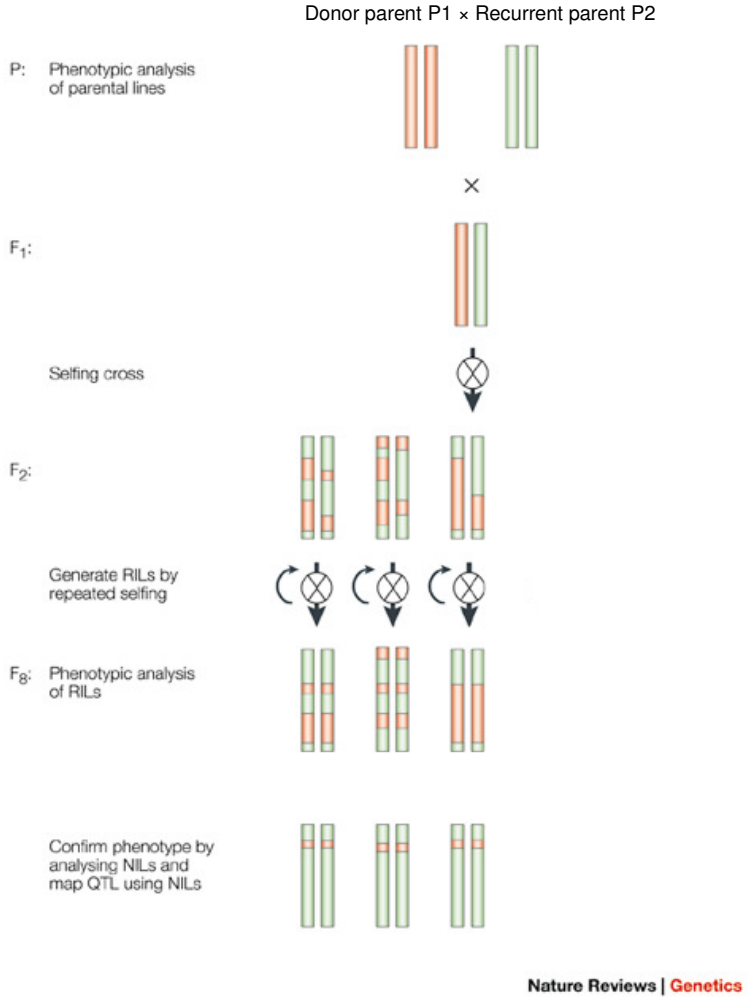
which need to be neutral and unlinked to give unbiased genetic diversity estimates. Currently, in pearl millet co-dominant RFLP, STS, SSR and dominant type of markers – AFPL and DaRT are available. The information on diversity in molecular markers provides the tool to study genetic structure of pearl millet (Hash et al. unpublished).

1.5.1 Developing pearl millet mapping populations

There are several ways of developing segregating mapping populations for QTL analysis. In plants, the use of mapping populations consisting of homozygous individuals is preferred because it allows uniform performance of replications and multiple analyses of the same population. Homozygous populations can be obtained by repeated selfing or sibling mating, like in the case of recombinant inbred lines (RIL), but also by induced chromosomal doubling of haploids, such as doubled haploid lines (DHL) (Rae et al. 1999, von Korff et al. 2004). RIL are considered advantageous over DHL because of their higher recombination frequency in the population, resulting from multiple meiotic events that occur during repeated selfing (Jansen 2003). Common RIL population is formed by crossing two inbred strains followed by repeated selfing or sibling mating to create a new inbred line whose genome is a mosaic of the parental genomes (Fig 1). As each RIL is an inbred strain, and so can be propagated eternally, a panel of RILs has a number of advantages for genetic mapping: i) genotype of the line need to be genotyped only once, ii) multiple individuals from each line can be phenotyped to reduce individual, environmental and measurement variability. Though, mapping analyses of RIL population can be biased due to the masking effects of major QTL and epistatic interactions of multiple QTLs (Ungerer et al. 2003).

ICRISAT pearl millet populations intended for genetic studies are usually based on F1 selfing cycles. These F1 progenies are selfed to produce F2 and the cycles of selfing continue usually up to F6 population of lines (RIL) to assure their reasonably low heterozygosity (basic crossing scheme viz Fig. 1).

Figure 1. Basic scheme of recombinant inbred lines (RIL) development by selfing: two inbred parental lines (P1 - donor parent and P2 - recurrent parent) are crossed to prepare F1. F1 generation individuals are selfed giving rise to F2 recombinant lines with high heterozygosity. Individuals in F2 are then selfed several times to assure reasonably low heterozigosity of advanced selfed lines (RILs). Near isogenic lines (NIL) could be further developed by several cycles of marker assisted backcrossing of lines containing desired portion of DNA to the recurrent parental line.



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Initial pearl millet marker map was based on RFLP markers (Liu et al. 1992, 1994) (extended by Devos et al. (2000) and Qi et al. (2004)) and resulted in identification of 180 heterologous loci distributed among the expected 7 linkage groups. Subsequently, additional SSR markers information allowed identification of quantitative trait loci (QTL) associated with downy mildew resistance (Jones et al. 1995, 2002, Breese et al. 2002, Gulia 2004), rust and blast resistance (Morgan et al. 1998), terminal drought tolerance and grain and stover yield components (Yadav et al. 2002, 2003, 2004) and for characters involved in domestication (Poncet et al. 2002). The first and only commercial pearl millet hybrid (HHB 67-2) until now incorporating resistance to downy mildew through marker-assisted breeding (MAB) was released collaboratively by ICRISAT and the Haryana Agricultural University (HAU), India in January 2005 (ICRISAT 2005).

1.5.2 Identification of QTL for terminal drought tolerance

The mapping for identification of QTL influencing terminal drought tolerance was performed on two elite and highly drought resistant *Iniadi*-based inbred lines; PRLT 2/89-33 and 863B (Yadav et al. 1999a, b) which were crossed to drought sensitive lines H77/822-2 and ICMB841 respectively to generate RIL populations as mentioned above. In both populations, substantial portion of the variation in drought resistance mapped to the linkage group 2 (in both populations) and 6 (in population based on 863B×ICMB841) (Yadav et al. 1999a, b, Bidinger et al. 2007). Based on this QTL information there were initiated different marker-assisted backcrossing schemes to transfer LG2 QTL from drought resistant parent to the genetic backgrounds of drought sensitive lines (Hash et al. 1999). The advantage of NILs over RILs population is that the NIL analysis is not biased by possible QTL interactions and effects of major QTL as mentioned above. Moreover, these NILs can be readily used in farmers' systems as they combine the advantages of genotypic background of locally adapted plant materials with the small portion of chromosome (QTL) from donor genotype improving adapted genotype's drought resistance. Developed NILs are also precious material for basic research on the mechanisms of drought resistance, because they allow precise analysis of the important drought resistance mechanisms underlying the introgressed QTL (Kholová et al. 2010a, b, c).

Recently, advanced precise mapping population intended for dissection of LG2 drought tolerant QTL mechanisms is being developed. This "high resolution cross" population is based on the cross of the most drought tolerant NIL line ICMR01029 with ICMR01004 (which is basically downy mildew resistant form of H77/822-2). The lines originated from this cross should allow precise mapping the LG2 QTL region and contribute to further understanding the particular mechanisms involved in the complex machinery of drought resistance (Hash et al. unpublished, but population already developed).

Outline of the thesis:

- Characterization of pearl millet (*Pennisetum glaucum* (L.) R. Br.) genotypes know to contrast for terminal drought tolerance for various physiological, morphological, biochemical and anatomical traits.

- Identification of key traits and mechanisms putatively involved in the terminal drought tolerance in terms of yield.

- Confirmation of trait importance for terminal drought tolerance in NIL containing QTL for yield benefits under drought.

- Mapping the selected key traits in the RIL population.

2. OVERVIEW AND RESULTS

This essential part of the thesis consists of four key research publications relevant to the thesis topic which have been peer-reviewed and published in international journals and additional four key poster publications extending the publications contents which were presented at international conferences. This chapter presents a brief summary of each publication and its relevance to the topic.

Articles

2.1. Exploiting the functionality of root systems for dry, saline, and nutrient deficient environments in a changing climate

Vadez V, Krishnamurthy L, Kashiwagi J, Kholová J, Devi JM, Sharma KK, Bhatnagar-Matur P, Hoisington DA, Hash CT, Bidinger FR, Keatinge JDH. SAT e-journal 4 (1): 1-61. 2007.

This review sums up the current opinion about root functioning in varying environmental conditions and appointing up that the usefulness of certain root traits is limited and strongly dependent on the target environment. Furthermore, the work done on root system investigations in ICRISAT mandate crops (i.e. chickpea, pigeonpea, groundnut, sorghum and millet) is summed. Similarly, the potential use and constrains of the root traits in breeding for drought, salinity and nutrient deficiency tolerance in semi-arid tropics agricultural systems is discussed. It is emphasized that to incorporate roots in the breeding programs the knowledge of root functioning would be more useful rather than root morphology descriptive traits. It is also appointed out, that roots characteristics are a component traits which can explain the plants` abiotic stress response only partially and that the extended knowledge integrating the plants behavior in soil-atmosphere continuum should be understood before the particular traits could be incorporated into breeding programs.

Exploiting the functionality of root systems for dry, saline, and nutrient deficient environments in a changing climate

Vadez V, Krishnamurthy L, Kashiwagi JW, Kholová J, Devi JM, Sharma KK, Bhatnagar-Mathur P, Hoisington DA, Hash CT, Bidinger FR, Keatinge JDH

Introduction

Increasing episodes of drought, lack of sufficient nutrients, exposure to toxic minerals, and soil compaction are just a few examples of the environmental constraints that the roots are exposed to during plant growth. Understanding how roots respond to these stresses is crucial for improving crop production under such conditions. Yet, investigating roots is a very difficult task and, therefore, very little is known about the precise role that the roots play in contributing to plant adaptation to hostile environments. It is assumed that while the root depth and abundance would contribute to drought tolerance, profuse rooting would enhance nutrient capture, and where the membrane transporters would exclude salts from the root cells. However, a great deal is still unknown about how these mechanisms actually operate; for example which particular characteristics of roots and root hydraulics actually contributes to water uptake in a way that confers increased tolerance, how the stress signaling from the roots affects the physiological relations in the shoot and those between the shoot and the root, how water and nutrient absorption relate to one another when both are limiting, or how roots avoid the loading of salt in xylem vessels.

In this paper, our intention is not an exhaustive review of roots, but to highlight a few research topics related to abiotic stresses - mostly drought stress, but also nutrient limitation (especially phosphorus) and salt stress - where roots and their hydraulics are at the center stage. First, we provide an update on root structure, root hydraulics, and modes of water and nutrient absorption, mainly focusing on how inter- and intra-specific variations in these aspects can modify the way roots respond to a range of abiotic stresses. We then review scattered reports across a range of crops showing the contribution of roots to stress tolerance, and then report our own assessment of the role of roots using near isogenic lines (NILs) containing a terminal drought tolerance QTLs. We next review the breeding efforts on roots, some aspects of genetics, and report recent work at ICRISAT where the DREB1A gene appears to positively affect root growth in transgenic groundnut under drought conditions. We follow by looking at the role of roots in nutrient acquisition, and how water and nutrient uptake issues need to be addressed holistically. Then, we look at roots from the angle of salinity tolerance, reviewing where roots can contribute to salt tolerance. The following part is on root functionality and we argue that further progress on roots should concentrate of measuring both volume and kinetics of water uptake rather than root morphological traits. Finally, we review how water use efficiency (WUE) and other mechanisms involved in water saving in the soil profile, can eventually allow roots to sustain water uptake. This is considered from the angle of the chemical and hydraulic signaling taking place between roots and shoots. Based on the above, we conclude by proposing research avenues to unlock our knowledge on roots, in a way that eventually allows breeding for improved root characteristics in the face of current climate uncertainty.

Roots and stress tolerance – A review of past efforts

The composite transport model - Besides the fact that roots supply water to the plant and contribute to the overall plant water balance, relatively little is known about the processes and regulations of water uptake. It is well established that the hydrostatic pressure created by transpiration from the shoot is transmitted to the xylem vessels of the shoot and the roots, which drives water in the root cylinder toward the xylem vessels (Tyree, 1997; Steudle, 1995). It is also clear that the hydrostatic pressure is not the only factor responsible for water uptake, which also involves specialized membrane transporters (aquaporins) (Chrispeels and Maurel,1994, Tyerman et al., 2002, Javot and Maurel, 2002). Indeed, under no transpiration, water can be taken up by roots through an osmotic gradient (Steudle, 2000a). Therefore, the current model of water uptake through the root cylinder to the xylem, the composite transport model (Steudle, 2000a), is such that water is taken up via three major pathways: (i) an apoplastic pathway where water travels through the apoplast of the cells in the root cortex, toward the endodermis and the xylem vessels; (ii) a pathway of symplastic water transfer where water goes through cells and remains in the cytoplasm, traveling in the membrane continuum (endoplasmic reticulum and plasmodesmata); and (iii) a pathway through the vacuoles of cells (Steudle and Petersen, 1998; Steudle, 2000b) (Figure 1). It is considered that (ii) and (iii) represent the cell-to-cell pathway, as these components are difficult to separate and both are using membrane

transporters (aquaporins). This pathway usually offers a large resistance to water flow in contrast to the apoplastic pathway, which predominates when transpiration demand is high (Steudle, 2000a&b).

Regulation of radial resistance and abiotic stresses - Under various stresses such as drought, salinity, nutrient deficiency, root aging, or environmental conditions such as temperature, humidity, or light, the resistance to water flow varies (Steudle and Henzler, 1995), and, for instance, usually increases under water deficit (Steudle, 2000a). Most of that resistance is located in the root cylinder (radial resistance), whereas xylem vessels normally offer much less resistance (axial resistance) (Steudle, 2000a). In the root cylinder, the cell-to-cell pathway is a highly regulated movement, involving the crossing of many membranes through membrane transporters (aquaporins, Tyerman et al., 2002, Javot and Maurel, 2002), which usually offers a large resistance to water flow. Therefore, the understanding of which components of the composite model (Steudle, 2001) predominate under non-stressed conditions, and how these components change under a range of abiotic stresses, are crucial in understanding how plants regulate the rate of water and nutrient supply and eventually support transpiration and growth. Several reports have shown intra- and inter-specific differences in the relative proportion of water traveling through each of these pathways (Steudle and Frensch, 1996; Yadav et al., 1996; Steudle and Petersen, 1998, Steudle, 1993, Jackson et al., 2000). Intra-specific differences in the hydraulic properties of roots would affect the rate of soil water use, or would lower the root length density needed to absorb a given amount of water. The water traveling through the apoplastic pathways also lacks a "filtering" effect from the cells (the reflection coefficients of nutrients is usually small or close to zero), thereby taking along a number of nutrients such as salt (Azaizeh et al., 1992) or ABA (Hartung et al., 1998; Freundl et al., 2000) ("solvent drag"). In summary, the predominance of either one of the pathways could have a dramatic influence on the regulation of water uptake, with or without water stress. It also could have dramatic effects on the absorption of toxic salts (see below the section on salinity). Since, nutrient stress also affects the resistance provided by roots to the water flow; a nutrient deficiency would also affect the plant by influencing its water balance.

Roots as a consequence of an evolutionary strategy - Before going any further, we feel that it is important to "demystify" the importance of root for stress adaptation, in particular drought. For instance, many desert plants have been reported not to have a deep root system, whereas a deep rooting would become a more common trend in less extreme dry areas (Kummerow, 1980). In fact, the importance of any aspect of rooting pattern (depth, depth distribution, root length density, etc.) is totally relative to the distribution and amounts of water or nutrients in the soil profile. For example, an increased root depth/root volume is useful only where there is significant water available to exploit by increasing soil volume explored by roots. An increased root length density (RLD) is important only where there are significant amounts of water which is tightly bound to the soil matrix and does not readily move in response to local gradients created by root extraction – e.g. montmorillonitic clay soils. Also plant strategies for water uptake vary; some desert plants such as cacti have extensive but shallow systems to quickly capture large amounts of rainfall and nutrients from soil surface layers because they can store this for long periods, whereas others such as the creosote bush have roots to as much as 20 m, to tap water very deep in the soil profile where there is limited competition for water from other species. So, we believe that rooting aspects in most plants are evolutionary strategies to exploit environmental opportunities. We should therefore approach the roots of crops in the same way to exploit their diversity and their adaptive potential. What follows is a summary of the work on roots in ICRISAT´s mandate crops and few others, mostly focused on the adaptation to drought.

Roots in chickpea – In South Asia chickpea is mostly grown during the postrainy season in deep clay soil and depends on the residual moisture contained in the soil profile, therefore facing water deficit in the latest part of the growth cycle. In this context of terminal drought, breeding for root traits appears to be the right approach and Kashiwagi and colleagues (2006) have shown the importance of roots for seed yield under terminal drought conditions in chickpea. This work has been a major effort at ICRISAT for the past 20 years (Saxena, 1984, Johansen et al., 1997, Krishnamurthy et al., 1999) where a better adaptation of plants to terminal drought has been shown to be due to deeper rooting and higher root length density (RLD) in the deep layers. However, no work has been done to improve the nutrient uptake by chickpea plants. It has been reported that chickpea was able to allocate more roots to the deeper soil layers under conditions of stress than other legumes (Benjamin and Nielsen, 2005), or than more sensitive genotypes (Kashiwagi et al., 2006). However, this was so only when the phenology of the genotype was well suited to the test environment. For example, the chickpea genotypes K1189 and ICC898 had adequate RLD compared to ICC4958 and Annigeri in the work by Kashiwagi and colleagues (2006), but their yields were poor under terminal drought, mostly because

they were longer duration varieties. As such, the putatively beneficial effect of roots on terminal drought yield was overridden by the effect of crop phenology. Also, the testing of a mapping population developed between two elite parental lines of chickpea varying for their root volume showed that the differences in RLD would not always translate in a yield increase (Serraj et al., 2004), especially in locations where the season length is higher and the evaporative demand lower such as in North India (Krishnamurthy et al., 2004), thereby, showing that parameters other than roots also played a more crucial role. Therefore, roots are only one component of the overall performance of chickpea under terminal drought conditions, and needs to be addressed together with other traits. Similar principles are very likely to prevail in other crops.

Roots in groundnut - Despite the paucity of studies on roots, it has been shown that roots are expected to play an important role in drought adaptation in the light textured and deep soils of the South West US (Ketring et al., 1982, 1984; Pandey et al., 1984), where a relation between root depth and pod yield has been established (Robertson et al. 1980, Boote et al., 1982). However, only a few genotypes were tested in these experiments, even though differences in the rooting depth were found (Krauss and Deacon, 1994). As for the putative role of root for nutrient uptake in nutrient poor soils, virtually no work has been made in groundnut in that respect. A few studies in the late 70's and early 80's reported root responses to water stress and indicated that the growth of roots increased upon water deficit (Allen et al., 1976), in particular rooting depth (Lenka and Misra, 1973; Narasimham et al., 1977, Ketring and Reid, 1993). Ketring and Reid (1993) found that groundnut was able to establish both a deep and laterally spreading root system fairly early during the growing cycle, providing adaptation to drought occurrence during and later in the season. By contrast, Robertson et al (1980) did not find any RLD differences at shallow soil depths between well-irrigated and water stressed conditions. Meisner and Karnok (1992), contrary to previous studies cited above, found that root growth decreased upon water deficit, though not as much in the deeper layer where water was still available. In summary, rooting characteristics appear to vary in groundnut but the dynamics of root growth under water deficit are still unclear. To date, data are still lacking to conclude which root trait, in which soil, environment, and stress type, could contributes to drought tolerance in groundnut.

Roots in pigeonpea - Virtually nothing is published on roots in pigeonpea under water stress, except for a few reports from the late 70's (Narayanan and Sheldrake, 1975, 1976, Arihara et al., 1991). It is assumed that pigeonpea is deep-rooted and that confers drought tolerance because the crop is usually grown on deep soils and completes its life cycle on residual moisture. More work has been accomplished in pigeonpea in relation to its ability to absorb nutrient having low solubility such as P, thanks to the secretion of pissidyc acid (Ae et al., 1991). Recent data on the hydraulic characteristics of pigeonpea roots, in particular the ability for hydraulic lift, might be an interesting asset for both nutrient and water (Sekiya and Yano, 2002, 2004 – See related paragraph). As we will see below, the capacity for hydraulic lift may be at the price of a well-developed endodermis, which may allow excess salt to flow-in freely and cause salt stress sensitivity. In any case, pigeonpea is a legume crop where, probably a lot more work on roots is needed to fully exploit the potential and particularities of its roots. Yet, studies on pigeonpea root traits have remain largely anecdotal; how roots of pigeonpea contribute to its adaptation to dry environment, how its ability to take up low solubility nutrient would interact with water uptake under water deficit, are virtually unknown. Like chickpea, the large variations in flowering time across the pigeonpea germplasm would require a comprehensive consideration of both phenology and roots.

Roots in sorghum – Sorghum is considered as a drought tolerant crop whose well-known deep roots are assumed to play a key role in its drought adaptation. To the best of our knowledge, no work has targeted the roots of sorghum to enhance to nutrient absorption in low fertility environments. Although, a lot of drought-related studies have been carried out with sorghum, surprisingly very limited work has been done on the roots. Only a few reports have presented evidence of genotypic variation for root traits (Bhan et al., 1973, Mayaki et al., 1976, Jordan et al., 1979), and these studies have focused on only a few breeding lines with a limited genetic base. Genotypic variations for root traits have been found in other studies using solution culture (Blum et al., 1977), or in small pots (Abd-Ellatif et al., 1978), but the results should be considered with caution. A more recent study showed that a drought tolerant sorghum line possessed roots at least 40 cm deeper than a drought sensitive one (Salih et al., 1999). This agrees with some of our own observations showing deeper rooting of staygreen lines under drought conditions (Vadez et al., 2005) (Figure 2). In fact, most of the drought-related work in sorghum has focused on the staygreen trait which is known to be extremely complex (Borrell and Hammer, 2000). Different hypotheses have been advanced to explain staygreen; these include the N balance between leaves and grain (Van Oosterom et al., 2006a&b,

2007), or differences in transpiration efficiency (Borrell et al., 2000). It has been shown that the staygreen characteristic of two maize hybrids would correlate with a higher N uptake during grain filling in the staygreen type (Rajcan and Toollenaar, 1999). Surprisingly, no one has hypothesized that N uptake differences could result from water uptake differences during grain filling. More work is certainly needed in this direction, since it has been shown that root growth continues well into the grain filling stage in hybrid sorghum (Bower, 1972, cited by Jordan et al, 1979).

Roots in pearl millet – Like sorghum, pearl millet is also a deep rooted and a drought-adapted crop. Unfortunately, few studies have explored the genetic variation for root traits and none has attempted to use these differences in breeding. Data from Chopart (1983) indicate that the rooting depth of pearl millet in deep sandy soils can reach at least 200 cm and that the root front can increase as much as 3.5 cm per day between 15 and 50 days after sowing. Bruck et al (2003) found no genotypic differences in the root depth of 5 pearl millet varieties, but found genotypic differences in the RLD, especially at depths between 50 and 175 cm, with RLD as high as 0.30 cm per cm^3 at 125 cm depth. In such case, root expansion would be both for water and nutrient capture, in the erratic rainfall and poor fertility conditions under which it is cultivated in the Sahel. At ICRISAT, we have assessed the rooting depth and RLD in long PVC tubes (2.4 m long, 16 cm diameter) in hybrids based on parental lines contrasting for terminal drought tolerance and in near isogenic lines with and without terminal drought tolerance QTLs. We found that the terminal drought tolerant lines do have a relatively more profuse rooting in the deeper layers than the sensitive lines (Vadez et al., 2005) (Figure 3). Our current hypothesis is that a slight increase in deep rooting would help sustain higher water uptake during the post anthesis period, which in turn would contribute to better grain filling, under environments in which water is available in deeper soil layers.

Roots in other crops - Roots have also been investigated in other crops, although with a similar limited focus and a "non-sustained approach". These include white clover (Blaikie and Mason, 1993), lentils (Silim et al., 1993a, 1993b), wheat (Gregory and Eastham, 1996), cotton (Taylor and Klepper, 1975; Quisenberry et al., 1981), oats (Carrigan and Frey, 1980), rice (Champoux et al., 1995; Yadav et al., 1997; Price et al., 1999, 2000) and maize (Jenison et al., 1981; Guingo et al., 1998; Tuberosa, 2002, 2003), or simply not investigated although terminal drought conditions would prevail (Frahm et al., 2004). For example, upland rice was considered more adapted to drought conditions than lowland rice because it has a deeper and more prolific root system (Steponkus et al, 1980). In broad bean, deep cultivation enhanced water extraction by promoting deeper root growth (Rowse and Barnes, 1979). The capacity of roots to penetrate a compacted soil layer (Bengough et al., 1997, Unger and Kaspar, 1994, Clark et al., 2003) has been given importance in wheat (Gemtos et al., 1999, 2000; Ishaq et al., 2001; Kubo et al., 2004), cotton (Coelho et al., 2000), soybean (Flowers and Lal, 1998), and rice (Ray et al., 1996). Roots have been looked at for a better phosphorus uptake in common bean (for a review, see Lynch and Brown, 2001), or specialized types of roots for P acquisition in *Lotus japonicus* (proteoid roots) (Lambers et al., 2006).

Roots for water supply and drought tolerance

Usual assumptions on roots for water-limited conditions - Under conditions of drought, it has long been considered (Miller, 1916, cited by Kashiwagi et al., 2006, O'Toole and Bland 1987) that an increased root depth would contribute to better drought tolerance. Under such conditions, Jordan and colleagues (1983) have shown that deeper rooting would increase crop yield under drought stress. It has been reported that an increased soil volume explored would increase crop yield under water-limited environments (Jones and Zur, 1984). Since sorghum is deeper rooted than maize, a theoretical analysis has shown that increasing the root depth of maize to that of sorghum would contribute to a yield increase in most dry years (Sinclair and Muchow, 2001). Ludlow and Muchow (1990) have reviewed 16 traits that potentially contribute to drought tolerance. The three most important traits included plant phenology, osmotic adjustment, and rooting depth. Although in these studies, the type of drought imposed was not fully described, it is understood that roots would have an essential role under terminal drought conditions, i.e., for those crops grown on residual soil moisture after the end of the rains, and where drought stress usually occurs after flowering. Whether roots contribute during intermittent drought still needs investigation, as there is virtually no published data on the topic. In any case, there is a consensus that root should contribute to a better adaptation to dry conditions.

Current status of breeding for roots - Very limited efforts to breed for root traits have been undertaken, mostly because of the difficulties involved, the incomplete knowledge of the key parameters in the rooting characteristics that contribute to drought tolerance, and a lack of the

knowledge of the range of variations available for root traits that can be used for breeding. Despite the importance given to roots in the drought scenario, few teams have undertaken breeding for root traits. Even if root QTL have been identified in certain crops such as rice (Champoux et al., 1995; Yadav et al., 1997; Price et al., 1997, 1999), no products have appeared. There is also some doubt on the contribution of root QTL to drought tolerance in rice (Price et al., 2002). In maize, where the root pulling force is well related to root length density (Merill and Rawlins, 1979; Sanguinetti et al 1998), Bolanos and colleagues (1993) have found a negative correlation between root pulling force and grain yield under drought conditions. In fact, no relation was found between the *Root-ABA1* QTL on maize bin 2.04, and grain yield (Giuliani et al., 2005). Hence, to breed for roots, not only is a lot of work needed to explore the diversity for root traits: (i) methods still need to be designed to have sufficient throughput to deal with large number of accessions and with sufficient heritability to permit breeding, (ii) there is also an important need to establish a sufficient relationship between the measurement of root traits and their impact on yield under water limited conditions.

Breeding efforts in chickpea – Some of these efforts have been made in chickpea (Serraj et al., 2004) where massive investments in labor have been made to measure roots in the field. Since field-based data is frequently associated with poor heritability that undermine the use of these traits for breeding, simpler systems have been designed for assessing variation in root traits, which consist of growing plants in 1.2 m tall and 16 cm diameter cylinders, and measuring RLD at every 15 cm depth interval at 35 days after sowing (Kashiwagi et al., 2006). Cylinder measurements show good agreement with depth and RLD determined in the field and have been used to explore the diversity for these traits in chickpea (Kashiwagi et al., 2006). Also by using this method, root depth and RLD are being phenotyped in RIL populations and QTLs identified. In fact, a major putative QTL for RLD was identified in a population involving a profuse rooting parent ICC4958 and the contrasting Annigeri (Chandra et al., 2004).

Although it is critical for deciding breeding strategies, the available information about the genetics of root characteristics is still limited, except for some reports on heritability estimates compared to the progress on agronomical and physiological studies of root characteristics (Krishnamurthy et al., 2004; Kashiwagi et al., 2005). In chickpea, a major contribution of additive gene effects and additive × additive gene interactions on the root dry weight and root length density were reported (Kashiwagi et al., 2007). In addition, the consistent direction of the gene effects toward increasing root growth was also observed. Similar results were reported in common bean about gene components that control the expression of root dry weight and root surface area (Araujo et al., 2004). Similarly in cotton also, the gene effects of root characteristics showed that additive and additive × additive gene effects accounted for about 50% of the variation in root length in one of the two crosses tested at seedling stage (Eissa et al., 1983). Since, the root characteristics in both the legume crops including chickpea and common bean showed additive × additive epistasis, an advised selection procedure should be taken into account to exploit their interallelic interaction. This suggested that delaying selections to later generations and generating larger populations for selections could be important strategies for improving root systems of chickpea to exploit additive × additive interaction, as shown earlier (Upadhyaya and Nigam, 1998). By contrast, early-generation selection would be less effective. Further, it would be advantageous to backcross one or more times with recurrent parent before selection to enhance the probability of obtaining superior lines (Dudley, 1982). Since it is practically impossible to investigate a large population for RLD and RDW screening, marker assisted selection needs to be sought for proper screening of these characteristics.

Breeding efforts in maize - Breeding for root traits is on-going in maize, where QTLs for root traits have been identified (Tuberosa et al., 2002, 2003). For this, a hydroponic system has been used in which primary and seminal root growth was assessed at about 3 weeks after germination. An obvious criticism of such a system is whether root growth differences in hydroponics would result in consistent root growth differences in a soil/field environment, and whether these would eventually be reflected in differences in drought tolerance in the field. Although, previous work has shown a relation between seminal root traits in hydroponics and root lodging in the field (Landi et al., 1998; Sanguinetti et al., 1998), weak relations have been found between seminal root traits in hydroponics and root pulling resistance in the field (Landi et al., 2001), and between seminal root traits in hydroponics and field grain yield under water stress conditions ($r = 0.20$) (Tuberosa et al., 2002). In fact, this work even showed a weak, significant but negative relation between primary rooting in hydroponics and the grain yield under water stress in the field ($r = -0.27$). Even so, a QTL on marker CSU61b in bin 1.06 appeared to have a major effect on root traits in hydroponics, co-mapping with grain yield under both well-watered and water stress conditions (Tuberosa et al., 2002). Interestingly,

one of these QTLs, *Root-ABA1* on maize bin 2.04 was recently found responsible for both primary and seminal root growth and increased ABA concentration in the leaf (Giuliani et al., 2005, Landi et al., 2005). With the current advances in syntenic studies across the cereal species, more work is needed to clarify the functional role of roots in terminal drought tolerance QTL of pearl millet and staygreen QTL of sorghum, and to explore the putatively conserved genomic regions involved in rooting traits across cereal genomes.

Genetics of root systems - To promote the use of root traits in breeding programs, a better understanding of the genetics of root development is needed. In this respect, although the QTLs for root traits above may not relate well to better performance in field conditions, the work from Tuberosa and colleagues has the merit of shedding light on the genomic portions involved in early root development, an aspect that several authors indicate as important to cope with water deficit (Araki and IIjima, 1998; Jesko, 2001). This is a first step to understand the genetics of root development. In that respect, recent studies are now trying to tackle in a more systematic way how root growth is genetically controlled, which was not possible before (Hochcholdinger et al., 2004; Malamy, 2005; Kashiwagi et al., 2007). Root traits have also been targeted by genetic transformation in tomato, where an *Arabidopsis* gene related to the vacuolar H^+ pyrophosphatase (AVP1), led to an increased root growth under water deficit (Park et al., 2005), which was hypothesized to be related to a modification in the auxin fluxes. A recent study carried out at ICRISAT also shows the involvement of *DREB1A* transcription factor driven by a stress responsive promoter from the *rd29* gene of *Arabisopsis thaliana*, on the development of groundnut roots under drought stress conditions (Vadez et al., 2007). These transgenic plants of groundnut variety JL 24 were grown in 1.2 m long and 16 cm diameter cylinders under well-watered conditions for 30 days before withdrawing irrigation in half of the plants. Forty days later, upon drought treatment the root growth was dramatically found to increase in the transgenics, whereas roots remained unchanged in the non-transgenic plants (Figure 4). This resulted in a higher water uptake from the soil. This work suggests that *DREB1A* triggers native genes of groundnut that might be involved in root development, and needs further investigations.

Prospects for better exploiting the potential of root systems for drought - Overall, there have been a number of scattered studies on roots in different crops, documenting root systems and their putative contribution to drought tolerance. While these studies are of high value, they suggest a number of comments. First, a common feature in most of these studies is the very "static" manner in which the roots were assessed, i.e., destructive samplings at one or several points in time, giving virtually no information on the "dynamics" of root characteristics. From these studies, what particular root trait, or what particular aspect of root growth would contribute to a better adaptation to water deficit remain unclear. Second, the limited number of genotypes tested in each crop does not permit an exhaustive assessment of the range of variations available and the potential for breeding these traits. This drawback is mostly explained by the difficulty in studying roots, thus requiring a simplification of the methods used to evaluate a larger number of lines. Third, when testing the putative relation between differences in rooting traits and drought tolerance, genotype phenology (drought escape) was often the overriding factor explaining plant tolerance (Blum et al., 1977, Kashiwagi et al., 2006). Therefore, the exact contribution of roots to drought tolerance can only be tested once sufficient genetic variations in root traits are found within groups of genotypes sharing a similar phenology. Given these limitations and to remove the "static" approach used so far, we propose that our future approach on roots should focus on root functionality rather than morphology. We should first measure water uptake under water deficit, in a "dynamic" and precise way, in a large range of genotypes representative of the species' diversity. Such lysimetric system is shown in Figure 5. This should carefully consider the phenology of genotypes, and determine the relation between a given pattern of kinetics/volume of water uptake and drought tolerance. Once contrasting genotypes are identified, root developmental and morphological patterns can be investigated thoroughly.

Root for water supply and nutrient uptake in poor soil fertility of the SAT

The objective of this section is not to make an exhaustive review of the contribution of roots to nutrient uptake, especially phosphorus (P). There are several reviews and reports on the root traits related to P uptake (Lynch and Brown, 2001; Sinclair and Vadez, 2002; Hinsinger et al., 2003; Gahoonia and Nielsen, 2004, Lambers et al., 2006). Instead, we will focus on how roots can contribute to the acquisition of both water and nutrients, with a focus on P, in an integrative way rather than looking at roots for nutrients and for water separately.

Root architecture needed for water and nutrient uptake - Both nutrients and water are concomitantly limiting factors in many areas of the semi-arid tropics (SAT). It is increasingly becoming clear that in these areas, poor fertility is a primary factor for poor crop performance rather than water stress (Payne et al., 1990). These authors found that the poor fertility limited root development in pearl millet that was unable to capture the water contained in the profile and water drainage occurred below the root zone. As a consequence, plants suffered from drought stress when rains receded, although water was available deeper in the profile (Payne et al., 1990). Root establishment in poor fertility soil is essential to ensure full use of available water. To acquire nutrients, the development of secondary roots is needed mostly in the soil surface layers where the nutrients usually concentrate and their absorption is made easier because of higher microbial activity such as in bean (Lynch and Brown, 2001; Lynch and Beebe, 1995; Liao et al., 2001) and wheat (Manske et al., 2000). To acquire water, in contrast, profuse rooting in the deeper soil layers would be required. It appears from a recent study that P acquisition is less in deeper-rooted plants than in shallow-rooted plants (Ge et al., 2000), thus indicating that shallow and deep rooting are rather antagonistic developments (Lynch and Brown, 2001), as suggested earlier by Chopart (1983). Yet, there is a need to identify rooting patterns that allow both nutrient acquisition and water uptake. The use of molecular markers for these two traits – water and nutrient acquisition – might be useful to break this negative linkage, at least partially, if effective QTL for these two traits can be identified.

How to maintain nutrient uptake in soils that frequently dry - In addition to the fact that , as Lynch and Brown (2001) admit, a "nutrient foraging" phenotype would have a poorer capacity for water uptake from deep in the profile, such a phenotype may also not fit in environments where the top surface is likely to be dry for long periods. Therefore, the hypothesis that a shallow rooting pattern contributes to an enhanced nutrient acquisition in nutrient deficient environment needs to be revisited when top soil drying is a common feature. Some work would also be needed to assess the volumetric soil moisture threshold where nutrient acquisition is no longer possible. The question then remains, how to ensure superior nutrient uptake in these poor nutrient environments? A shallow root system may still be valid for rainfed crops of the SAT where the top soil would be re-wetted periodically and in particular in unfertilized soils where most of the nitrogen would be present in the top surface and would need to be absorbed before being leached down the soil profile. A more profuse root system in this case might also contribute to both water and nutrient acquisition. For instance, it has been shown that pearl millet roots can expand both horizontally for over a meter and vertically in a sparse stand (Bruck et al. 2003a,b). Helping early plant establishment may be also a way to ensure that a minimum root development has occurred to take full benefit of the on-going rains. The microdosing method used in West Africa (Tabo et al., 2005) would be one more option. A more recent work shows that a minute application of P close to the root of pearl millet seedlings helps plant establishment and growth under P limited environments (Valluru et al., 2007), and pearl millet seed coating with P is also showing very similar results (unpublished results). Yet, the presence of water around the seed is a prerequisite for seedlings to take up nutrients, and the question of nutrient absorption in nutrient and water scarce environments remains unresolved. The hydraulic lift may be part of the answer.

Hydraulic lift – This is an interesting root feature that could be relevant for the absorption of nutrients in dry top soil. This phenomenon (Caldwell et al., 1991) has been reported in different crops and particularly pigeonpea (Wan et al., 2000; Sekiya and Yano, 2002) and it is related to the morphology of the root system, in particular, the presence or absence of an endodermis. Under conditions of high transpirational demand, the pressure gradient in the root (lower water potential than in the soil) is in favor of water absorption by the roots. During the night, when there is no transpiration and only a modest osmotic gradient, the soil water potential is usually lower than the potential in the roots. Unless there is a particular mechanism in place, water would normally flow back to the soil, following pressure gradients. That backflow is normally prevented by the endodermis which acts as a barrier to the flow of water from the root to the soil (Freundl et al., 2000). For deep rooted crops, the roots are in contact with wet soil and the osmotic gradient is sufficient to allow water uptake by the deepest roots. By contrast, the shallow roots are exposed to a dry soil, and the pressure gradient between roots lacking an endodermis and soils in these layers allows water to flow back to the soil. This phenomenon is called the hydraulic lift and consists of lifting water from the deep layers to the top layers. Such a feature might help take up nutrients from the rhizosphere in the top soil in environments where drying is frequent. A species like pigeonpea, in which hydraulic lift has been reported, and which is also known to perform well under low soil P (Ae et al., 1991), might be of great interest. Last but not the least, an interesting study with maize hybrids showed that the drought tolerant line was able to hydraulically lift water from the deep and wet soil layers to the shallow and

dry soil layers during the night (Wan et al., 2000). The capacity for hydraulic lift is directly related to differences in the anatomy of the root cylinder (Figure 1) and likely related to the absence of an endodermis (Steudle, 2000a).

Relation between nutrient deficiency and WUE – To achieve a high WUE, plants need to maintain a low CO2 concentration in the stomatal chamber, which can be possible if the photosynthetic rate is high. Nutrient deficient plants (in particular N and P) can have decreased rates of photosynthesis, explaining a putatively close association between water and nutrients with regards to WUE. To account for poor fertility, sparse planting densities are commonly used which dramatically increase the evaporation component of the crop's evapotranspiration, and decreases water use overall (Payne 2000). Moreover, in nutrient depleted environments, one factor involved in the calculation of WUE (Tanner and Sinclair, 1983) can also be decreased by low fertility ("m" factor explained by Payne 2000, citing DeWit, 1958). Other evidence indicate that WUE drops in different crops cultivated under nutrient deficient conditions (Bruck et al., 2003a,b). Finally, under nutrient limited conditions, it has been shown that the hydraulic conductance of plants decreases (Clarkson et al., 2000), although no evidence of any genetic differences in this decrease have been reported. Therefore, it is clear that the nutrient and water limitations interact closely, and that the root hydraulic conductance is involved.

Root characteristics and salt stress tolerance

In this part also, the purpose is not an extensive review on salt stress tolerance, but to focus only where roots could be of importance, focusing on certain aspects that, we feel, have not received lots of attention.

How roots interact with salt stress? - Under saline conditions, roots are obviously the plant organs exposed to salt stress. There are different ways in which roots play an important role in the plant response to salt stress: (i) avoiding the entry of sodium in the root cell or favoring its exclusion in the root medium; (ii) avoiding its loading in the xylem vessels, to prevent its build up in the shoot tissues; and (iii) signaling to the shoot via hormones such as ABA. Here, we will not review exhaustively the exclusion of Na from the root cells since (i) has received much attention and reviews are available (Tester and Davenport, 2003; Munns, 2002; Munns et al., 2002). We would look at (ii) and (iii) where much less work has been done, and where again the root architecture as described initially appears to matter.

Roots for excluding Na from the plant - Sodium (Na) exclusion from the shoot is indeed the major trait considered important to confer salinity tolerance in several crops. As a consequence, a lot of the work currently focused on improving the capacity of roots to deal with Na exclusion (item (i) above), either by exploiting the natural variation for this trait, like in wheat (Munns et al., 2002, Munns and James, 2003), or in rice (Gregorio et al., 1993), or through genetic transformation where there is a plethora of reports (eg: Apse et al. 1999; Shi et al. 2003; Vinocur and Altman, 2005; Denby and Gehring, 2005; Chinnusamy et al., 2005, Mathuis, 2006 and most citations there in). In this respect, breeding is currently on-going at IRRI, where salt-exclusion QTLs have been found and are in the process of being introgressed in locally adapted lines to confer them the adaptation to salty conditions. Nevertheless, whether salt exclusion from the shoot is the key factor explaining differences in salt stress tolerance is still an issue that requires clarification since very few studies have investigated the relation between a accumulation in the shoot and salt tolerance based on yield evaluation. We recently reported no such relation in a large set of chickpea genotypes (Vadez et al., 2007). In fact, the reason for the differences in salt accumulation in the shoot in many studies, in particular those using transgenics, may be the use of hydroponic systems, which are also known to affect the structure of the root systems, since hydroponically grown plants lack an exodermis in contrast to aeroponically grown plants (Freundl et al., 2000; Hose et al., 2001) and therefore the related hydraulics. For instance, salinity appears to induce the subberization of the hypo- and endodermis (Shannon et al., 1994), or the development of the exodermis (Reinhardt and Rost, 1995). More arguments follow thereafter, to justify a closer look at how the root structure may explain a great deal of how much salt eventually reach the shoot.

Loading of salt in the root xylem and relation with the composite transport model - Much less has been done to avoid the loading of salt in plant organs and we feel that it is an important issue to consider. Here, the composite transport model of water uptake may help explain genotypic differences in the loading of salt in the xylem. As we saw earlier, plants take up water from the soil

through an apoplastic or cellular pathway (Steudle, 2000b) (Figure 1). In the apoplastic flow, the reflection coefficient of the minerals is close to zero, and minerals are dragged in the water flow until the endodermis, as previously found with ABA (Hartung et al., 1998; Freundl et al., 2000), or the exodermis (Hose et al., 2001). There is reason to believe that crop genotypes having a less developed endodermis, or no/loose Casparian band, and a predominant apoplastic pathway for water uptake (such as maize) may allow salt loading into the xylem. By contrast, plants with a well developed endodermis, or favoring a cell-to-cell pathway for water uptake (like barley, Steudle 2000a), may have a more efficient system to filter salt before they reach the xylem. We believe that further work is needed to test whether contrasting materials for salt tolerance are related with putative differences in the way they take up water from the root hydraulic standpoint. Little work has been done to explore that hypothesis, although reports show that indeed a higher apoplastic water uptake was related to a higher accumulation of salt, in intravarietal selections of line IR36 with different degrees of salt accumulation (Yadav et al., 1996; Yeo et al., 1999). In another report, most salt tolerant genotypes of *Prosopis strombulifera* had an early suberization of the endodermal cells (Reinoso et al., 2004). An interesting "coincidence" is that plants displaying the hydraulic lift behavior reported above would also lack the capacity to "filter" salt through the endodermis. Pigeonpea is one such example, and it happens to be extremely sensitive to salinity compared to other crops (Srivastava et al., 2006). Therefore, an investigation of salt tolerance with regard to particular differences in the root morphology such as the presence of Casparian bands, or subberization of the cells at the level of the endodermis, may provide interesting insights. Looking into those mechanisms may also help understand why the effects of salt stress are higher under high VPD conditions, since under such conditions, the proportion of water channeled through the apoplast would be higher (Steudle, 2000a).

Root signaling under salt stress - A third area where roots are involved and where relatively little attention has been paid is related to signaling. As in the case of drought, plants respond to salt stress by producing ABA that result in stomatal closure and reduced water/salt uptake (Fricke et al., 2006). Work on sorghum and salt stress has shown that ABA was responsible in the adaptation to salt stress when plants were pre-treated with ABA (Amzallag et al., 1990), and suggest that part of the plant adaptation to salt could be mediated by differences in the root production of ABA. Also, salt stress is reported to decrease the hydraulic conductance of roots (Tyerman et al., 1989). As for drought, there seems to be both chemical and hydraulic signals involved in the response to salt stress. A better understanding of these would help prioritize the approach to increase tolerance to salt stress. In any case, these signals would contribute to a decrease in the transpiration rate. This would have two antagonistic effects: (i) a beneficial effect of decreasing the influx of salt accompanying the water flux into the root; and (ii) a limitation to the transpiration water to support carbon fixation and, therefore, a loss in biomass accumulation. We can clearly see that an optimal biomass production under salt stress would become a tradeoff between both aspects. Work is needed to determine how each of these antagonistic effects vary across genotypes reported to differ in salt tolerance. For instance, we have recently started work to measure the apparent Na concentration in the xylem and found very large differences between groundnut and pigeonpea genotypes that vary for tolerance. Yet, we have shown that the rate of transpiration drops relatively more upon salt stress in salt tolerant groundnut genotypes than in sensitive ones. By contrast, salt tolerant groundnuts compensate for more limited transpiration rate by increasing their transpiration efficiency (TE) to a relatively greater extent. Therefore, the salt-tolerant genotypes of groundnut, apparently manage to reduce their transpiration stream (and the related Na flux), but compensate the carbon fixation loss by increasing their levels of TE relatively more than salt sensitive genotypes.

Root dynamics – Toward capturing volume and kinetics of water uptake

We know little on the range of variations for root traits, their development pattern, and their contribution to drought tolerance. In most of the previous studies, knowledge has increased mostly on root morphology (Mc Cully, 1995), and traits such as RLD, depth, or weight, rather than root functions (water uptake, growth kinetics), have been measured (e.g. in Merill and Rawlins, 1979). Yet, water uptake is perhaps the most important component of a simple crop growth model defined by Passioura (1977) (Y = T x TE x HI, where Y is the yield, T is transpiration and accounts for the amount of water taken up by roots, TE is transpiration efficiency, and HI is the harvest index). So, the first requirement of roots is a high water uptake.

Root length density and water uptake - How much water is taken up would obviously relate somewhat to the RLD, but this link is still unclear because of the lack of data comparing the two

parameters. Several authors concluded that RLD and water uptake is related (Passioura, 1983; Monteith, 1986, Lafolie et al., 1991). This view is challenged by other studies showing poor relations between water uptake and RLD across several cereals and legumes (Hamblin and Tennant, 1987; Dardanelli et al., 1997; Katayama et al., 2000, Amato and Ritchie, 2002). In fact, it appears that cereals and legumes have large differences in their specific root water uptake, because of finer roots in the cereals than in legumes. Nevertheless, the relation between RLD and water uptake remains weak even after considering cereals and legumes separately. The above authors conclude that legumes have more abundant metaxylem vessels, which decrease their axial resistance to water flow, explaining the higher rate of water intake per unit root length. However, it has also been shown that a small length of roots in deep layers where water is plentiful would be sufficient to amply supply water to the plant when the top soil is dry (Gregory et al., 1978; Sharp and Davies, 1985). This would logically offset the linear relation between water uptake and RLD. It would also dismiss the hypothesis of differences in axial resistance limiting the rate of water flow across cereal and legumes, in agreement with Steudle's hypothesis (2000a&b). In any case, the lack of relation between water uptake and RLD agrees well with our data on groundnut (unpublished data). By contrast, we found a good relation between water uptake and RLD in *DREB1A* groundnut transgenics, where a higher water uptake of transgenic plants under water deficit was well related to higher RLD below the 40 cm depth (Vadez et al., 2007). Hence, there are clearly some controversies over the water uptake and RLD relationship. Finally, water uptake should be the prime target as suggested previously (McIntyre et al., 1995; Dardanelli et al., 1997) and such water uptake is unlikely to be dependent on differences of axial resistance to water flow. New models have been designed to take this into account (Dardanelli et al., 2004).

Water uptake and phenology - Under drought conditions, the primary factor contributing to better yield is a suitable phenology, adjusted to the water available from rainfall or soil moisture to allow the crop to complete its life cycle (drought escape mechanism) (Serraj et al., 2004). Several studies indicate that "superior" root traits contribute to drought tolerance of genotypes provided these have a suitable phenology (Blum et al., 1977, Kashiwagi et al., 2006). Therefore, while measuring the volume of water taken up by roots is certainly an important factor, understanding the kinetics of water uptake, and how this kinetics relates to the phenological stage of a plant, are equally important issues. This view is shared by Boote et al. (1982, cited in Meisner and Ketring, 1992), who argue that sufficient amounts of water at key times during the plant cycle is more important than across the whole cycle. We hypothesize that these key stages may be the reproductive stages and the later stages of grain filling. Previous work on roots indicates that root growth can persist at very different stages and under different conditions such as drought (Chopart, 1983; Hafner, 1993; Ketring and Reid, 1993), although genotypic assessment for this is lacking. A key missing link in these studies is how the reported root growth relates to differences in water uptake, and how much the water uptake varies among genotypes over the growth cycle Therefore, our working hypothesis is that differences in root growth under drought during reproduction and the latest part of grain filling would result in differences in water uptake, in turn resulting in differences in reproduction (seed number) and better grain filling (see next two paragraphs). We therefore suggest that the genotypic differences for water uptake during these key periods would be extremely difficult to determine by measuring only the roots, especially because of the usual large experimental errors in root measurements (Figure 2 & 3).

Water uptake and plant reproduction – Plant reproductive stages is extremely sensitive to any type of stress (Boyer and Westgate, 2004). Here, we consider the reproductive stages as the sequence of events between the emergence of a flower bud to the beginning of grain filling. It is important to understand the kinetics of water supply under stress during these stages, the existence of any genotypic difference in the kinetics, how such differences finally relate to yield differences. Our recent data show that groundnut plants grown in long and large PVC cylinders and exposed to water stress during flowering had very distinct patterns of water use, where some genotypes had a "liberal" behavior and maximized transpiration during the first 10 days following withdrawal of irrigation, but ran short of water during later stages (Figure 6). Others had a "conservative" use of water, limited their transpiration quickly after withdrawing irrigation, but were able to extract water for a longer period of time. The latter genotypes also had higher ABA content, both under well-watered and under water stressed conditions (unpublished data). Although we did not test whether these differences in kinetics had any bearing on the relative yield, but the data suggests that the stress intensity suffered by plants during their reproduction, probably varied across lines in relation with the differences in the kinetics of water uptake and in ABA. More work is needed to elucidate these differences.

Water uptake and grain filling - Differences in water uptake during grain filling would affect

photosynthesis and consequently the supply of carbohydrates to the maturing grains. For instance, a good relation between RLD in the deep layer and the HI (indicative of grain filling) was observed, especially under severe drought conditions (Kashiwagi et al., 2006). A similar phenomenon may also prevail in sorghum where the staygreen phenotype correlates with better grain filling. We consider that the maintenance of physiologically active and green leaves under terminal moisture stress possibly provided a minimum water uptake to sustain growth under these conditions, which is in agreement with a deeper rooting of staygreen genotypes under water stressed conditions (Vadez et al., 2005) (Figure 3). Such water uptake would in turn maintain photosynthetic activity and carbohydrate supply to the growing grain. We are currently testing a similar hypothesis to study the putative role of root water uptake during the grain filling in pearl millet genotypes introgressed with a terminal drought tolerance QTL that contributes to an enhanced panicle harvest index (PNHI). A better grain filling might be explained by enhanced water uptake toward the late stages of grain filling. We hypothesize that the water needed to sustain grain filling may be relatively small and due to minute differences in the root development (depth, RLD). Such differences would be difficult to capture by a physical assessment of roots, but could be measured by an assessment of water uptake, which would "integrate" the benefit of slight RLD differences over time. Work would also be needed to determine the threshold amount of water required to sustain grain filling.

Water uptake and soil characteristic – One difficulty to assess previous work on root also relates to the large differences in soils used to investigate roots. In this review, we pay a particular attention to the hydraulics of roots, as a way to explain part of the plants response to a range of stresses. The hydraulics of roots under conditions of receding moisture is obviously closely related to the hydraulic properties of the soil, such as the soil porosity. A recent theoretical analysis concluded that the transpiration response of plants is relatively uniform across a range of soils in which transpiration decline usually occurs when about 60% of the volumetric soil water has been depleted (Sinclair, 2005). However, the soil type would influence its hydraulic properties in a way that would determine the hydraulic integrity of the soil-plant-atmosphere continuum. Here, how intention is not to review that complex issue and we rather relate to recent reviews where the hydraulic properties of the soil and of the plants are taken holistically (Taylor and Klepper, 1978; Sperry et al., 1998, 2002; Jackson et al., 2000; Passioura, 2002; Sinclair, 2005). We also argue here that the use of modeling to predict soil water extraction (e.g. Jamieson and Ewert, 1999; Dardanelli et al., 2004) may ease the understanding of the role of soil in water extraction by plants exposed to progressive drying.

The metabolic / development cost of root – Whether the metabolic and development cost of roots is "expensive" for the plant, and whether the overall carbon/nutrient balance between roots and shoots can significantly impact the economic yield are still subject to debate. For instance, Passioura (1983) hypothesized that yield could be increased by decreasing roots as they represent a high energy investment. Van der Werf (1988) calculated that the ATP cost of producing one gram of root was equivalent to the maintenance cost of that same gram for 10 days. This cost could even be higher under stress conditions since, Eissentstat (1992, 1997) estimated that root carbon cost could reach about 40% of total plant cost under phosphorus stress. This would explain the high turnover of root systems, i.e., the fact that plants shed roots to limit their metabolic cost which represents a net carbon contribution to the soil (Krauss and Deacon, 1994). Siddique and colleagues (1990) found that wheat genotypes with high HI would have lower root/shoot ratios, indicating less investment in roots. In fact, the turnover of roots can be relatively rapid, with a half life of 30-40 days in groundnut (Krauss and Deacon, 1994). Therefore, even if the root/shoot ratio at a given point in time in many species is only between 10 and 40%, a complete turnover of roots in about 40 days would bring the root/shoot ratio close to 100% over the entire life cycle. This would be a substantial part of plant carbohydrate and protein investment. While this is certainly an important characteristic for the long term sustainability of low input agro-ecosystems, in relation to the organic matter returned to the soil, it is potentially an immediate yield decreasing factor in case the development of large root systems is not needed. Yet under conditions of limited soil P or limited plant available water, this investment may be necessary to support shoot growth. Therefore, a critical need is to assess the target stress conditions under which a significant investment in root mass would contribute to a better drought adaptation. Interestingly, it has been shown in several studies that elevated CO_2 would contribute to an increased root growth (Rogers et al., 1992, 1996). Work would also be needed to investigate whether differences in the root hydraulics, i.e., conferring differences in resistance of roots to water flow, could minimize the requirement in terms of RLD to capture water.

Water saving behavior – Relation with leaf conductance to water

In the three-component yield architecture model presented above (Passioura, 1977), water uptake and

water use efficiency (WUE) are called "drought avoidance" parameters, i.e., plants would "avoid" drought by either tapping into more water from the soil, or by using that water better (Serraj et al., 2004). Eventually, a higher WUE would save water in the soil profile, which would help sustain water uptake by plants. The root-related drought avoidance is closely intertwined with the WUE-related drought avoidance, although these components have been considered to be independent. Therefore, more work is needed to better understand their links and complementarities.

Can higher TE contribute to saving water in the soil profile? - It has been previously stated that there was little hope of finding differences in the intrinsic transpiration efficiency (TE, the instantaneous rate of carbon fixation divided by the instantaneous rate of transpiration, an important component of WUE in plants) in plant genotypes of a given species (Tanner and Sinclair, 1983). However, genetic variations for TE have been found in various crops like wheat (Ehdaie et al., 1991; Condon et al., 2002, Rebetzke et al., 2002, Richards et al., 2002) cowpea (Hall et al., 1992; Ismael and Hall, 1992), bean (Elheringer et al., 1991), and groundnut (Hubick et al., 1986; Wright et al., 1994, Bindhu madava et al, 1999 Krishnamurthy et al., 2007). These differences are explained by more active mesophyll efficiency (Uday Kumar et al., 1998), or a lower stomatal conductance such as in the wheat cultivar Quarrion (Condon et al, 2002) or in transgenic groundnut (Bhatnagar Mathur et al., 2007). In a recent large screening of 440 representative groundnut germplasm and breeding lines, we found a 4-fold range of variation for TE (unpublished results), a range of variation which has not been reported before. The question remains whether a better TE contributes to water saving in the profile that can be used by roots during grain filling.

A maximum rate of transpiration to save water in the soil profile - Another aspect of water saving relates to the control of the overall water loss at the leaf level. Recent upstream work on the *ERECTA* gene, involved in the regulation of TE in *Arabidopsis*, shows that *ERECTA* plays a role not only on the regulation of the photosynthetic system, but also on the stomatal conductance (through stomata density) (Masles et al., 2005). We have observed before that certain species such as pearl millet in semi-arid conditions do maximize transpiration even if the vapor pressure deficit (VPD) is high (above 2.5 kPa) (Squire, 1979; Henson and Mahalakshmi, 1985). In the work reviewed by Bidinger and Hash (2004), no attention was paid to possible genetic variations in this strategy, although recent modeling data show that a maximum daily transpiration rate would indeed contribute to saving water in the soil profile and would increase TE (Sinclair et al., 2005). We recently found that a major difference among pearl millet genotypes having similar phenology but differing for terminal drought tolerance was indeed a lower rate of water loss per unit of leaf area (transpiration over a period of 1-2 days divided by leaf area) in terminal drought tolerant genotypes (unpublished data). These differences have been measured under well-watered conditions, indicating that this trait is constitutive. These results have been observed very consistently across experiments, either on whole plants taken at different stages or on detached leaves over short periods of time. This index, which reflects the leaf´s stomatal conductance over a period of time, would save water and make it available for the later stages of the crop cycle, in particular grain filling. It may not be a coincidence that the phenotype used to identify QTLs for terminal drought tolerance was the panicle harvest index, a direct measurement of grain filling in plants. Water saving from a maximum rate of transpiration would decrease the proportion of water used before anthesis, and fits well with an old hypothesis by Passioura (1983) that a higher proportion of water loss after anthesis would contribute to better grain filling under water stress conditions, a hypothesis recently revived by Hammer and colleagues in sorghum (2007).

Sensitivity of stomata to VPD to save water in the soil profile - Pearl millet has been found to be sensitive to high VPD levels, usually above 3-4 kPa where the stomata close to avoid wasteful water loss (Squire, 1979). This is a well-known characteristic in crops growing in dry environments where stomata close when the evaporative demand is too high to be supported by the maximum water supply by the roots. However, possible genotypic variations for the sensitivity to VPD have not been studied, especially at intermediate VPD levels (2-3 kPa) where it is still assumed that genotypes would maintain their stomata fully open. Recent studies on a long known "slow-wilting" genotype of soybean (PI416937) used in breeding drought tolerant varieties indicates that the transpiration increased linearly in response to increases in VPD until about 2 kPA in all genotypes. Above these levels, transpiration rates remained essentially constant. In genotype PI416937, at least a partial stomatal closure did occur above 2.0 kPa, whereas other genotypes maintained a linear increase in transpiration up to VPD values of about 3.5 kPa (Sinclair et al., 2007). A consequence of this trait is that the leaf canopy temperature would increase under well-watered conditions, making it a fairly easy trait to measure using infrared thermometers, provided it is measured at the adequate VPD above

2 kPa. In fact, there is some indirect evidence of this "slow-wilting" trait in the canopy temperature literature. For instance, we believe that the differences in the canopy temperature between genotypes Senegal Bulk and HMP559 reported in Singh and Kanemasu (1983) are likely to be due to either differences in the sensitivity of stomata to VPD in these lines, or differences in their rate of water loss per unit leaf area. In our recent work, we have gathered evidence of such contrasting behavior in pearl millet genotypes that differ in terminal drought tolerance (unpublished). Therefore, further work is needed to assess whether this trait exists in other crops, and to use it for breeding varieties with water saving behavior.

Relation with hydraulic conductance - Signaling aspects

We have seen above that differences in how leaves would regulate their water loss would indirectly save water in the soil profile and allow roots to take up water for longer periods of time. In turn, roots are the sensors of drought or salt stresses and can relay the signal to shoots through hormones such as ABA (Davies and Zhang, 1991, Davies et al., 2000), through hydraulic signals in the case of more severe stresses (Sperry et al., 2002), or through an integration of chemical and hydraulic signals (Tardieu and Davies, 1993, Comstock, 2002). Such signaling would eventually contribute to decreasing stomatal conductance and would mechanically act on increasing the overall water use efficiency of plants (Farquhar et al., 1982, 1988, 1989, Condon et al., 2002).

Can differences in root hydraulic conductance explain a maximized transpiration rate? - The reason for differences in the rate of water loss per unit leaf area, or in the sensitivity of stomata to VPD (see above) are unclear and may involve some sort of differences in the overall plant conductivity to water. Assuming that there is no limitation in the axial conductance to water, the main "points" where conductivity can vary are at the leaf-atmosphere interface or at the root-rhizosphere interface. As far as roots are concerned, differences in the two later traits above could be explained by constitutive differences in the hydraulic conductance of roots. In fact, only hydraulic signals from the roots could explain the rapidity of the response to an increased VPD. Hence, it would be very interesting to look at the contrast for terminal drought tolerance in pearl millet from the angle of root hydraulic conductance; for example, by looking at the relative contribution of the cell-to-cell and apoplastic pathways to the root hydraulic conductance. Indirect assessment of this could be done by measuring the effect of mercuric chloride, a specific inhibitor of aquaporin on the rate of transpiration in plants (Maggio and Joly, 1995, Maurel, 1997; Maurel and Chrispel, 2001; Tyerman et al., 2002; Martre et al., 2002).

Work has also been done about 25 years ago on the contribution of small xylem vessels to high axial hydraulic resistance (Richards and Passioura, 1981a&b; Passioura, 1983). In this work, certain wheat genotypes had smaller xylem vessels that contributed to their increased WUE. In any case, it would also be interesting to compare the size of xylem vessels in pearl millet genotypes that contrast for terminal drought tolerance and rate of water loss per unit leaf area.

Root signaling to control stomata movement - The contribution of roots to the water use efficiency of transpiring organs may be through signaling. Roots are the primary organ exposed to a range of abiotic stresses and the signaling of these stresses to the shoot through ABA is well established (Zhang and Davies, 1991a&b, Stoll et al., 2000). It is hypothesized that the signaling takes two steps: (i) a first step at early stages of drought stress when ABA is transported to the shoot causing a drop in stomatal conductance and leaf expansion rate, and (ii) a second step at higher stress intensities where hydraulic signals are received by the shoot and contribute to *de novo* synthesis of ABA in the leaves, thereby accentuating the effect of ABA (Saab and Sharp, 1989). Such signaling also occurs under conditions of rapid stress imposition to avoid cavitation in the xylem vessels (Sperry et al., 2002). The ABA-related drop in stomatal conductance would contribute to an increase in TE. ABA also contributes to the development of roots (Saab et al., 1990; Sharp et al., 1994; Munns and Cramer, 1996; Spollen et al., 2000) and then to the water uptake. Hence, it would be very important to study the signaling from the roots to understand how water use efficiency is regulated in the shoots. It was interesting to note that in our work where groundnut genotypes were tested in long PVC lysimeters (Figure 4), the two genotypes showing the "conservative" behavior also had a high level of ABA in their leaves, even under well-watered conditions. We also found that pearl millet genotypes having a lower rate of water loss per unit leaf area had a higher ABA concentration in the leaves under well-watered conditions. The origin of this ABA and the role of roots in these differences require further investigation.

Conclusion

Roots play a central role in their response to many abiotic stresses, either directly or indirectly through their involvement in signaling. We have indicated in this review that many aspects of the plant response to drought, nutrient, and salt stress can be studied from the angle of the root structure and hydraulics, especially in the way a plant acquires water. In the case of drought stress, it is evident that there is a large deficit of knowledge on the contribution of roots to tolerance to water deficits, but that filling in this gap will likely require a dramatic improvement in the methods used to investigate roots. Our inclination is to focus more on measuring water uptake by roots and relatively less on understand the root structural development needed to increase water uptake. Obviously, such an approach needs to have a sufficiently high throughput to allow the assessment of large number of genotypes. Regarding the role of roots in response to nutrient stress, there is a need to look at both water and nutrient stress in a comprehensive way, as our target is the semi-arid tropic where both stresses are concomitant. It is also likely that root structure differences will partly explain differences in the salt tolerance of plants.

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Figure 1. Representation of a root cylinder and the pathways for water and nutrient movements in roots (reproduced from Steudle, 2000b, with permission from the author). The vacuolar and symplastic pathways represent together the cell-to-cell pathway and cannot be easily distinguished.

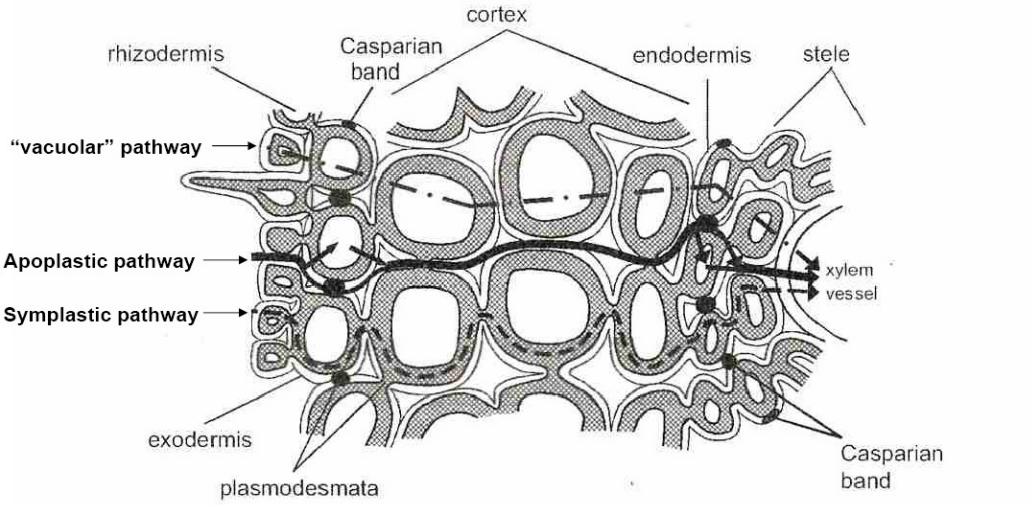

Figure 2. Root DW at different depths under drought conditions, expressed in % of the root dry weight in the 0-30 cm layer, in senescent (blue bars) and staygreen sorghum (red bars) (RSG 04012 is a re-selection from a cross between E36-1 and R16). Staygreen materials tend to root deeper than senescent ones. Experimental (SE) errors at measuring roots are usually very large.

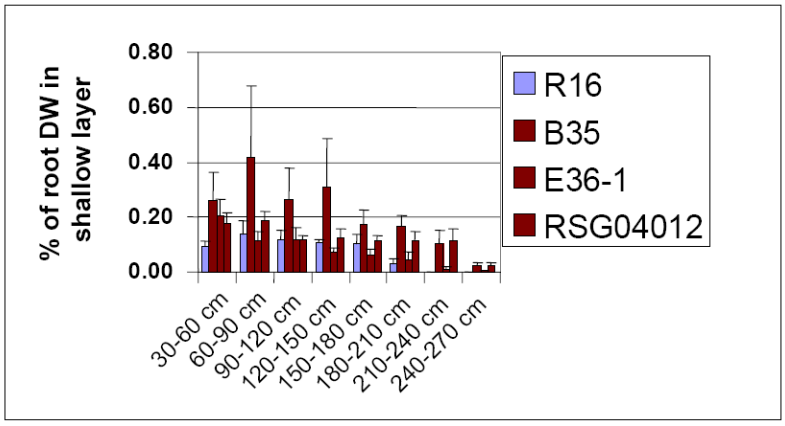

Figure 3. Root DW at different depths, expressed in % of the root dry weight in the 0-30 cm layer, in drought sensitive and tolerant pearl millet (ICMR 01029 and ICMR 01031 have H77/833-2 background and are introgressed with a terminal drought tolerance QTL from donor PRLT 2/89-33). Bars represent SE.

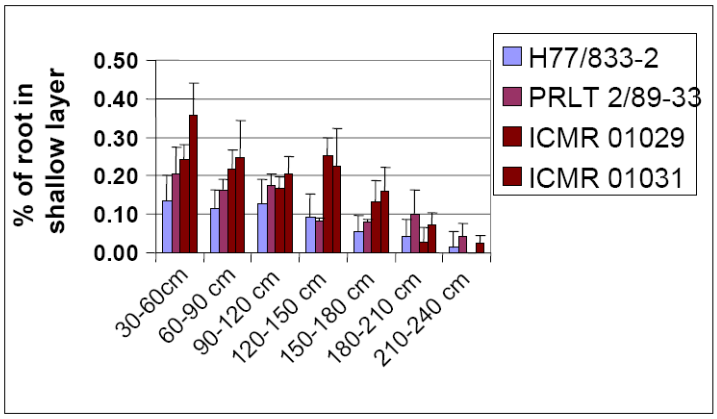

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Figure 4. Root dry weight (g plant^{-1}) in 5 transgenic events and wild-type JL24 grown in PVC tubes (1.2 m long, 16-cm diameter) under well watered conditions (WW) and exposed to water stress (WS) from 30 days after sowing. Bars represent SE.

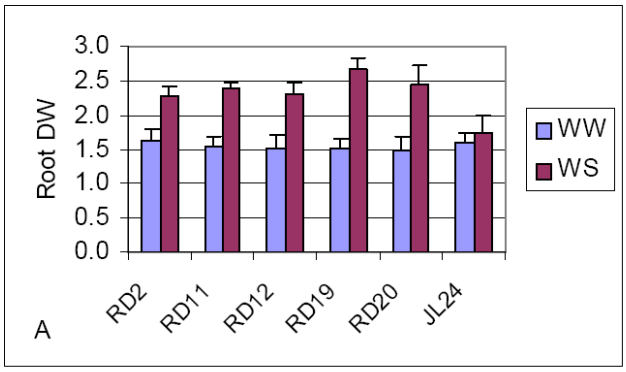

Figure 5. Lysimetric system newly used at ICRISAT to evaluate plant water uptake in PVC tubes (1.2 m long, 20-cm diameter). The length and diameter of the tubes are designed so that the soil volume available to each plant is equivalent to the soil volume available under field conditions at current sowing densities (for groundnut and chickpea). The system is used here for the evaluation of transgenics in P2 facilities. A larger system has been set up outdoors for germplasm screening.

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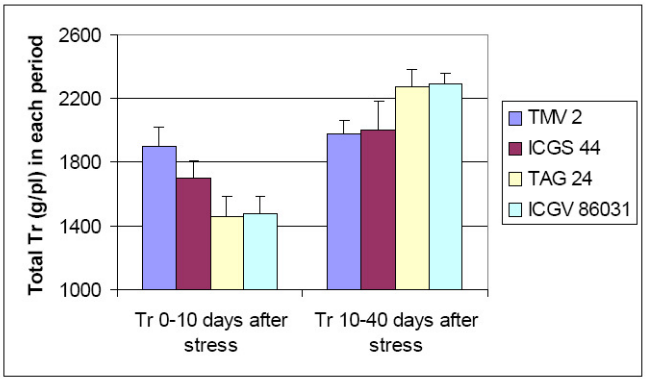
Figure 6. Cumulated evapotranspiration (g plant^{-1}) in four groundnut cultivars in the first 10 days after being exposed to stress, or between 10 and 40 days after being exposed to stress. Plants were grown in PVC tubes (1.2 m long, 16-cm diameter) under well watered conditions until 30 days after sowing and then exposed to water stress (WS) for 40 days. Bars represent SE.



2.2. Root research for drought tolerance in legumes: Quo vadis?

Vadez V, Rao S, Kholová J, Krishnamurthy L, Kashiwagi J, Ratnakumar P, Sharma KK, Bhatnagar-Matur P, Basu, PS, Journal of Food Legumes 21(2): 77-85. 2008

This review describes the various approaches that have been made to assess and improve crops drought tolerance focusing on root characteristics in legumes, especially chickpea. Among many factors that are associated with drought tolerance in legumes, root traits have been considered to be the most important attributes enabling the plant to mine water efficiently from deeper soil layers under dry environments. The potential of lysimetric systems developed at ICRISAT to ease the research of the root variability is discussed. Also, the perspective of drought tolerance improvement through transgenosis and use of wild relatives is drawn. Nevertheless, it is appointed out, that the research till date represents more of statical approach being currently used for research of drought, which is in fact dynamic process. In the review the examples of other SAT crops drought tolerance research are taken and it is highlighted the innovative dynamic understanding of crops water use should be applied. The example of pearl millet is used here to emphasize that the comprehensive understanding of root water uptake and shoot water loss could help to progress with legume drought tolerance research as well and so setting the theoretical base ground for innovative research approaches applicable not exclusively only for legumes.

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Root research for drought tolerance in legumes: *Quo vadis?*

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ABSTRACT

Improving crop productivity under conditions of abiotic constraints in field is one of the major concerns in many areas of the world where legumes are grown. The legumes are generally grown under water-limiting conditions and as a result, these crops often encounter drought situation that reduces productivity to a large extent. Among many factors that are associated with drought tolerance in legume crops, root traits have been considered to be the most important attributes enabling the plant to mine water efficiently from deeper soil layer under dry environments. Most of the methods used to evaluate roots are time consuming, that provide valuable information about the root morphology but they do not reflect the dynamic characteristics of roots and root systems. Considerable amount of genetic variability with respect to root traits involving length, dry weight, root length density (RLD), *etc.*, has been observed. Apart from the observed morphological variation in roots which has specific significance of adaptation, their functional aspects involving direct water uptake and their related kinetics are equally important. A large lysimetric system has been developed at ICRISAT to make progress in this direction. Compared to other legumes, root traits in chickpea has been thoroughly investigated and consequently, preliminary breeding works have been initiated using root traits. Root measurements in plants grown in cylinders showed almost similar relationship with depth and RLD determined in the field and thus have been used to explore diversity for these traits in chickpea. The possible diversity for root traits has been analyzed in wild relatives and transgenics as well. Using this method, root depth and RLD are being phenotyped in several recombinant inbred lines (RIL) populations. A major putative QTL for RLD was identified in a population involving a profuse rooting parent ICC 4958 and the contrasting Annigeri. These QTLs will be a faster and easier replacement technique against time-consuming phenotyping of roots and will be the way forward to introgress superior root characteristics. Efforts are also being made to use wild relatives of *Cicer* for inheriting some adaptive traits. In chickpea, preliminary work is being carried out to test the response to drought stress of DREB*1A* and P5CSF transgenic events using a lysimetric system. The work demonstrated a modest increase in water uptake but not yield under water stress in one transgenic event of DREB1A and one P5CSF event. In groundnut, *DREB1A* triggers native genes that might be involved in root development. In this review, the progress made so far on roots in legume crops has been elucidated which might explore possibilities of breeding genotypes to inherit efficient root system in legumes.

Dr. Vincent Vadez is a French national working as a Senior scientist and leading the Crop Physiology Laboratory at ICRISAT, working on the plant response to water deficit in mandated crops of ICRISAT such as groundnut, chickpea, pearl millet and sorghum. His research at ICRISAT focuses on traits that are closely related to the yield architecture and that can have a benefit under water limited environments. Major focus of his studies is on transpiration efficiency (TE, i.e. the amount of biomass produced per unit of water transpired) with a specific goal to obtain QTL for TE that would then be introgressed into locally adapted germplasm. Presently, challenges of his research team are to explore the genetic diversity for the trait TE towards the development of suitable mapping populations, to understand the interaction between TE and the environmental conditions and to assess the potential of introgressing such traits from wild Arachis relatives. His current interest is to focus on water uptake by roots, where the goal is also to obtain QTL for an increased and efficient water uptake towards yield increase under water limited environments. Based on the intensive research done for the past several years, his group is presently attempting to understand the relation between "physical" root traits (*e.g.* depth, length density, volume, etc.) and functional root traits (water uptake, pattern of uptake over time under stress), and their relation to yield. The diversity and QTL mapping of such traits are being explored.

INTRODUCTION

Drought is one of the most common abiotic stresses reducing the yield of many crops including legumes. Improving the tolerance of crops under water limited environments is prerequisite if agricultural production is to keep pace with the expected demographic increase. Beyond productivity, resilience of crops to water limited environments, *i.e.,* the capacity to yield even under very harsh conditions will be increasingly important. Extensive literatures are available describing roots as the potential trait to improve yield and resilience under drought. Yet, very little achievements have been made specifically in root based breeding. This could be

due to inherent difficulties in assessing root traits in a large set of genotypes that can be meaningful for plant breeders. Indeed, most studies on roots involve cumbersome methods which are time-consuming and the desired precision level with respect to measurement of rooting differences is hardly maintained, thereby limiting their application in breeding in spite of the fact that significant progress has been made in this direction (Kashiwagi *et al.* 2006). Therefore, there is a need for massive refinement and quantum leap in methodologies for assessing root traits in diverse germplasm to increase the throughput in a way that fulfills the needs of the breeding community.

Breeding for roots not only requires efficient and improved methods of screening but also in-depth knowledge about the specific function of the roots extracting water from soil from the deeper layers under water limited conditions. Indeed, most of the earlier workers attempted to relate the observed rooting differences among genotypes with higher water uptake, which may contribute towards higher yield. However, the relation between rooting behaviour and water uptake remains controversial (Passioura 1983, Hamblin and Tennant 1987, Amato and Ritchie 2002). Water uptake is considered to be a crucial factor during key stages like flowering and grain filling (Boyer and Westgate 1984), and small differences in water uptake at these stages can bring large yield benefits (Boote *et al.* 1982). The measurements of roots carried out so far do not allow answering these questions. Differences in water uptake could be measured and used in breeding but prior to that, methods are needed for direct and precise measurement of genotype's seasonal pattern of water uptake.

Water uptake by roots would also depend on the pattern of water loss by the shoot. In fact, differences in water uptake at key stages could be due to a sparing use of soil moisture earlier on when the soil is wet. Therefore, work on water uptake by roots should not disregard the role of shoot in controlling plant water loss, in particular under well-watered conditions. How such traits vary in plant species, and the way they contribute to water availability at key stages leading to better yield under drought conditions, have not virtually been tested.

This review describes the progress made in roots in few legumes and limitations of the current systems being applied to assess roots, and will address the fundamental questions whether observed root morphological differences actually translate into differences in water uptake. We will finally propose a set of improvements that are needed for such kind of studies and then try to focus upon the new lysimetric system that is set up at ICRISAT.

Review and Update in Root Research

Chickpea

In South Asia, chickpea is mostly grown during the post-rainy season in deep clay soil, thus, facing water deficit in later part of the growth cycle. Under terminal drought, breeding for root traits is a logical approach. The work on roots in chickpea has been a major effort at ICRISAT for the past 20 years (Saxena 1984, Johansen *et al.* 1997, Krishnamurthy *et al.* 1999, Kashiwagi *et al.* 2006). Their work could successfully establish better adaptation of plants with deep root and high root length density (RLD) under terminal drought conditions. However, having deeper and more profuse roots often may not suffice the requirement of chickpea to contribute more yields. For example, the chickpea genotypes K 1189 and ICC 898 with fairly profusely roots had shown poor yields under terminal drought, mostly because of their long duration. As such, the putatively beneficial effect of roots on terminal drought yield was overridden by the dominating effect of crop phenology. Also, the testing of a mapping population developed between two elite parental lines of chickpea with varying root volume showed that the differences in RLD would not always translate in yield increase over locations (Serraj *et al.* 2004). This was especially the case in locations of northern latitudes where the season is longer and the evaporative demands lower (Krishnamurthy *et al.* 2004). Therefore, roots are only one of the components among all others, which influence overall performance of chickpea under terminal drought conditions.

Groundnut

Roots are expected to play an important role in adaptation to drought in the light-textured and deep soils of the South West US (Ketring *et al.* 1982, Pandey *et al.* 1984), where relation between root depth and pod yield has been established (Robertson et al. 1980, Boote *et* al. 1982). However, such observation was based upon few studies involving only few genotypes. There appears to be some controversy on the response of root to stress. Root growth was reported to increase upon water deficit (Allen *et al.* 1976), in particular rooting depth (Lenka and Misra 1973, Ketring and Reid 1993). By contrast, Robertson et al. (1980) did not find any RLD differences at shallow soil depths between well-irrigated and water stressed conditions. Similarly, Meisner and Karnok (1992) found that root growth decreased upon water deficit, though not as much in the deeper layer where water was still available. In fact, Ketring and Reid (1993) found that groundnut was able to establish both a deep and laterally spreading root system fairly early during the growing cycle, providing adaptation to drought occurrence during and later in the season. In summary, root characteristics appear to vary in groundnut but the dynamics of root growth under water deficit are still unclear. The above works were performed under different soil conditions, with an expected strong effect on the root growth and response to stress. Based on this, it is still unclear which root trait could contribute to better yield under water limited environment in groundnut, and in which soil, environment, and stress type.

Pigeonpea

Virtually nothing is published on roots in pigeonpea under water stress, except few reports (Arihara *et al.* 1991). It is simply assumed that pigeonpea is deep-rooted, which imparts tolerance to drought during pod setting and grain filling process of the crop cycle that are taking place under residual moisture. Yet, pigeonpea is a crop where a lot of questions on roots are still without an answer, and where more work is to be done for characterizing the root traits to exploit their full potential for breeding programme which demands inheriting drought tolerance characters for better adaptability under water-limiting conditions. Are roots involved in the drought resilience of pigeonpea, or is it an adaptation of the shoot system? Are hybrids better endowed with roots? Have short duration pigeonpea "lost" their roots and is there a prospect to "breed them back"? Like chickpea, the large variations in flowering time across the pigeonpea germplasm would require a comprehensive consideration of both phenology and roots.

Progress in Breeding

Some breeding efforts have been made in chickpea (Serraj *et al.* 2004) where massive investments in labour have been made to measure roots in the field. Since field-based data are frequently associated with poor heritability that undermine the use of these traits for breeding, simpler systems have been designed for assessing variation in root traits, which consist of growing plants in 1.2 m tall and 16 cm diameter cylinders, and measuring RLD at every 15 cm depth interval at 35 days after sowing (Kashiwagi *et al.* 2005). Cylinder measurements show good agreement with depth and RLD as determined in the field and have been used to explore the diversity for these traits in chickpea (Kashiwagi *et al.* 2006, 2007). Using this method, root depth and RLD are being phenotyped in several RIL populations. A major putative QTL for RLD was identified in a population involving a profuse rooting parent ICC 4958 and the contrasting Annigeri (Chandra *et al.* 2004). These QTLs provide faster and easier replacement against time-consuming phenotyping of roots and will be the way forward to introgress superior root characteristics in superior background.

Although critical for deciding breeding strategies, the available information about the genetics of root characteristics is still limited, except for some reports on heritability estimates (Krishnamurthy *et al.* 2004, Kashiwagi *et al.* 2005). In chickpea, a major contribution of additive gene effects and additive × additive gene interactions in root dry weight and root length density has been reported (Kashiwagi *et al.* 2007). In addition, the consistent direction of the gene effects toward increasing root growth was also observed. Similar results were reported in common bean about gene components that control the expression of root dry weight and root surface area (Araujo *et al.* 2004). Since, the root characteristics in both the legume crops including chickpea and common bean showed additive × additive epistasis, an advised selection procedure to exploit their inter-allelic interaction would be to delay selection to later generations and generating larger populations for selection, as shown earlier (Upadhyaya and Nigam 1998). By contrast, early-generation selection would be less effective.

No effort has been made to breed for roots in groundnut and pigeonpea. In fact, there is no prior knowledge on the range of genetic diversity for root traits in these crops and no knowledge about a putative relation between differences in rooting and drought adaptation. Such work should be done first before deciding whether focus on breeding for roots in these crops is needed.

Any Scope from the Wild Relatives

There are few scattered and non-convincing reports advocating the use of wild relatives of chickpea to improve the overall tolerance to drought (Singh *et al.* 1998, Toker *et al.* 2007). Unfortunately, there is virtually no proof of this concept demonstrating an actual improvement in the tolerance of cultivated chickpea. One of the difficulties to assess the relevance of wild germplasm in improving tolerance of cultigen is the difficulty to compare performance of wild and cultivated species. Wild chickpea, for instance, are usually long duration, showing poorer vegetative growth than the cultivated type. Wild peanut also have long duration, sometimes behave like perennials, are capable of vegetative multiplication. In any case, most wild relatives have poor agronomic characteristics, making comparison on the basis of yield virtually irrelevant. Therefore, their comparison to the cultigens can only be done on the basis of traits known to have a relation to a better adaptation to water limitation, and on the knowledge of the environment where such trait can be beneficial.

In chickpea, efforts have been made to characterize roots using different accessions of *Cicer* species (Krishnamurthy *et al.* 2003). The root system of wild relatives was found to be less deep than that of the cultivated ones (Krishnamurthy *et al.* 2003). Therefore, it was concluded that wild chickpea had limited potential for improving the tolerance of the cultigen through their root system. However, re-analysis of these data showed that the root/shoot ratio of the wild relatives was among the highest, and superior to most cultivated ones (Fig. 1). This would imply that each gram of shoot tissue, *i.e.,* each surface unit of leaf area, is supported by a larger length of roots compared to the cultivated species, which could have a real importance under certain drought conditions. Work would then be needed to determine under which water-limited agronomic environments, such a higher partitioning to roots is needed to achieve better yield. In such case, wild relatives could become a potential donor of genes for that particular phenotype.

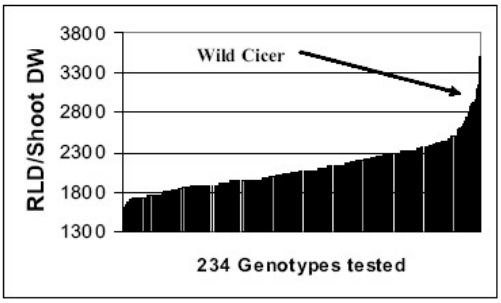

Fig. 1. Root length density (RDL) per shoot dry weight ratio (cm cm^{-3} g^{-1}) in 234 genotypes of chickpea (*Cicer arietinum* L.), including some accessions from wild *Cicer* species. Data are means of three replicated plants. See Krishnamurthy *et al.* (2003) for details on the root extraction procedure.

In case of groundnut and pigeonpea, the evaluation of rooting characteristics in the wild relatives has not been undertaken.

Any Scope from the Transgenics

There is only one report on transgenics in which an *Arabidopsis* gene related to the vacuolar H^+ pyrophosphatase (AVP1) led to an increased root growth under water deficit conditions (Park *et al.* 2005), which is hypothesized to be related to a modification in the auxin fluxes.

In chickpea, preliminary work is being carried out at ICRISAT to test the response to drought stress of DREB1A and P5CSF transgenic events, using a lysimetric system by which water uptake is assessed rather than root morphological characteristics (see below). So far, only a modest increase in water uptake under water stress conditions has been found in one transgenic event of DREB1A and one P5CSF event. However, the observed differences did not lead to any significant yield increase (our unpublished data).

Contrary to chickpea, a recent study carried out at ICRISAT shows the involvement of *DREB1A* transcription factor driven by a stress responsive promoter from the *rd29* gene of *Arabidopsis thaliana*, on the development of groundnut roots under drought stress conditions (Vadez *et al.* 2007a). These transgenic plants of groundnut variety JL 24 were grown in 1.2 m long and 16 cm diameter cylinders under well-watered conditions for 30 days before withdrawing irrigation in half of the plants. Forty days after stopping irrigation in the drought treatment, the root growth was found to dramatically increase in the transgenics, whereas roots remained unchanged in the non-transgenic plants. This resulted in a higher water uptake from the soil (Fig. 2). This work suggests that *DREB1A* triggers native genes of groundnut that might be involved in root development, and needs further investigations.

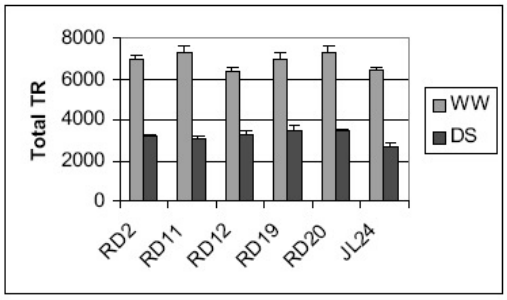

Fig. 2. Total evapo-transpiration over a 40-days period (g $plant^{-1}$) in 5 transgenic events and a wild type parent (JL 24) grown in PVC cylinders of 16 cm diameter and 1.20 m long. Plants were exposed to well-watered (WW) by compensating water loss or to drought stress (DS) by saturating the soil profile at 30 days after sowing and preventing further irrigation. Data are means (bars indicate SE) of 5 replicated cylinders with one plant in each cylinder.

Usual Assumptions on Roots and Limitations of Current Methods

Current assumptions

An increase in rooting depth has often been equated to increased drought tolerance (Miller 1916, cited by Kashiwagi et al. 2006, Jordan et al. 1983, O'Toole and Bland 1987). Jones and Zur (1984) reported that an increased soil volume explored would increase crop yield under water-limited environments. There is some theoretical analysis from modeling showing that increasing the root depth of maize to the rooting depth of sorghum would contribute to yield increase in most dry years (Sinclair and Muchow 2001). There is no such work in legumes, although rooting depth is one of the three traits that potentially contribute to drought tolerance among the 16 traits reviewed by Ludlow and Muchow (1990). Although in these studies, the type of drought imposed was not fully described, it is understood that roots would have an essential role under terminal drought conditions, *i.e.*, for those crops grown on residual soil moisture after the end of the rains, and where drought stress usually occurs after flowering. Whether roots contribute during intermittent drought still needs investigation, as there is virtually no published data on the topic. Such work would be needed in the case of groundnut and pigeonpea that are usually rainfed crops, both in Africa and Asia and can face episodes of drought throughout the cropping cycle.

Limitations to available studies on roots and the methods used

Although there has been a number of studies on roots in different crops, the lack of use into breeding programmes suggest some limitation to the works that were performed: (i) most of these studies assessed roots in a very "static" manner,

i.e., destructive samplings at one or several points in time, giving virtually no information on the "dynamics" of root characteristics, and it is not clear what particular root trait, or what particular aspect of root growth would contribute to a better adaptation to water deficit; (ii) all the studies on roots used limited number of genotypes except few (Kashiwagi *et al.* 2006), giving poor idea on the range of variations available to breed; This drawback is mostly explained by the difficulty in studying roots, thus, requiring a simplification of the methods used to evaluate a larger number of lines; (iii) some of the reported work clearly show that the putative relation between rooting traits and drought tolerance is often overridden by escape mechanisms related to genotype's phenology (Blum *et al.* 1977, Kashiwagi *et al.* 2006). Therefore, the contribution of roots to tolerance can only be truly tested once genetic variation in root traits is found within groups of genotypes sharing a similar phenology; (iv) Above all, most studies on roots published so far have relied on a fundamental assumption that increased root length density would equate with higher water uptake (see below) and therefore, on yield.

What Progress is Needed in Root Research?

Test whether differences in root length density relate to differences in water uptake

How much water is taken up would obviously relate somewhat to the RLD, but this link is still unclear because of the lack of data comparing the two parameters. Several authors concluded that RLD and water uptake is related (Passioura 1983, Monteith 1986, Lafolie *et al.* 1991). This view is challenged by other studies showing poor relation between water uptake and RLD across several cereals and legumes (Hamblin and Tennant 1987, Dardanelli *et al.* 1997, Katayama *et al.* 2000, Amato and Ritchie 2002). Even after considering legumes and cereals separately, legumes appear to have higher rate of water intake per unit root length compared to cereals, which would be explained by more abundant metaxylem vessels, which decrease their axial resistance to water flow. In any case, whether the RLD differences in chickpea relate to differences in water uptake or not need to be tested in view of its possible impact on yield. In fact, the lack of relation between water uptake and RLD agrees well with our data on groundnut (Fig. 3), where we run regression with root dry weight. By contrast, we found good relation between water uptake and root dry weight in *DREB1A* groundnut transgenics, where higher water uptake of transgenic plants under water deficit was well related to higher RLD below the 40 cm depth (Vadez et al. 2007a – Fig. 4). Our interpretation is that the root hydraulic conductance of groundnut varies likely across genotypes, which would explain that similar weights of roots, assumed to be related to proportional RLD, could lead to totally different water uptake. The good relation between RLD and water uptake found in transgenics would probably mean that the genetic transformation with DREB1A did not affect root hydraulics.

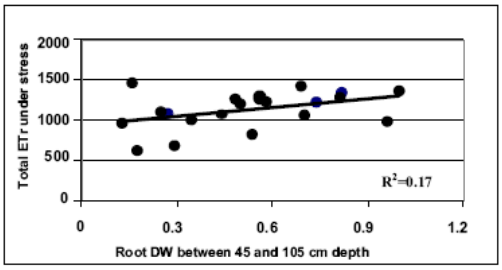

Fig. 3. Lack of a clear relationship between root dry weight (g plant^{-1}) within the 45-105 cm depth and the sum of the evapotranspiration, Etr (g plant^{-1}) when plants were undergoing water stress (after evapotranspiration of drought stressed plants fell below 50% of that in control plants). Each data point represents a replicated cylinder with one plant per cylinder. The 20 data points are 4 genotypes and 5 replications per genotype.

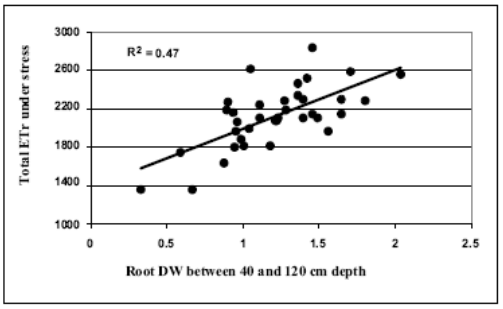

Fig. 4. A fairly clear relationship between root dry weight (DW) within the 45-120 cm depth (in g plant^{-1}) and the sum of the evapotranspiration when plants were undergoing water stress (after evapotranspiration of drought stressed plants fell below 50% of that in control plants). Each data point represents a replicated cylinder with one plant per cylinder. The 20 data points are 6 genotypes (5 transgenics events and one wild type) and 6 replications per genotype. See Vadez *et al.* (2007) for details on the experimental procedure.

The need to assess root "dynamically"

Following the above debate on root length density and its putative relation to water uptake, it has also been shown that a small length of roots in deep layers where water is plentiful would be sufficient to fully supply water to the plant when the top soil is dry (Gregory *et al.* 1978, Sharp and Davies 1985). This would logically offset the linear relation between water uptake and RLD. This hypothesis suggests that the assessment of RLD over the entire profile would be less relevant than the assessment at specific depth. Kashiwagi and colleagues showed that the overall RLD was related to seed yield in two experimental years and that RLD at depth was significantly related to seed yield under harsher conditions

only, although the overall RLD was also significantly related to seed yield in that year.

In any case, there are clearly some controversies over the water uptake and RLD relationship and several of our datasets on germplasm (unpublished) shows that root dry weight and water uptake are little related. Again, the benefit of deeper / more profuse rooting system remains to support enhanced/ sustained water uptake, in particular for key developmental stages. The assessment of roots at a given time during the lifetime of the crop is unlikely to deliver the precise information that would be needed to truly assess the potential of roots. As suggested by other authors, water uptake should be the prime target as suggested previously (McIntyre *et al.* 1995, Dardanelli *et al.* 1997) and such water uptake should be assessed *in-vivo* and repeatedly in plants exposed to stress, in conditions that mimic the field conditions, in particular with regards to having a soil profile, and compare to well-watered plants. In that respect, our preliminary data (Fig. 5) indicate that different genotypes appear to deplete the soil profile water of the cylinder in a different manner after irrigation has been suppressed. In Fig. 5, genotype TMV2 appears to take up higher amount of water than TAG24 during the initial 8 days after irrigation was withheld. In the forthcoming 10 days (9-19 days after withdrawing irrigation), TAG24 took up more water on almost everyday than TMV2. Such figure indicates that the kinetics of water uptake vary among genotypes.

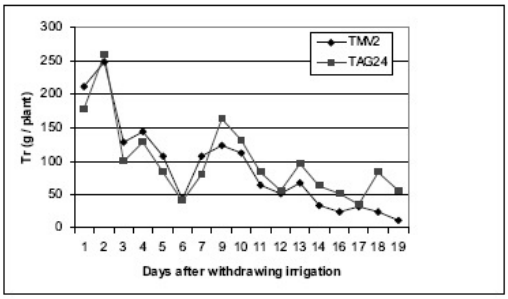

Fig. 5. Profile of transpiration, Tr (g plant^{-1}) in two groundnut genotypes grown in 1.2 m cylinders (16 cm diameter) after saturation of the soil profile and after irrigation has been withheld. Data are the mean of 5 replicated plants, one per cylinder.

The need to understand comprehensively root water uptake and shoot water loss

A better water uptake by roots at key stages like the grain filling period could be related to a sparing use of water by the shoot at earlier stages when the soil is wet. This type of behavior would permit saving of water in the soil profile and make it available for the later stages of the cropping cycle, *e.g.* during reproduction or grain filling. Although a more moderate water use would be related to less photosynthetic activity and therefore potential growth, this characteristic could be of value where water is limited. In fact, a modeling study showed that imposing a maximum rate of transpiration per day would contribute to water saving, increase in transpiration efficiency, and lead to yield benefit in most years in sorghum (Sinclair *et al.* 2005). There are possibly two ways in which maximum rate of transpiration can be achieved: (i) by having a lower stomatal conductance; (ii) or by limiting stomatal conductance when the vapor pressure deficit (VPD) is high.

In relation to mechanism (i) as indicated above, recent work on the *ERECTA* gene shows a role both on the regulation of photosynthesis, but also on the stomatal conductance (through stomatal density) (Masles *et al*. 2005). This would potentially lead to a limitation in the maximum rate of transpiration. We recently found that a major difference among pearl millet genotypes having similar phenology but differing in terminal drought tolerance was indeed due to a lower rate of water loss per unit of leaf area (transpiration over a period of 2 days divided by leaf area) in terminal drought tolerant genotypes (unpublished data). These differences were measured under well-watered conditions, indicating that this trait is constitutive. These results have been observed very consistently across experiments, either on whole plants taken at different stages or on detached leaves over short periods of time. This index, which reflects the leaf stomatal conductance over a period of time, would save water and make it available for the later stages of the crop cycle, in particular grain filling. In fact, the QTL for terminal drought tolerance that was identified was a QTL for yield and for a high panicle harvest index, which indirectly assesses grain filling in plants. Water saving from this lower stomatal conductance would decrease the proportion of water used before anthesis and make it available for grain filling. This would fit well an old hypothesis by Passioura (1983) that a higher proportion of water loss after anthesis would contribute to better grain filling under water stress conditions.

In relation to mechanism (ii) as mentioned above, the transpiration of certain genotypes of soybean has been shown to no longer increase, or to increase at a lower rate at vapor pressure deficit (VPD) above 2.0 KPa (Sinclair *et al.* 2008). This trait would limit soil moisture use when the VPD is high and when carbon fixation has a high water cost. Similar and additive to the above trait, it would make more soil water available for grain filling. We have observed before that certain species such as pearl millet in semi-arid conditions do maximize transpiration even if the vapor pressure deficit (VPD) is high (above 2.5 KPa) (Squire 1979). In the work reviewed by Bidinger and Hash (2004), no attention was paid to possible genetic variations in this strategy. Our recent data fully agree with the above reported data on soybean and show that pearl millet genotypes differ in their transpiration response to VPD (Vadez

et al. 2007b), with the terminal drought tolerance QTL donor having a considerably slowed transpiration at high VPD, whereas genotypes not holding this QTL would have transpiration responding linearly to VPD increases above 2.0 KPa.

The way forward to progress in root research

Basic considerations

Given these limitations and to remove the "static" approach used so far, we propose that our future approach on roots should focus on root functionality rather than morphology. We mean to say the focus be given to measuring water uptake under water deficit, in a "dynamic" and precise way, in a large range of genotypes representative of the species' diversity. This should carefully consider the phenology of genotypes, and determine the relation between a given pattern of kinetics/volume of water uptake and the performance under stress (based either on biomass or yield). Further, the evaluation of water uptake under stress should be compared to a fully irrigated control. Once contrasting genotypes are identified, the root developmental and morphological patterns could be investigated thoroughly to understand the genetics of development patterns.

The lysimetric system set up at ICRISAT

Such lysimetric system is shown in Fig. 6 and 7. It takes into account the above considerations. Additionally, we have paid utmost attention to develop a system that mimics the field conditions as closely as possible. The cylinders on Fig. 6 are 1.20 m long and 20 cm in diameters (thereafter called "small tubes"). The dimensions of the tubes have been determined in a way that the volume of soil available to each individual plant grown in each tube corresponds to the volume of soil available under current sowing density of groundnut and chickpea (approximately 25 – 30 plant /m²). A large scale lysimetric system has been set up with over 2300 cylinders. The major purpose is to translate the capacity to precisely measure water uptake differences into a throughput that can fit the need of breeding program (Fig. 7). Another system is being developed for more loosely planted crops such as pigeonpea. In that system, the tubes are 2.0 m long and 25 cm diameter (thereafter called "long tubes") and 1200 are available.

The soil packing has been done using soil sieved in particles smaller than 1 cm. This allowed us to control the bulk density to approximately 1.4, which is standard value for Alfisol. To ensure that moisture is available in all parts of the cylinders, 40 kg of dry soil were initially filled in each cylinder. We checked that the soil level was similar in all tubes. A previous assessment of the water needed to fill the profile before drainage determined that the soil water holding capacity of the soil was approximately 20%. Therefore, 8 liter of water was added to the first 40 kg of soil. An additional 10 kg of dry soil were filled to each cylinder soon after the water had penetrated the profile and watering with 2 L was done subsequently. At that stage, the cylinders were almost filled at the desired level, *i.e.* approximately 5 cm from the top. A top up using dry soil was done to ensure that all cylinders would be filled at the same level. This top up varied between 500g and about 1 kg, *i.e.* less than 1% variation across cylinders, for which all the cylinders had a very similar bulk density, close to 1.4.

At planting, the soil is wetted and seeds planted at a rate of 2/cylinders, later on thinned to 1 plant per cylinder. Watering is done at regular interval. This system is most suited to tailor a number of drought regimes to a range of crops (Fig. 7). In our preliminary experiments, we have imposed the last

Fig. 6. Lysimetric system newly used at ICRISAT to evaluate plant water uptake in PVC tubes (1.2 m long, 20-cm diameter), filled with Alfisol at a bulk density of about 1.4. The length and diameter of the tubes are designed so that the soil volume available to each plant is equivalent to the soil volume available under field conditions at current sowing densities (for groundnut and chickpea). The system is used here for the evaluation of transgenics in P2 facilities.

Fig. 7. Overall view of the lysimetric system developed at ICRISAT. The tubes are 1.2 m long with 20 cm diameter. They are filled with Alfisol at a set bulk density of 1.4. Each trench can accommodate approximately 700 cylinders. It takes about one day to 4 persons for weighing about 600 cylinders.

irrigation (to saturate the soil profile) at about one week after flowering. At that stage, low density polyethylene beads were applied to all cylinders (600 ml per cylinder to have a bead layer of approximately 2 cm). The purpose of the beads was to limit soil evaporation and our data show that about 90% of the soil evaporation is prevented. Therefore, the regular weighing of the cylinders provides essentially transpiration data. The beads also allow pegging in the case of groundnut.

CONCLUSION

Although some work has been attempted to exploit the potential of roots to improve the adaptation of crops to water limiting environments, it is little in comparison to the number of studies on other plant parts. So far, the methods used to evaluate roots are time consuming and provide information that, though valuable, does not reflect the dynamic characteristics of roots and root systems. In this review, we advocate that more focus should be given on the functionality of roots rather than their morphology, in particular by looking at direct water uptake measurements and their related kinetics. Additionally, we argue that the capacity of roots to take up water should also be looked at in the context of the capacity of shoot to limit their water loss, in a comprehensive manner. To progress in that direction, a large lysimetric system has been developed at ICRISAT.

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2.3 Constitutive water conserving mechanisms are correlated with the terminal drought tolerance of pearl millet [*Pennisetum glaucum* (L.) R. Br.]

Kholová J, Hash CT, Kakkera A, Kočová M, Vadez V. Journal of Experimental Botany. 61(2): 369-377. 2010.

The paper presents an innovative approach for understanding the drought tolerance mechanisms in the orphaned crop as is pearl millet. The purpose of this work was to characterize pearl millet lines contrasting for terminal drought tolerance for various physiological traits and identify divergent key traits which could be involved in the drought tolerance mechanisms. The basic hypothesis underlying this work was that terminal drought tolerant plant do have access to some water to fill up their grains. Therefore, any mechanism that would contribute to saving water earlier on during the plant cycle would indirectly contribute to this. Here we explored the possibility that tolerant plants would have a lower conductance, or would have different threshold of soil moisture where the transpiration starts declining.
Two terminal drought tolerant (PRLT 2/89-33 and 863B-P2), two terminal drought sensitive (H77/833-2 and ICMB841-P3) and several near-isogenic lines (NILs), introgressed with a terminal drought tolerance quantitative trait locus (QTL) from the donor parent PRLT 2/89-33 into H77/833-2 genetic background, were tested. We could clearly distinguish tolerant and sensitive genotypes based on following physiological traits; i) upon water deficit exposure, tolerant genotypes had lower fraction of transpirable soil water (FTSW) when their transpiration started to decline, ii) in well-watered conditions, tolerant genotypes exhibited lower transpiration rate on full plant basis as well as in detached leaves experiments, iii) transpiration rates of examined genotypes were not related to their stomata densities.

Our results demonstrate that constitutive traits controlling leaf water loss under well-watered conditions correlate with the terminal drought tolerance of pearl millet. These traits may influence the amount of water available in the soil during the critical stage of grain filling under terminal drought.

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RESEARCH PAPER

Constitutive water-conserving mechanisms are correlated with the terminal drought tolerance of pearl millet [*Pennisetum glaucum* (L.) R. Br.]

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Abstract

Pearl millet, a key staple crop of the semi-arid tropics, is mostly grown in water-limited conditions, and improving its performance depends on how genotypes manage limited water resources. This study investigates whether the control of water loss under non-limiting water conditions is involved in the terminal drought tolerance of pearl millet. Two pairs of tolerant×sensitive pearl millet genotypes, PRLT 2/89-33–H77/833-2 and 863B-P2–ICMB 841-P3, and near-isogenic lines (NILs), introgressed with a terminal drought tolerance quantitative trait locus (QTL) from the donor parent PRLT 2/89-33 into H77/833-2 (NILs-QTL), were tested. Upon exposure to water deficit, transpiration began to decline at lower fractions of transpirable soil water (FTSW) in tolerant than in sensitive genotypes, and NILs-QTL followed the pattern of the tolerant parents. The transpiration rate (Tr, in g water loss cm^{-2} d^{-1}) under well-watered conditions was lower in tolerant than in sensitive parental genotypes, and the Tr of NILs-QTL followed the pattern of the tolerant parents. In addition, Tr measured in detached leaves (g water loss cm^{-2} h^{-1}) from field-grown plants of the parental lines showed lower Tr values in tolerant parents. Defoliation led to an increase in Tr that was higher in sensitive than in tolerant genotypes. The differences in Tr between genotypes was not related to the stomatal density. These results demonstrate that constitutive traits controlling leaf water loss under well-watered conditions correlate with the terminal drought tolerance of pearl millet. Such traits may lead to more water being available for grain filling under terminal drought.

Key words: Drought stress, fraction of transpirable soil water, pearl millet, stomatal density, transpiration rate.

Introduction

Water deficit is one of the major abiotic factors limiting crop productivity in the semi-arid tropics, and climate change is likely to make drought stresses even more severe in the future. Therefore, sustainable and equitable global food security is, at least in part, dependent on the development of crop plants with better adaptation to water-limited environments.

Under drought, the leaf gas exchange of plants is reduced and this leads to lower biomass accumulation and grain yield. Previous work in several crops shows genotypic differences in how leaf gas exchange responds to water stress, with certain genotypes being capable of sustaining plant transpiration until the soil becomes fairly dry, whereas others react with a decline in transpiration when the soil is still relatively wet. This has been documented in maize (Ray and Sinclair, 1997), soybean (Vadez and Sinclair, 2001; Hufstetler et al., 2007), and groundnut (Bhatnagar-Mathur *et al.*, 2007). The relevance of either type of behaviour for performance under drought conditions in the field would depend on the pattern of drought: a decline in transpiration at high soil moisture would allow some water saving and would be beneficial in the case of long drought spells, but the related decrease in light capture and carbon fixation would eventually be reflected in a yield penalty in

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Abbreviations: FTSW, fraction of transpirable soil water; NIL, near-isogenic line; SD, stomatal density; Tr, transpiration rate.

conditions of short drought spells. Therefore, the soil moisture threshold [the fraction of transpirable soil water (FTSW)] where transpiration declines is extremely useful to understand and forecast genotypic behaviour in the face of a water deficit (Sinclair and Ludlow, 1986; Sadras and Milroy, 1996; Ray and Sinclair, 1997).

One aspect of water management that is often overlooked relates to the control of the overall water loss at the leaf level when water is available. A conservative use of water, even if soil moisture is sufficient to supply plant water demand fully, would maintain water in the soil profile for a longer period of time and might be advantageous under conditions of a long drought spell and/or terminal drought. The control of leaf area (LA) and leaf conductance are the main factors determining plant water losses. At a given LA, stomata regulation is the prime actor for the control of that water loss. A low stomata conductance, which could be in part related to a difference in the stomatal density (SD), would probably confer such a conservative pattern of water use. Recent findings on genes involved in the regulation of transpiration efficiency in *Arabidopsis* shows that a single gene, *ERECTA*, is involved in the regulation of SD and mesophyll cell proliferation (Masle *et al.*, 2005). Differences in stomatal conductance would have a direct impact on the gas exchange rate (Henson *et al.*, 1983; Muchow and Sinclair 1989; Masle *et al.*, 2005). Yet, in pearl millet there are limited data on the possible variation of that trait and how it can relate to differences in tolerance.

Stomatal conductance is heavily regulated, being influenced by many factors internal and external to the leaf. Stomatal conductance is not only linked to internal biochemical processes but is influenced by a range of physical factors such as the hydraulic conductance of xylem (Sperry *et al.*, 2005) whose short-term variation can be explained by physicochemical processes such as cavitation (Salleo *et al.*, 2001), wall collapse (Cochard *et al.*, 2004), changes of water viscosity with temperature (Cochard *et al.*, 2004), changes of wall permeability with sap chemical composition (Zwieniecky *et al.*, 2001), or leaf architecture (Tsuda and Tyree, 2000; Sack *et al.*, 2003). Yet, no clear relationship was established between stomatal conductance under well-watered conditions and tolerance to terminal drought. Since stomatal conductance is highly variable and is difficult to compare on many genotypes, methods are needed for comparing conductance of water in a way that is independent of environmental fluctuations.

Previous data (Black and Squire, 1979) showed that stomatal conductance in pearl millet was capable of adjustment in response to LA restriction or to a change in the LA ratio (LAR) (Henson and Mahalakshmi, 1985). In the study of Black and Squire (1979), a restriction of photosynthetically active LA led to an increased stomatal conductance of the remaining LA, showing the capacity of stomata to adjust to changes in LA. So pearl millet stomatal conductance and LA appear to be closely related. Thus, while work on pearl millet has focused on understanding how the leaf canopy develops to maximize water use (Bidinger and Hash, 2003), or considers the reduction of LA under drought as an adaptation (Wallace *et al.*, 1993), it is argued here that it is important to study how both conductance and LA interact in the control of water loss.

Lines of pearl millet contrasting in yield under terminal drought conditions are known (Bidinger *et al.*, 1987). Quantitative trait loci (QTLs) for that trait have been identified (Yadav *et al.*, 2002) and confirmed in another genetic background (Yadav *et al.*, 2004). Near-isogenic lines (NILs) containing a major terminal drought tolerance QTL on linkage group 2 have been generated and these lines have confirmed the role of the QTL in achieving a higher yield under terminal drought conditions (Serraj *et al.*, 2005). The major effect of the QTL is to improve grain filling, but the underlying mechanisms are not known. Root growth under drought varies among these contrasting lines (Vadez *et al.*, 2007). However, differences in how plants regulate their water loss have not been investigated.

The overall objective of the present study was to assess whether pearl millet genotypes varying for a QTL responsible for terminal drought tolerance differed in how plants controlled leaf water loss. Specific objectives were to: (i) compare whether these pearl millet genotypes differed in their response to a progressive exposure to water deficit; (ii) assess whether they differ in the regulation of their leaf water loss when water is non-limiting; and (iii) test how the rate of water loss changes upon alteration of the LA (LAR in $cm^2\ g^{-1}$).

Materials and methods

Genetic material

Parental lines: Two pairs of pearl millet (*Pennisetum americanum* L.) genotypes contrasting in tolerance under drought stress [PRLT 2/89-33 (tolerant) versus H77/833-2 (sensitive) and 863B-P2 (tolerant) versus ICMB 841-P3 (sensitive)] were selected for the study based on previous experiments (Yadav *et al.*, 2004; Serraj *et al.*, 2005). Tolerance/sensitivity was assessed on test-cross hybrids of these inbred parental lines, developed by crossing the inbred parental lines to male-sterile line tester 843A for PRLT 2/89-33 and H77/833-2 and to male-sterile line tester H77/833-2 for 863B-P2 and ICMB 841-P3 (Stegmeier *et al.*, 1998). Tolerance of these hybrids was based on yield under terminal drought stress in several years of field trials and on the panicle harvest index (PNHI), an index that assesses the success of spikelet fertilization and the degree of grain filling (Bidinger *et al.*, 1987). Sensitive genotypes H77/833-2 and ICMB 841-P3 are of North Indian origin and are heat-resistant parental genotypes of many commercial hybrids of this area. Tolerant genotypes PRLT 2/89-33 and 863B-P2 derive from the ICRISAT Bold Seeded Early Composite, which is an elite breeding population based on Iniadi landrace germplasm from West Africa.

Near-isogenic lines: From the two crosses between tolerant/sensitive pairs reported above, a major QTL for terminal drought tolerance was identified on linkage group 2 (Yadav *et al.*, 2002, 2004). To develop the QTL introgression lines in the background of H77/833-2, the latter was crossed to PRLT 2/89-33 followed by four backcrosses with H77/833-2 to recover most of its genetic background. At each backcross the assessment of the presence or absence of the terminal drought tolerance QTL was made using simple sequence repeat (SSR) flanking markers on LG2 (*Xpsmp2059*, *Xpsmp2066*, and *Xpsmp2237*). Two steps of selfing

were performed to generate inbred NILs-QTL. Test-cross hybrids of the NILs-QTL, made using the same male-sterile line 843A, were produced with five introgression lines ICMR 1029, ICMR 1031, ICMR 2041, ICMR 2042, and ICMR 2044. The test-cross hybrids involving ICMR 1029, ICMR 1031, and ICMR 2041 were previously found to be superior in terms of their yield under terminal drought tolerance, whereas ICMR 2042 and ICMR 2044 had a yield response in the field that was similar to that of the test-cross hybrid from the sensitive parent H77/833-2 (Serraj *et al.*, 2005).

Plant growth and response to drought

Plants were grown in pots during May–June 2007 and January–February 2008 in a glasshouse under near-optimal conditions [day/night temperature 32/25 °C, relative humidity oscillating between 40% and 80% during the course of the day, and the resulting vapour pressure deficit (VPD) varying between 2.86 kPa and 0.63 kPa]. The soil used was an Alfisol collected from ICRISAT's farm and had a pH of ~7. The soil was mixed with sand and manure (5:3:1). Pots were filled with 5 kg and 9 kg of the Alfisol:sand:manure mixture for the assessment at the vegetative and reproductive stage, respectively (details provided below). The soil was amended with N, P, and K by mixing di-ammonium phosphate and muriated potash at a rate of 300 mg kg^{-1} and 200 mg kg^{-1}. In addition plants were top dressed with 1 4g urea per plant at 4 weeks after sowing.

The transpiration response of plant to progressive exposure to water deficit was assessed in parental lines [PRLT 2/89-33 (tolerant) and H77/833-2 (sensitive), 863B-P2 (tolerant) and ICMB 841-P3 (sensitive)] in May–June 2007. The assessment was initiated before the flag leaf stage in Experiment 1 (30 d after sowing) and after the panicles were fully emerged in Experiment 2 (40 d after sowing). For the sake of brevity, the former stage of assessment is hereafter called the 'vegetative stage' whereas the latter is referred to as the 'reproductive stage'. These two experiments were designed to estimate whether the FTSW threshold where transpiration declines varied with genotypes and phenological stages. Experiment 3, carried out in January–February 2008, was similar to Experiments 1 and 2, and used PRLT 2/89-33, H77/833-2, and four QTL introgression lines (ICMR1029, ICMR 1031, ICMR 2042, and ICMR 2044) to assess a putative relationship between the FTSW threshold for transpiration decline and the known performance of NILs-QTL in the field and the putative presence/absence of the introgressed QTL. Based on results from Experiments 1 and 2, Experiment 3 was performed at the vegetative developmental stage only.

For each experiment there were two sets of plants with six replicates of each genotype. At the time of imposing the treatment, depending on development stage, pots were saturated with water and allowed to drain overnight. The following morning, plants were bagged in a plastic bag wrapped around the stem, and pots were subsequently weighed. Pot weight was thereafter taken every day in the morning. Two water treatments were imposed: a well-watered and a water stress treatment. The WW set of plants was maintained close to 80% field capacity by bringing the pot weight to that level (i.e. 100 g and 200 g below the saturated weight for the 5 kg and 9 kg pots, respectively) every day. The WS set of plants was exposed to a gradual water stress by partially compensating water loss from transpiration, i.e. plants were allowed to lose no more than 70 g and 100 g of water on each day for the vegetative and reproductive stage assessment, respectively. The difference in re-watering was related to the pot size and allowed the imposition of relatively similar kinetics of stress imposition in these pots varying in size and therefore in water availability. Any transpiration in excess of these maximum daily water losses allowed was added back to the pots, as previously described (Vadez and Sinclair, 2001).

The transpiration values were normalized to facilitate comparison. First, the transpiration ratio (TR) was calculated by dividing the transpiration of each individual plant of a given genotype by the average WW transpiration of that genotype. Secondly, the TR was normalized by dividing each TR value over time by the average of the TR value for the first 3 d of the experiment when there was still no water limitation. This second normalization gave the normalized transpiration ratio (NTR), which accounted for plant to plant variation in transpiration within each genotype. When the NTR of stressed plants fell below 0.10, i.e. when the transpiration of WS plants was <10% of that of WW plants, all the plants were harvested and LA and the dry weights of their parts were measured.

After harvest, the FTSW for each day of the experiment was calculated. The FTSW values represent the portion of remaining volumetric soil water available for transpiration on each day of the experiment and were used as the indicator of stress (Ritchie, 1981). FTSW on each day n was calculated as:

$$(\text{Pot weight day } n - \text{Final pot weight}) / (\text{Initial pot weight} - \text{Final pot weight})$$

Rate of water loss per unit of leaf area

The rate of water loss per unit of LA was assessed under well-watered conditions over the course of 3 d. It was assumed that the LA changes during this period would not differ across genotypes and this index would 'integrate' the behaviour of stomata over the 3 d. To do so, the daily transpiration of control plants was averaged over the last 3 d before being harvested. At harvest the LA was measured and the transpiration data used for conversion of transpiration to water loss per unit of LA and per day (Tr, transpiration rate in g cm^{-2} d^{-1}). The purpose of this was not to obtain an absolute value of Tr but rather a comparative estimate for different genotypes. The Tr was measured before the panicles emerged ('vegetative phase') and after the panicles had emerged ('reproductive phase').

All four parental lines (H77/833-2, PRLT 2/89-33, ICMB 841-P3, and 863B-P2) were compared for their Tr at the vegetative and reproductive stage, using plants of the WW treatment of Experiments 1 and 2. Tr was also evaluated in NILs-QTL (ICMR1029, ICMR1031, and ICMR1041) along with two parental lines (H77/833-2, PRLT 2/89-33) in Experiment 4, using six replicated plants per genotypes. Plants of Experiment 4 were grown under well-watered conditions in a glasshouse in August 2008, following the conditions described above and used for the assessment of Tr. The arrangement in the glasshouse was such that pots were widely spaced with ~25–30 cm between each pot. Therefore, the plant density of the experimental set-up was <5 plants m^{-2}, which limited leaf shading of the bottom leaves.

In Experiment 5, Tr was measured on detached leaves that were sampled from the well-watered block of a field experiment where the four parental lines were assessed for yield under terminal drought (data not shown). It had previously been determined with container-grown plants that the Tr measured from whole plants was consistent with the detached leaf Tr (DLT) from detached leaves from these plants (data not shown). The first fully developed leaves of plants grown in the field were used to determine the DLT. The leaves were placed in test tubes with 0.1 mM EDTA to prevent the clogging of xylem vessels and tightly covered with plastic film (Parafilm) and aluminium foil to prevent evaporation. Subsequently, leaves were acclimated for 30 min in a growth chamber (30 °C, 80% relative humidity, 600 μm light intensity at the leaf level), and the tubes were then weighed. The leaves were weighed every hour, and after 3 h they were harvested, LA was determined, and, finally, DLT was calculated as the Tr (g water cm^{-2} h^{-1}).

Influence of defoliation on the Tr

In Experiment 6, tests were conducted to determine whether the Tr (g cm^{-2} d^{-1}) was affected by partial defoliation of the plants.

Saturated pots with plants of the parental line hybrids were bagged with foil to avoid soil evaporation and placed in a growth chamber under standard conditions (30 °C, 80% relative humidity, 600 µm light intensity at the canopy level). Previous assessment had shown that the transpiration in the chamber was relatively constant throughout the day except for ~2 h after light set. After 1 d of acclimation to these conditions, plant transpiration was measured during 3 h by weighing every hour, and subsequently plants were partially defoliated. Every second leaf from the top was eliminated, leading to an LA reduction of 57, 50, 53, and 53% in PRLT 2/89-33, H77/833-2, 863B-P2, and ICMB 841-P3, respectively. It took about an hour for defoliation of the plants and then transpiration was estimated during another 3 h. Finally, reduced LA and remaining LA were measured and used for calculation of the Tr (g cm^{-2} h^{-1}) before and after LA reduction. The percentage change in Tr was also calculated by dividing each individual Tr value after defoliation by the mean Tr for each respective genotype before defoliation.

Stomatal density

SD was estimated on the first fully developed leaf of well-watered plants of the four parental lines. Colourless nail polish was spread on the abaxial leaf side of the most fully expanded leaves sampled before the panicle emerged from WW plants of Experiment 1. The leaf print was observed under the light microscope (magnification 20×10, area 9.86 mm^2). The number of stomata was counted at three randomly chosen places, avoiding large vessels in the leaf print.

Statistical analysis

The experimental design for Experiments 1–3 was a randomized complete block design with two water treatments (WW and WS) as main factors and genotypes as subfactors with six replications. The experimental design for Experiments 4–6 was a randomized complete block design with one treatment as main factor (WW in Experiments 4 and 5 and defoliation in Experiment 6) and genotypes as subfactor, with six (Experiment 4), five (Experiment 5), and four replications (Experiment 6). Analyses of variance (ANOVAs) were done with the statistical program package CoStat version 6.204 (CoHort Software, Monterey, CA, USA). One-way ANOVA was carried out to test for genotypic differences within treatment (LSD in Table 5) and to compare genotype means across treatment where the Tukey–Kramer test was then used for the analysis of differences between genotype means across treatments (letters in Table 1 and 2). For the FTSW threshold analysis, SAS (SAS Institute, Inc., 1988, Cary, NC, USA) was used. Each NTR value was plotted to a corresponding FTSW value for each day of the experiment, and the FTSW thresholds where NTR initiated its decline were determined using a plateau regression procedure as described previously (Ray and Sinclair, 1998). This analysis provided a confidence interval for each threshold value.

Results

Effect of drought exposure on growth parameters

Water deficit reduced biomass in Experiments 1 and 2 (Tables 1, 2). The most drought-affected parts of the plants were the stem and the panicle at both growth stages assessed, in agreement with previous work (Winkel *et al.*, 2001), compared with root and leaf biomass, which were the

Table 1. Distribution of dry mass between plant parts in Experiment 1 (vegetative stage)

The plant parts examined were root dry weight (RDW), stem dry weight (SDW), panicle dry weight (PDW), leaf dry weight (LDW), total dry weight (TDW), and leaf parameters: leaf area (LA), specific leaf area (SLA) of four pearl millet genotypes [H77/833-2 (sensitive) and PRLT 2/89-33 (tolerant), ICMB 841-P3 (sensitive) and 863B-P2 (tolerant)]. The values are shown with ±SE; n=6. Lower case letters following means discriminate genotype means between treatments.

Treatment	Genotype	RDW (g)	SDW (g)	PDW (g)	LDW (g)	LA (cm^2)	SLA (cm^2/g)	TDW (g)
Control	PRLT 2/89-33	5.24±0.611 a	20.19±1.91 a	2.74±1.04 a	7.08±0.55 a	1352±115 a	191.0±4.8 a	34.8±3.12 a
	H77/833-2	14.50±0.58 a	20.08±1.83 a	5.42±0.79 a	6.45±0.48 a	1246±98 a	193.3±2.7 a	41.60±3.92 a
	863B-P2	10.11±2.11 a	22.66±1.62 a	5.42±2.45	10.93±0.86 a	2276±182 a	208.2±6.5 a	47.61±2.85 a
	ICMB 841-P3	3.93±0.71 a	16.20±0.92 a	3.38±0.34 a	6.80±0.62 a	1552±152 a	228.4±8.8 a	30.30±1.83 a
Drought	PRLT 2/89-33	3.58±0.27 b	18.74±1.49 a	2.41±1.05 a	6.78±0.26 a	969±184 b	124.0±1.9 b	30.55±1.73 a
	H77/833-2	5.02±0.87 b	10.40±1.29 b	3.24±0.36 b	3.94±0.45 b	599.1±41.1 b	158.2±13.0 b	21.75±2.94 b
	863B-P2	4.97±0.93 a	12.95±1.14 b	–	10.61±1.25 a	1462±174 b	159.5±14.7 b	27.16±2.02 b
	ICMB 841-P3	6.25±1.62 a	15.29±0.93 a	1.37±0.37 b	6.21±0.33 a	1087±19 b	174.2±2.6 b	28.65±2.75 a

Table 2. Distribution of dry mass between plant parts in Experiment 2 (reproductive stage)

The plant parts examined were root dry weight (RDW), stem dry weight (SDW), panicle dry weight (PDW), leaf dry weight (LDW), total dry weight (TDW), and leaf parameters: leaf area (LA), specific leaf area (SLA) of four pearl millet genotypes [H77/833-2 (sensitive) and PRLT 2/89-33 (tolerant), ICMB 841-P3 (sensitive) and 863B-P2 (tolerant)]. Lower case letters following means discriminate genotype means between treatments.

Treatment	Genotype	RDW (g)	SDW (g)	PDW (g)	LDW (g)	LA (cm^2)	SLA (cm^2/g)	TDW (g)
Control	PRLT 2/89-33	15.54±1.50 a	31.52±4.06 a	6.18±1.81 a	11.63±1.22 a	2132±240 a	183.2±5.7 a	66.00±5.70 a
	H77/833-2	12.49±1.55 a	23.05±1.88 a	19.89±1.89 a	7.57±0.47 a	1328±122 a	174.7±8.8 a	63.32±4.72 a
	863B-P2	24.88±2.97 a	37.32±1.60 a	12.17±1.81 a	20.09±1.70 a	3924±455 a	200.3±19.5 a	96.40±6.33 a
	ICMB 841-P3	23.12±3.72 a	36.19±4.37 a	14.63±2.61 a	12.08±1.42 a	2680± 351 a	221.3±9.3 a	86.9 ±7.56 a
Drought	PRLT 2/89 33	17.71±3.37 a	27.32±2.29 a	5.33±1.54 a	13.55±0.91 a	738±72 b	55.5±6.1 b	62.13±2.74 a
	H77/833 2	10.59±1.95 a	23.95±2.25 a	16.89±2.37 a	7.98±0.77 a	762±108 b	97.8±11.3 b	57.63±7.52 a
	863B-P2	29.19±3.32 a	35.86±4.31 a	13.21±0.75 a	20.37±3.19 a	1902±291 b	94.1±6.5 b	98.63±8.10 a
	ICMB 841-P3	20.69±3.43 a	38.01±2.81 a	17.86±2.11 a	13.82±1.20 a	1267 ±199 b	91.1±11.3 b	87.61± 5.32 a

least affected by drought. Although leaf dry weight (LDW) changed little under drought, LA decreased 50–75% compared with control conditions, at both stages of assessment. This was related to leaf thickening in part indicated by a 30–50% decrease of the specific leaf area (SLA) in all the genotypes under drought conditions at the vegetative stage and reproductive stage. 863B-P2 had the highest LA and LDW values, followed by ICMB 841-P3 along with PRLT 2/89-33, while H77/833-2 had the lowest LA and LDW values.

It was also found at the vegetative stage that, while the SLA decreased (leaves thickened) in all genotypes, this trend was more marked in the tolerant parents (PRLT 2/89-33 and 863B-P2) than in their respective sensitive partner. In addition, it was found that as SLA increased Tr (g water $cm^{-2}d^{-1}$, see below) decreased.

Transpiration response to soil drying

At the vegetative stage, the transpiration started declining at FTSW values ranging between 0.49 and 0.30 (Fig. 1a, b). In fact, threshold values were higher for sensitive H77/833-2 and ICMB 841B-P2 than for PRLT 2/89-33 and 863B-P2 at the vegetative stage. In contrast, at the reproductive stage the transpiration dropped at similar FTSW values ranging between 0.26 and 0.35 (Fig. 1c, d) in all genotypes (Table 3).

Based on the above results, this dry down at the vegetative stage only was reproduced with the first parental pair PRLT-2/89-33 and H77/833-2 along with their NILs-QTL (Fig. 2). Again parental genotypes differed and the sensitive parent H77/833-2 had a higher FTSW threshold than tolerant PRLT-2/89-33. Moreover, the FTSW for superior NILs-QTL ICMR 1029 and ICMR 1031 was similar to that of the tolerant parent (PRLT-2/89-33), whereas the FTSW threshold of sensitive NILs-QTL ICMR 2042 and ICMR 2044 was similar to that of the sensitive parent (H77/833-2) (Table 4).

Rate of water loss per unit leaf area and time–transpiration rate

The Tr (g cm^{-2} d^{-1}) was lower in tolerant PRLT 2/89033 than in H77/83302 at both the vegetative and reproductive stage (P <0.01). Similar results were found with the other pair of parental lines, with the Tr being also lower in tolerant 863B-P2 than in sensitive ICMB 841-P3 (Fig. 3a, b). These results fully confirmed data that were obtained in previous experiments (data not shown).

This measurement of Tr was repeated at the vegetative stage with parental lines PRLT 2/89-33 and H77/833-2 and three NILs-QTL (ICMR 1029, ICMR 1031, and ICMR 2041). Again the Tr was lower in tolerant PRLT 2/89-33. In addition, the Tr in the NILs-QTL was lower than in the sensitive parent H77/833-2 and similar to the tolerant PRLT2/89-33 (Fig. 4) (P <0.01).

Prior to assessing Tr in detached leaves from plants grown in the field, tests were carried out to determine whether the Trs of detached leaves and whole plants were

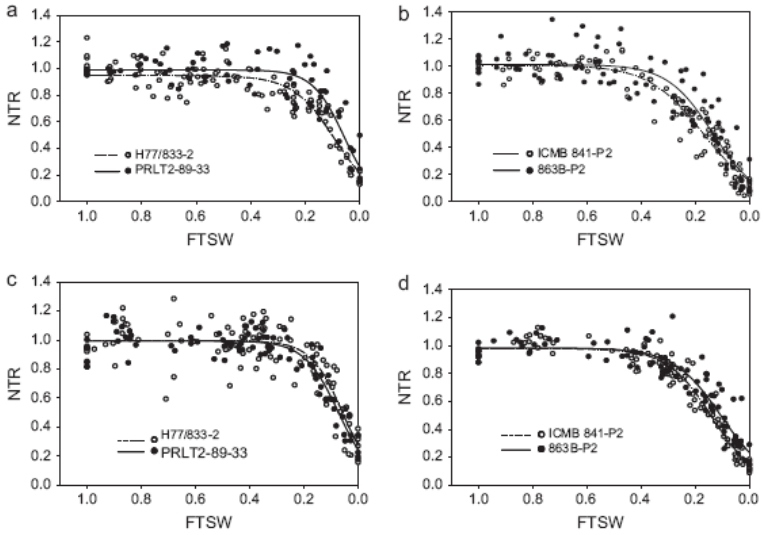

Fig. 1. Relationship between the normalized transpiration rate (NTR) and the fraction of transpirable soil water (FTSW) of two pearl millet genotype pairs: H77/833-2 and PRLT 2/89-33, and ICMB 841-P3 and 863B-P2 (H77/833-2, ICMB 841-P3—sensitive; PRLT 2/89-33, 863B-P2—tolerant) during the vegetative (a, b) and reproductive (c, d) stage in 2007. The FTSW thresholds where transpiration initiated its decline were calculated with a plateau regression procedure from SAS. Then the regression lines of the relationships between NTR and FTSW were drawn by fitting NTR to FTSW data above and below the respective threshold for transpiration decline in each genotype and assessment stage.

Table 3. Statistical analysis of data from Figs 1a, b, 2 showing the FTSW threshold where transpiration declines upon exposure to progressive water deficit in vegetative and reproductive stage (Experiments 1 and 2) in several pearl millet genotypes

Data are the means of six replicated plants per genotype.

Genotype	FTSW threshold	Approximate SE	95% CI
Vegetative stage			
PRLT 2/89-33	0.3036	0.0353	0.2333–0.3738
H77/833-2	0.4923	0.0476	0.3977–0.5869
ICMB 841-P2	0.4146	0.0158	0.3849–0.4478
863B-P2	0.3267	0.0176	0.2918–0.3616
Reproductive stage			
PRLT 2/89-33	0.2645	0.0227	0.2194–0.3096
H77/833-2	0.2489	0.0258	0.1977–0.3001
ICMB 841-P2	0.3553	0.00836	0.3387–0.3791
863B-P2	0.3565	0.0141	0.3285–0.3846

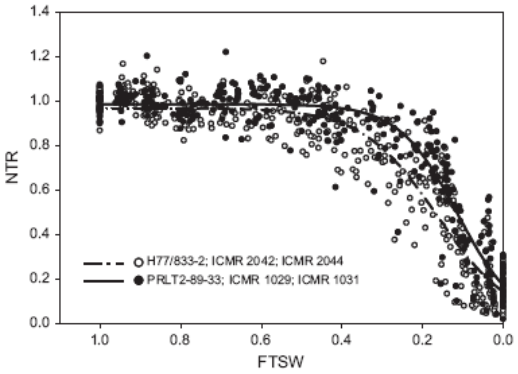

Fig. 2. Relationship between the normalized transpiration ratio (NTR) and the fraction of transpirable soil water (FTSW) of two pearl millet genotype pairs: H77/833-2 and PRLT 2/89-33 and their four NILs-QTL [ICMR 1029 and ICMR 1031 that yielded similarly to tolerant PRLT 2/89-33 under terminal drought and ICMR2042 and ICMR 2044 that yielded similarly to sensitive H77/833-2 (Serraj *et al.*, 2005)] during the vegetative developmental stage in 2008 (Experiment 3).

similar. To do so, the Tr of detached leaves and the Tr of whole plants were simultaneously followed in two genotypes contrasting in this trait (PRLT2/89-33 and H77/833-2) during 3 h of the daylight cycle under glasshouse conditions. The Tr of detached leaves (0.0033 g cm^{-2} h^{-1}) was similar to that from whole plants (0.0030 g cm^{-2} h^{-1}), averaged across genotypes for both Tr measurements. In addition, Tr was lower in PRLT2/89-33 than in H77/833-2 in both cases (P <0.001).

The detached leaf protocol was used to assess the transpiration rate in detached leaves sampled from the field, and a lower Tr was found in PRLT2/89-33 than in H77/833-2 and a lower Tr in 863B-P2 than in ICMB 841-P3 (Fig. 5) (P <0.01).

Table 4. Statistical analysis of data from Figs 2 showing the FTSW threshold where transpiration declines upon exposure to progressive water deficit in several pearl millet genotypes, including NIL-QTL materials

Data are the means of six replicated plants per genotype.

Genotype	FTSW threshold	SE	Approximate 95% CI
H77/833-2	0.3772	0.017	0.3436 –0.4108
ICMR 1029	0.2965	0.0153	0.2662 – 0.3269
ICMR 1031	0.2982	0.0137	0.2711–0.3253
ICMR 2042	0.4699	0.0181	0.4340–0.5058
ICMR 2044	0.411	0.0148	0.3817–0.4403
PRLT 2/89-33	0.304	0.00954	0.2851–0.3228

Effect of defoliation on transpiration rate

The reduction of LA and thus the disruption of the LAR resulted in an increase in Tr in all the genotypes, except ICMB 841-P3 (Table 5). The relationship between LAR and Tr was fitted with an exponential equation (P <0.001, data not shown). After defoliation, neither the Tr nor the percentage increase in Tr were any different between genotypes at P <0.05 (Table 5).

Stomatal density

The numbers of stomata in an area of 9.86 mm^2 on the first fully developed leaf from plants of Experiment 1 were counted, and no differences in SD were found between genotypes (Table 5).

Discussion

Differences in transpiration rate

Crucial results for understanding the drought tolerance strategy of millet emerged from the comparison of Tr under well-watered conditions. Both tolerant genotypes (PRLT 2/89-33 and 863B-P2) showed lower Tr compared with their sensitive partners (H77/833-2 and ICMB 841-P3) in both developmental stages. In addition, Tr variability was also found on detached leaves sampled from the field. Evidence for the role of Tr in the terminal drought tolerance QTL was confirmed, with all tolerant NILs exhibiting a Tr similar to the tolerant parent and lower than the sensitive parent. These results show a relationship between the terminal drought tolerance of PRLT 289/33 and NILs and their lower rate of water loss per unit of LA under well-watered conditions. This trait would conserve soil moisture for later stages, in particular during the grain filling period. It would have great value under terminal drought conditions and in environments where soil evaporation is limited. This interpretation would fit well the fact that the

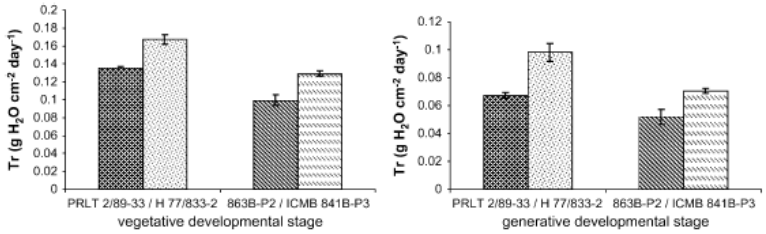

Fig. 3. Transpiration rate (Tr) of pearl millet parental genotype pairs: H77/833-2 and PRLT 2/89-33, ICMB 841-P3 and 863B-P2; (H77/833-2, ICMB 841-P3 - sensitive, PRLT 2/89-33, 863B-P2 - tolerant). Tr was compared among vegetative (3a) and reproductive stage (3b) of development in 2007. Bars indicate the SE (n=6).

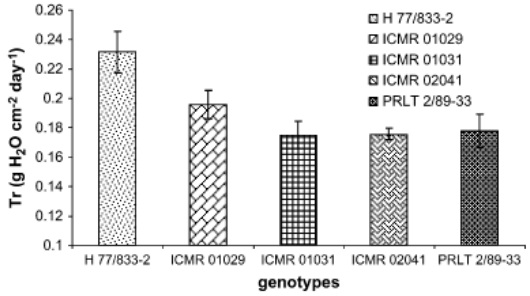

Fig. 4. Transpiration rate (Tr) of parental genotype H77/833-2 (sensitive) and PRLT 2/89-33 (tolerant) and their NILs-QTL (ICMR 1029, ICMR 1031, and ICMR 2041) in the vegetative stage of development in 2008. Bars indicate the SE (n=6).

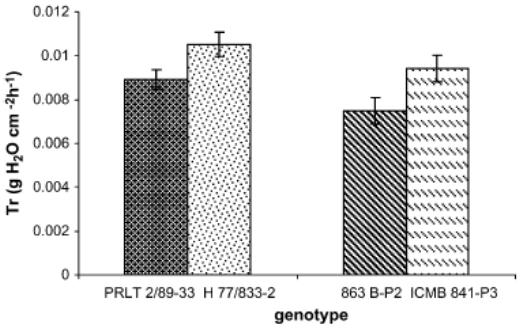

Fig. 5. Average of the transpiration rate (Tr, g cm^{-2} h^{-1}) of detached leaves of four genotypes (H77/833-2 and ICMB 841-P3—sensitive; PRLT 2/89-33 and 863B-P2—tolerant) grown under well-watered conditions in the field. The transpiration rate was assessed over a 3 h period in a glasshouse. Bars indicate the SE (n=6).

terminal drought tolerance QTL is responsible for a better PNHI, i.e. a proxy for grain filling. Such data have not been reported so far despite the fact that the importance of plant water management in well-watered conditions has previously been discussed a number of times (Mortlock *et al.*, 2001; Condon *et al.*, 2002; Sinclair *et al.*, 2005, 2007). More work is needed to test the hypothesis that Tr differences would lead to more water availability during grain filling.

Table 5. Transpiration rate (Tr) at full leaf area (LA) and at reduced LA, percentage Tr increase, and average stomatal density in genotypes H77/833-2, PRLT 2/89-33, ICMB 841-P3 and 863B-P2

Stomatal density data were collected from the first fully developed leaves from greenhouse Experiment 1. Stomata were counted on set areas of 9.86 mm^2. Data are means ±SE of four replicated plants for the Tr measurement and of six replicated stomatal density assessments.

Genotype	Tr of full LA (g H_2O cm^{-2} h^{-1})	Tr of reduced LA (g H_2O cm^{-2} h^{-1})	% Tr increase	Stomatal density (per 9.86 mm^2)
PRLT 2/89-33	0.014±0.001	0.021±0.003	44±9	9.51±0.70
H77/2 833-2	0.019±0.002	0.031±0.005	77±25	11.50±0.58
863B-P2	0.011±0.001	0.017±0.002	52±10	10.07±0.54
ICMB 841-P3	0.017±0.003	0.020±0.003	31±7	9.95±0.66
LSD (P < 0.05)	0.005	0.011	33	2.02

The aim of this study to at least partially explain differences in plant water management by examining the number of stomata did not confirm the initial hypothesis. It was expected that variability in SD might provide an explanation for previously observed differences in Tr, as was shown in Masle *et al.* (2005) who provided evidence that the gene *ERECTA* had an impact on SD. This seems not to be the case for pearl millet. Thus, if stomata number might play a role in drought tolerance (Muchow and Sinclair. 1989), the present results agree with the previous assertions that stomatal regulation rather than SD is more important for regulating water loss in pearl millet (Henson *al.*, 1981; Liu *et al.*, 2003; Zhang *et al.*, 2005). There, the use of Tr as 'an integrated proxy' for stomatal conductance appeared to be a simple and successful screen to discriminate tolerant and sensitive genotypes.

Effect of defoliation on the transpiration rate

The Tr may not only be dependent upon internal biochemistry of plants but may also be influenced by physical characteristics of plants' internal architecture (Salleo *et al.*, 2001; Zwieniecky *et al.*, 2001; Cochard *et al.*, 2004; Sperry *et al.*, 2005). When the LAR was experimentally altered to test the

possibility of short-term adjustment, the Tr of the remaining LA adjusted very quickly to the exponential function. This would suggest that a hydraulic control of the change in Tr is involved in such a rapid change of the stomatal opening.

Differences in FTSW threshold

A previous study has shown that pot size had no influence on the FTSW threshold (Ray and Sinclair, 1998) and the present protocol appeared adequate for comparing genotypic response to soil water deficit. It was found that the FTSW threshold of tolerant parental genotypes was lower compared with sensitive genotypes in the vegetative developmental stage. This meant that the transpiration dropped upon progressive soil drying in relatively dryer soil in the tolerant lines than in the sensitive lines. This genotypic variability in transpiration response to soil drying was in agreement with data obtained in groundnut (Bhatnagar-Mathur *et al.*, 2007). In addition, the response measured in one contrasting parental pair and their NILs-QTL provided evidence that the FTSW threshold obtained for superior NILs-QTL was similar to that of tolerant PRLT 2/89-33 and the QTL donor parent. In contrast, the FTSW threshold obtained for NILs-QTL that did not yield better than H77/833-2 in the field was indeed similar to that of sensitive H77/833-2. These data provide evidence for a role for the QTL in explaining the differences in these thresholds and hence the role of these threshold differences in understanding the variability between lines in their terminal drought tolerance. The reasons for these differences are intriguing, given that: (i) tolerant genotypes have a lower Tr under well-watered conditions, which would denote a more 'conservative' water use; and (ii) tolerant genotypes have a lower FTSW threshold for transpiration decline under drought which indicates that they attempt to maximize water use. Both could in fact be related. Indeed a lower Tr in tolerant lines under well-watered conditions would lead to lower daily transpiration, which would logically drive the TR of drought-exposed plants upwards, and consequently the NTR. Therefore, the maintenance of an NTR under drought conditions at a level close to that of well-watered plants, which leads to having a lower FTSW threshold for the beginning of the transpiration drop, might simply be a consequence of the lower rate of water loss per unit of LA (Tr) in the well-watered plants of tolerant genotypes. In fact, this agrees well with the fact that the presence/absence of the QTL appeared to discriminate well for both a lower/higher Tr and lower/higher FTSW threshold. The same interpretation could be obtained from similar data in transgenic groundnut (Bhatnagar-Mathur *et al.*, 2007). The only unexplained issue is the fact that the FTSW thresholds were not different at the reproductive stage whereas Tr was still different.

Conclusion

This study showed that genotypes contrasting in terms of terminal drought tolerance, based on seed yield in field conditions, also contrasted in the control of leaf water loss under well-watered conditions. This trend was directly related to the presence or absence of a terminal drought tolerance QTL. The tolerant/QTL holder genotypes had a lower rate of water loss per unit LA (Tr, g water cm^{-2} d^{-1}). It is hypothesized that this characteristic would contribute to a more conservative water use in field conditions, making more water available for the grain filling stage, which would be very important for terminal drought conditions. This hypothesis remains to be tested. A lower Tr would also lead to having a lower FTSW threshold where transpiration declines upon progressive exposure to water deficit, making drought-stressed plant behave like well-watered plants until the soil has become dryer than for sensitive lines. Since Tr was measurable on whole plants but also on single detached leaves that could be collected from the field, Tr may be a very convenient trait to phenotype across a range of experimental conditions. Although more work is needed to understand better how Tr is regulated, Tr may be further considered as an insightful tool for selection screening in pearl millet breeding programmes.

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2.4 Terminal drought-tolerant pearl millet [*Pennisetum glaucum* (L.) R. Br.] have high leaf ABA and limit transpiration at high vapor pressure deficit.

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This work is a continuation of a previous paper (Kholová et al. 2010a) in which we found that tolerant genotypes had a lower transpiration rate under well watered conditions. We hypothesized that this would also lead plants exposed to progressive water deficit to maintain transpiration up to the level of unstressed plants until lower thresholds of soil moisture (dryer soil). Here we dissect these previously identified physiological traits important for terminal drought tolerance on the deeper level of plant organization. i.e. if the constitutive water conserving mechanisms of tolerant pearl millet genotypes relate to different levels of leaf abscisic acid (ABA), if plant hydraulics might be involved, and whether these could translate into overall differences in transpiration efficiency (TE). All these traits were followed in tolerant/sensitive pearl millet genotypes, including near-isogenic lines introgressed with a terminal drought tolerance QTL as in previous study.

Most genotypic differences were again identified in well-watered conditions, as expected, indicating that the traits considered are constitutive in essence. ABA levels in optimally watered plants were higher in tolerant genotypes, including NILs, than in sensitive genotypes, and ABA did not increase under drought. Tr in the well-watered conditions was lower in tolerant genotypes at all VPD levels. Almost all tolerant genotypes (with exception of one NIL) slowed down their Tr when VPD crossed a breakpoint of 1.4-1.9kPa, whereas sensitive genotypes showed no change in the Tr response across the whole VPD range.

It was concluded, that two water-saving (drought avoidance) mechanisms may operate under well-watered conditions in tolerant pearl millet: i) a low Tr even at low VPD conditions, which may relate to leaf ABA; ii) a sensitivity to higher VPD that further restricts Tr, which suggests the involvement of hydraulic signals. Both traits, thought didn't clearly reflect into TE differences, could, however, contribute to absolute water saving. This confirmation of water conserving drought avoidance mechanisms deserves consideration in the breeding for terminal drought tolerance.

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RESEARCH PAPER

Terminal drought-tolerant pearl millet [*Pennisetum glaucum* (L.) R. Br.] have high leaf ABA and limit transpiration at high vapour pressure deficit

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Abstract

It was previously shown that pearl millet genotypes carrying a terminal drought tolerance quantitative trait locus (QTL) had a lower transpiration rate (Tr; g cm^{-2} d^{-1}) under well-watered conditions than sensitive lines. Here experiments were carried out to test whether this relates to leaf abscisic acid (ABA) and Tr concentration at high vapour pressure deficit (VPD), and whether that leads to transpiration efficiency (TE) differences. These traits were measured in tolerant/sensitive pearl millet genotypes, including near-isogenic lines introgressed with a terminal drought tolerance QTL (NIL-QTLs). Most genotypic differences were found under well-watered conditions. ABA levels under well-watered conditions were higher in tolerant genotypes, including NIL-QTLs, than in sensitive genotypes, and ABA did not increase under water stress. Well-watered Tr was lower in tolerant than in sensitive genotypes at all VPD levels. Except for one line, Tr slowed down in tolerant lines above a breakpoint at 1.40–1.90 kPa, with the slope decreasing >50%, whereas sensitive lines showed no change in that Tr response across the whole VPD range. It is concluded that two water-saving (avoidance) mechanisms may operate under well-watered conditions in tolerant pearl millet: (i) a low Tr even at low VPD conditions, which may relate to leaf ABA; and (ii) a sensitivity to higher VPD that further restricts Tr, which suggests the involvement of hydraulic signals. Both traits, which did not lead to TE differences, could contribute to absolute water saving seen in part due to dry weight increase differences. This water saved would become critical for grain filling and deserves consideration in the breeding of terminal drought-tolerant lines.

Key words: ABA, pearl millet, terminal drought stress, transpiration rate, transpiration efficiency, vapour pressure deficit.

Introduction

Water deficit is one of the major factors limiting global crop production. Much research on drought tolerance has focused on the characterization of plants during water deficit (Farquhar and Richards, 1984; Howell, 2001; Condon *et al.*, 2004; Blum, 2005). In pearl millet, stomata play an important role in minimizing crop water use in pre-anthesis water deficit (Winkel *et al.*, 2001). However, controlling leaf water losses when water is non-limiting for plant development may also be a suitable adaptation strategy. It was recently shown that pearl millet genotypes carrying a terminal drought tolerance quantitative trait locus (QTL) have a lower rate of water loss per unit leaf area under well-watered (WW) conditions [transpiraion rate (Tr), in g cm^{-2} d^{-1}] (Kholová *et al.*, 2008, 2009). This water-saving mechanism operating under non-stressed conditions could leave water available in the soil profile for

Abbreviations: ABA, abscisic acid; FTSW, fraction of transpirable soil water; NIL-QTLs, near-isogenic lines containing quantitative trait loci, TE, transpiration efficiency; Tr, transpiration rate; VPD, vapour pressure deficit.

grain filling and could be beneficial for terminal stress conditions. Yet, how certain pearl millet plants achieve low Tr is still unclear. This water-saving mechanism from a lower leaf conductance could relate to high leaf abscisic acid (ABA) differences or to lower Tr at high vapour pressure deficit (VPD) levels, and these both may be related (Thompson *et al.*, 2007). Lower leaf conductance could then lead to differences in transpiration efficiency (TE, g biomass kg^{-1} water transpired) (Condon *et al.*, 2004).

The ABA content in the leaves could be involved in lowering the Tr differences. Indeed, ABA is known to be part of a complex mechanism of stomata regulation. Large inter/intraspecific variation in ABA levels has been reported (Conti *et al.*, 1994; Mugo, 1999; Chandrasekar *et al.*, 2000; Li and Wang, 2003; Yin *et al.*, 2005; Zhang *et al.*, 2005). In wheat and some woody plants, a higher ABA level was correlated with drought tolerance (Chandrasekar *et al.*, 2000; Li and Wang, 2003; Yin *et al.*, 2005; Zhang *et al.*, 2005), although no such correlation was reported in maize and sunflower (Conti *et al.*, 1994; Mugo, 1999; Cellier *et al.*, 1998, 2000). Therefore, the ABA–tolerance link is, as expected, highly crop and environment specific. Here, leaf ABA contents in pearl millet genotypes differing in terminal drought tolerance and Tr are compared.

The Tr previously found to discriminate drought-tolerant and drought-sensitive pearl millet genotypes (Kholová *et al.*, 2009) 'integrates' the regulation of stomata over a substantial length of time, i.e. 1–2 d. However, it may not pinpoint transient genotypic differences in stomata regulation occurring during the course of the day. As such, the Tr assessment does not indicate whether Tr differences between genotypes are constant during the day or whether transient changes in environmental conditions lead to transient larger Tr differences between genotypes. One such possibility are the changes in VPD. In areas where pearl millet is grown, a high VPD during the hotter hours of the day leads to a closure of stomata even under WW conditions (Black and Squire, 1979; Squire *et al.*, 1979; Henson and Mahalakshmi, 1985). However, no attention was paid to possible genetic variation in this response to VPD. This phenomenon has only recently been reported in soybean (Sinclair *et al.*, 2007) and it was assessed here in pearl millet genotypes that differ in Tr.

Several studies have reported a relationship between an enhanced ABA level and TE in gene-manipulated plants (Ashwart *et al.*, 2005, Thompson *et al.*, 2007). Zhang *et al.* (2005) subjected wheat genotypes originating from 'wet' and 'dry' regions to drought stress and found that plants with 'dry land origin' had higher ABA levels and subsequently better water use efficiency. In addition, modelling data show that a limited Tr at high VPD would indeed contribute to saving water in the soil profile and might increase the TE (Sinclair *et al.*, 2005). So, putative differences in the Tr response to VPD and in leaf ABA could lead to differences in TE.

Two objectives of this study were to investigate leaf ABA content and Tr under different VPDs in pearl millet lines differing in Tr under WW conditions. A third objective was then to assess whether putative differences in leaf ABA and Tr at high VPD could lead to differences in TE. Whether water-saving mechanisms should be looked at from the angle of water productivity and/or absolute plant water use is discussed.

Materials and methods

Plant material

Parental genotypes: Two pairs of inbred pearl millet [*Pennisetum glaucum* (L.) R. Br.] lines, the parents of mapping populations, differing in terminal drought tolerance [PRLT 2/89-33 (tolerant) and H 77/833-2 (sensitive), 863B-P2 (tolerant) and ICMB 841-P3 (sensitive)] were selected for the study. This contrast was based on previous experiments (Yadav *et al.*, 2002; Serraj *et al.*, 2005), where tolerance/sensitivity was assessed on testcross hybrids of these inbred parental lines. These hybrids were developed by crossing the PRLT 2/89-33 and H 77/833-2 inbred parental lines to a male-sterile line tester, seed parent 843A, whereas hybrids of 863B-P2 and ICMB 841-P3 were developed by pollinating them with H 77/833-2 (Stegmeier *et al.*, 1998). Tolerance of these hybrids was based on grain and stover yield maintenance under managed terminal drought stress in several years of field trials, and on panicle harvest index (PNHI), an index that proxies for the success of spikelet fertility and the degree of grain filling (Bidinger *et al.*, 1987). The drought-sensitive parental genotypes (H 77/833-2 and ICMB 841-P3) are parents of F_1 hybrid cultivars that were commercially important in northwestern India. The drought-tolerant parental genotype PRLT 2/89-33 was derived from the ICRISAT Bold Seeded Early Composite, which is an elite breeding population based largely on Iniadi landrace germplasm from West Africa (Andrews and Anand Kumar, 1996), while 863B-P2 was bred by inbreeding within a sample of this landrace (Rai *et al.*, 2008). This Iniadi germplasm is generally known for better grain filling under terminal drought stress conditions.

Near-isogenic lines (NIL-QTLs): Using the two crosses described above, a major QTL for terminal drought tolerance was identified on pearl millet linkage group 2 (Yadav *et al.*, 2002; Bidinger *et al.*, 2007). To develop the QTL introgression lines in the background of sensitive parent H 77/833-2 (recurrent parent), it was crossed to the drought-tolerant donor parent PRLT 2/89-33. The resulting F_1 was backcrossed to recurrent parent H 77/833-2 for four generations. At each backcross, the presence or absence of the terminal drought tolerance QTL was determined using flanking RFLP (restriction fragment length polymorphism) markers on pearl millet linkage group 2. At the end of fourth generation, two steps of selfing and marker-assisted selection using flanking simple sequence repeat (SSR) markers *Xpsmp2059*, *Xpsmp2066*, and *Xpsmp2237* were performed to generate a set of NILs (ICMR 1029, ICMR 1031, ICMR 2041, ICMR 2042, and ICMR 2044) transferred with a drought tolerance QTL (hereafter referred to as NIL-QTLs). Testcross hybrids were produced for each of these lines by using them as pollinator lines on the same male-sterile line 843A. Hybrids involving ICMR 01029, ICMR 01031, and ICMR 02041 were previously found to be superior to the testcross hybrid developed with the sensitive parent H 77/833-2 for their yield maintenance under terminal drought in the field; hybrids of ICMR 02042 had a yield response in the field that was more like the testcross hybrid from the drought-sensitive parent H 77/833-2; hybrids of ICMR 02044 had intermediate yield response (Serraj *et al.*, 2005).

Plant growth and exposure to drought (drydown)

Description of experiments: Leaf ABA, the transpiration response to different VPD conditions, and TE were measured in seven experiments conducted during either the vegetative stage (25 days

after sowing prior to panicle emergence) in Experiments 1, 3, 5, 6, and 7, or the reproductive stage [40 days after sowing (DAS) when panicles were fully emerged] in Experiments 2 and 4 (Table 1). Leaf ABA was analysed in water-stressed (WS) and WW plants in Experiments 1, 2, and 5. TE was assessed in WS and WW plants in Experiments 3, 4, and 6. The transpiration response to VPD was tested in WW plants in Experiments 3, 6, and 7. Parental lines were tested in Experiments 1–4, while parental lines plus NIL-QTLs were tested in Experiments 5–7 (Table 1). Plants were grown in pots with Alfisol mixed with sand and manure (5:3:1) (5/9 kg pot^{-1} for the vegetative/reproductive stage) with one plant per pot in the glasshouse under optimal conditions (day/night temperature 35/25 °C, relative humidity oscillated between 50% and 80% during the day and the resulting VPD ranged between 2.81 kPa and 0.63 kPa).

Protocol for water stress imposition (drydown): Eighteen pots of each genotype were grown under WW conditions until the time of imposing the water treatment (25 or 40 DAS depending on the experiment). Six pots of each genotype were harvested at the time of treatment imposition for biomass assessment (pre-drydown biomass). The remaining 12 pots of each genotype were saturated with water and allowed to drain overnight. The following morning, the pots were bagged with a plastic bag wrapped around the stem and pots were subsequently weighed. Pots were weighed thereafter every day in the morning. Half of the plants were maintained under WW conditions by daily re-watering up to 80% field capacity, by bringing the pot weight to that level [100/200 g (vegetative/reproductive stage) below the saturated weight] every day. The other half of the plants were gradually exposed to WS by partially compensating water loss from transpiration, i.e. plants were allowed to lose no more than 70/100 g each day at the vegetative/reproductive stage (see the experimental design below). Therefore, any transpiration in excess of 70/100 g was added back to the pots, as previously described (Vadez and Sinclair, 2001). The experiment was terminated when, for a given genotype, the transpiration of WS plants was <10% of that of WW plants. The duration of the experiment was ~16–20 d, short enough to ignore the fresh weight increase in the pot weight. Plants were then harvested and leaf area and the dry weights of their parts were measured. After harvest, the fraction of transpirable soil water (FTSW) for each day of the experiment was calculated. The FTSW values that represented the portion of remaining transpirable soil water were used as the indicator of stress, so that experiments could be rigorously compared. FTSW of day $\underline{n}$ was calculated as:

$$(\text{pot weight of day } n - \text{final pot weight})/(\text{initial pot weight} - \text{final pot weight}).$$

The component 'initial pot weight–final pot weight' did not vary between genotypes (all extracted similar quantities of water from the pots). Since all plants transpired >70/100 g water d^{-1} (vegetative/reproductive stage), all the genotypes were at a similar FTSW throughout the experimental period and then exposed to similar stress intensities, at least from the viewpoint of the soil water content. The experimental design for Experiments 1–6 was a completely randomized design with water treatment as the main factor (WW and WS) and genotypes as subfactor in six replications.

Transpiration efficiency estimation

TE was measured in WW and WS plants of Experiments 3, 4, and 6, and was calculated as the production of biomass per amount of water transpired during the drydown as:

$$\text{TE} = (\text{final harvested biomass} - \text{predrydown biomass})/\text{water transpired}$$

Water transpired was the sum of daily transpiration measured in the drydown, assessed by regular weighing of pots and recording of water added. The final harvested biomass was that of WW and WS at the end of the drydown.

ABA assessment

Free ABA was estimated in the last fully expanded leaf of every plant (WW and WS), using two replicated samples per plant in Experiments 1 and 2 and one sample per plant in Experiment 5. The first sampling was at the beginning of the drydown when the soil of WS plants was still wet (FTSW=0.75/0.9 in Experiments 1 and 2) and the second when FTSW had reached 0.25/0.30. In Experiment 5, based on the results from Experiments 1 and 2,

Table 1. Description of the experiments, treatments used, material tested, stage of evaluation, measurement carried out, and date of sowing

Experiment	Treatment	Additional sets	Plant material	Growth stage	Trait measured	Date
1	WW–WS		Parents	Vegetative	ABA	February 2007
2	WW–WS		Parents	Reproductive	ABA	February 2007
3	WW–WS	Pre-drydown set	Parents	Vegetative	TE	
					Tr-VPD	May 2007
4	WW–WS	Pre-drydown set	Parents	Reproductive	TE	May 2007
5	WW–WS		Parents	Vegetative	ABA	July 2007
			ICMR 1029			
			ICMR 1031			
			ICMR 1041			
6	WW–WS	Pre-drydown set	Parents	Vegetative	TE	July 2007
			ICMR 1029		Tr-VPD	
			ICMR 1031			
			ICMR 1041			
7	WW		Parents	Vegetative	Tr-VPD	February 2009
			ICMR 1029			
			ICMR 1031			
			ICMR 2042			
			ICMR 2044			

samples were collected only once when the FTSW of WS plants had reached 0.25 The ethyl acetate fractionation technique of Ryu and Li (1994) was used for sample preparation; 150 mg of deep-frozen tissue, collected as a small strip from the middle portion of the leaf, avoiding the mid-rib, was ground with 2 ml of 80% methanol containing butylated hydroxy toluene (0.001%, w/v). The extract was mechanically shaken for 1 h at 4 °C, briefly centrifuged (13 000 *g*), and passed through a Sep-Pak column (C-18 cartridge). Clear extract was pressure-evaporated, the residue dissolved in water, and ethyl acetate was added. This mixture was partitioned into aqueous and organic phases to separate free ABA from inert ABA conjugates. The ethyl acetate fraction was collected, evaporated, and the residue dissolved in 0.5 ml of sample buffer. ABA was assayed by competitive enzyme-linked immunosorbent assay (cELISA) according to Weiler (1982) with modifications. Polyclonal antibodies were raised against antigen –(±) *cis-trans* ABA (Sigma Aldrich) previously conjugated through its C_1 carboxyl site to a carrier protein bovine serum albumin (BSA). Cross-reaction of polyclonal antibodies with BSA to avoid their non-specific reaction was carried out before its use for ELISA. After coating ELISA plates with antigen, plates were loaded with 1% milk powder to minimize non-specific binding. The antigen coating dilution (300 ng ml^{-1}), primary antibody dilution (1:50 000), and alkaline phosphatase-linked secondary antibody dilution (1:8000) were chosen so that under assay conditions, absorbance A_{405}=1.0 for the blank was obtained after ~1 h incubation with p-NPP substrate (0.1% w/v). Simultaneously with the samples, reference standards (1000 to 3.9 ng ABA ml^{-1}) were loaded in every ELISA plate. Several spiking experiments to detect recovery of known ABA concentrations were conducted to validate this technique (not shown).

Transpiration rate under different VPD regimes

This was assessed on WW plants, either under natural variations in VPD during the course of the day in the glasshouse or outdoors, or by setting VPD conditions at constant light intensities in controlled-environment growth chambers. The design for the transpiration response to VPD in Experiments 3, 6, and 7 was a completely randomized design with one factor only (WW), genotypes as subfactors, and six replications.

Response of Tr to natural conditions: The transpiration of WW plants of Experiment 3 was measured hourly over an entire day in the glasshouse 1 d prior to harvest. At harvest, the leaf area was measured and Tr expressed as water loss per unit of leaf area and time (g H_2O cm^{-2} h^{-1}). A similar assessment was made with WW plants from Experiment 7 using the NILs ICMR 01029, ICMR 01031, ICMR 02042, and ICMR 02044, and parental genotypes PRLT 2/89-33 and H 77/833-2. That experiment was conducted outdoors and the VPD data were recorded hourly.

Response of Tr to increasing VPD in growth chambers: Under constant light and VPD in the growth chamber, the transpiration of WW plants showed little variation during the course of the light period, except for the first 2 h when there was an ~15% increase in the Tr (data not shown). This interval in the light period showing constant transpiration was used to assess the changes in Tr of WW plants caused by a ladder of increasing VPD regimes starting from 0.85 kPa up to 3.43 kPa.

This method was applied to six plants per genotype ('pre-drydown' set), grown under WW conditions and at the same time as Experiments 3 and 6. At 23 DAS, these plants were saturated with water, allowed to drain overnight, bagged around the stem, and transferred to a growth chamber for 1 d of acclimation. The day following acclimation, their transpiration response to VPD was assessed.

To analyse the data, a broken stick analysis was done using the split line regression of Genstat (9.0), which provides a breakpoint value where the slope of the fitted regression changes and values of the different slopes.

Statistical analysis

Analyses of variance (ANOVAs) were done with the statistical program package CoStat version 6.204 (CoHort Software, Monterey, CA, USA). One-way ANOVA was carried out to test for genotypic differences within treatment, and to compare the treatment effect for each genotype. Means were analysed using the Tukey–Kramer test and LSD (at P=0.05).

Results

Leaf ABA content

Across the two experiments, PRLT 2/89-33 (tolerant) generally had significantly higher leaf ABA than H 77/833-2 [Fig. 1a, b (P <0.1 under WS in Experiment 5; Fig. 2)], except under WS conditions during the reproductive stage in Experiment 1 (Fig. 1b). The ABA level of all three NIL-QTLs under WW conditions was significantly higher than in H 77/833-2 and similar to that of PRLT 2/89-33 (Fig. 2). Under WS conditions, the ABA content of drought-stressed plants did not discriminate NIL-QTLs from sensitive H 77/833-2 as well as it did under WW conditions, and ABA content was higher in ICMR 01029 and ICMR 01031 than in H 77/833-2.

For the pair 863B-P2 (tolerant) and ICMB 841-P3 (susceptible), results were less consistent. Under WW conditions, 863B-P2 had significantly higher ABA than ICMB 841-P3 during the vegetative period, but a significantly lower ABA during the reproductive period. Under WS, differences were non-significant.

At both the vegetative and reproductive stages in Experiments 1 and 2, the drought treatment caused no significant increase in the ABA level in any of the genotypes except for ICMB 841-P3 at the vegetative stage (Fig. 1a, b). In Experiment 5, progressive exposure to water stress also did not cause any significant ABA increase in any of these genotypes.

Transpiration rate under different VPD conditions

The transpiration of WW plants in Experiment 3 was measured under VPD conditions that varied between 0.63 kPa and 2.81 kPa. The Tr was higher in sensitive H 77/833-2 than in tolerant genotype PRLT 2/89-33 at all times of measurement except the initial and final measurements. In addition, these differences were largest between 12.15 h and 14.15 h, i.e. the time of the day with the highest VPD values (Fig. 3a). The other pair showed a similar trend, with 863B-P2 generally having a lower Tr than ICMB 841-P3, although differences were not always significant (Fig. 3b).

A similar transpiration response to the natural VPD cycle was tested outdoors (Experiment 7; Fig. 4). The daily pattern of Tr closely followed the daily pattern of VPD. Genotypic differences in Tr did not occur until the VPD was above ~3 kPa. Hence, at 10.00 h, differences were non-significant. Above a VPD of 3 kPa, the increase in Tr was

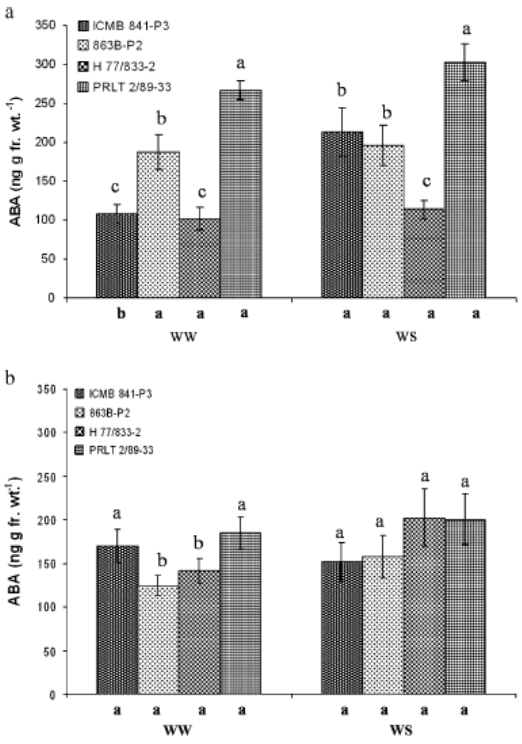

Fig. 1. ABA content of four pearl millet testcross hybrids [H 77/833-2, ICMB 841-P3 (sensitive), PRLT 2/89-33, 863B-P2 (tolerant)] grown in well-watered (WW) and water stress (WS) conditions and sampled at FTSW=0.25 at the vegetative stage (a) and at FTSW=0.30 at the reproductive stage (b). Experiments were conducted in January 2007 (Experiments 1 and 2). Values are the means (±SE) of six replicate plants per treatment and genotype. Genotypic means with the same letters above the bar within a treatment are not significantly different. Letters below the *x*-axis are for the genotypic comparison of means between treatments.

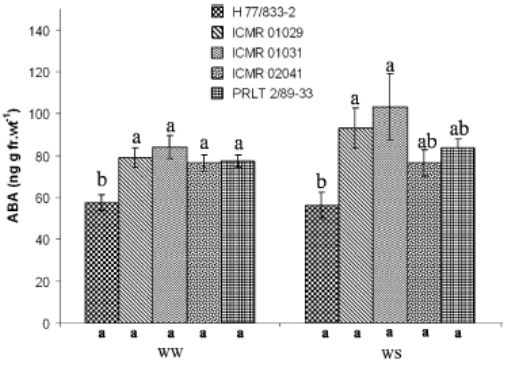

Fig. 2. ABA content of two pearl millet testcross hybrids of parental lines [H 77/833-2 (sensitive), PRLT 2/89-33 (tolerant)] and the testcross hybrids of their NIL-QTLs (ICMR 01029, ICMR 01031, and ICMR 02041) grown in well-watered conditions and under drought and sampled at FTSW=0.25 in the vegetative stage (Experiment 5, July 2007). Values are the means (±SE) of six replicate plants per treatment and genotype. Genotypic means with the same letters above the bar within a treatment are not significantly different. Letters below the *x*-axis are for the genotypic comparison of means between treatments.

lower in PRLT than in H77 and the largest Tr differences were found at the time of highest VPD, between 11.00 h and 15.00 h, when the VPD was >4 kPa. The Tr curves as a function of time of all four NIL-QTLs were in between those of the parental genotypes, i.e. always below that of H 77/833-2 (Tr differences at each time point were significant at P <0.1). In particular, drought-tolerant NIL-QTLs ICMR 01029 and ICMR 01031 were closer to the pattern of PRLT 2/89-33. Drought-sensitive NIL-QTL ICMR 02042 showed a pattern of Tr closer to that of H 77/833-2.

In the growth chamber in Experiment 3, the slope of the transpiration response to VPD was unchanged in H 77/833-2 (Fig. 5a) and ICMB 841-P3 (Fig. 5b) across the whole range of VPD. In contrast, this slope showed a breakpoint at 1.91 kPa in PRLT 2/89-33 and at 1.75 kPa in 863B-P2. For these two tolerant genotypes, the slope significantly decreased past their respective VPD breakpoints, indicating a slowdown in the transpiration response to VPD. Tr was also lower in PRLT 2/89-33 than in H77/833-2 even at VPD <2 kPa.

A similar assessment repeated with WW plants in Experiment 6 again showed the absence of a breakpoint in the transpiration response to VPD for H 77/833-2, whereas the Tr showed a breakdown in the response to VPD at 1.89 kPa in PRLT 2/89-33, and a significantly lower slope past this breakdown than before (Fig. 6). The NILs (ICMR 01029 and ICMR 01031) showed a breakdown in the Tr response similar to PRLT 2/89-33. One NIL showed no breakdown in the transpiration response (ICMR 02041). The reduction in the slope of the transpiration response to VPD above the breakpoint was in the order of 50–65%.

Transpiration efficiency

At the vegetative stage under WW conditions, TE was higher in H 77/833-2 than in PRLT 2/89-33 (Table 2), although that was not confirmed in Experiment 6 (Table 3). Tolerant genotype 863B-P2 had higher TE values than sensitive ICMB 841-P3, although that might have been due to experimental artefact. Surprisingly, the drought treatment did not cause any significant changes in TE (Table 2). At the vegetative stage under WS conditions and at the reproductive stage under both WW and WS conditions, there were no significant TE differences between any of the tolerant and sensitive genotypes.

TE was also examined in the parental genotypes and the NIL-QTLs during the vegetative growth stage (Experiment 6, Table 3). There was no TE change due to water stress and no TE differences between genotypes.

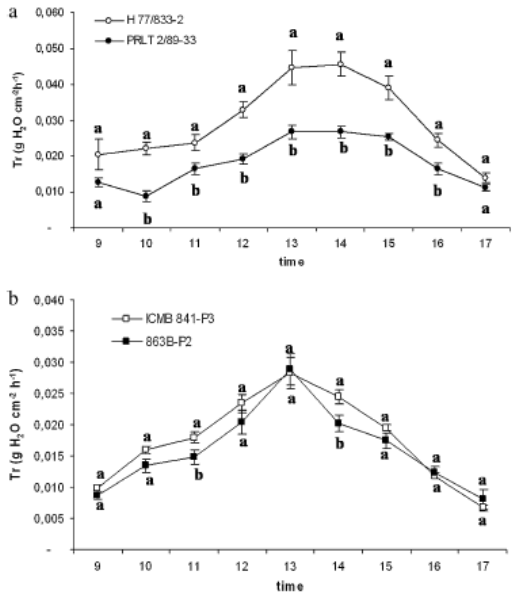

Fig. 3. Transpiration rate (Tr, g cm^{-2} h^{-1}) under well-watered conditions of two pearl millet testcross hybrids pairs: H 77/833-2 and PRLT 2/89-33 (a), and ICMB 841-P3 and 863B-P2 (b) [H 77/833-2, ICMB 841-P3 (sensitive), PRLT 2/89-33, 863B-P2 (tolerant)] over the course of a day. Plants were assessed at the vegetative stage (Experiment 3, May 2007). Each point expresses hourly Tr mean values. Values are the means (±SE) of six plants per treatment and genotype. Genotypic means with the same letters are not significantly different.

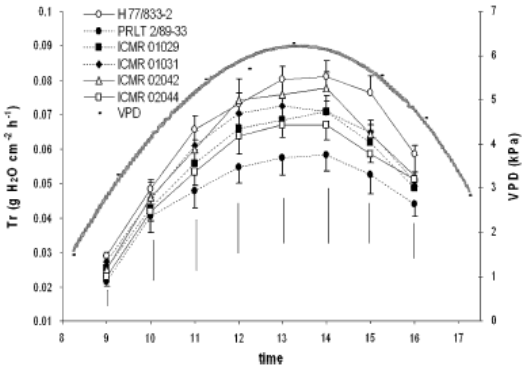

Fig. 4. Transpiration rate (Tr, g cm^{-2} h^{-1}) under well-watered conditions and VPD of pearl millet testcross hybrids of parental lines: H 77/833-2 (drought sensitive) and PRLT 2/89-33 (drought tolerant) and the testcross hybrids of their four NILs (ICMR 01029, ICMR 01031, ICMR 02042, and ICMR 02044) over the course of a day. Plants were assessed at the vegetative stage (Experiment 7, February 2009). Values are the means (± 1/2 SE) of five plants per treatment and genotype. The bar at each measurement time indicates the LSD for genotypic means.

Discussion

Three important results were obtained, and all under WW conditions: (i) the drought-tolerant genotypes, including NIL-QTLs, had higher leaf ABA contents than the drought-sensitive parent H 77/833-2; (ii) tolerant genotypes were responsive to VPD and restricted transpiration at high VPD compared with sensitive genotypes, by slowing down the transpiration response to VPD at high VPD; and (iii) the Tr (g cm^{-2} d^{-1}) were lower in tolerant genotypes even under low VPD conditions. Leaf ABA and the transpiration response to VPD under WW conditions discriminated tolerant and sensitive materials, although to a lesser extent in the 863B-P2–ICMB 841-P3 pair. Overall, TE did not differ between genotypes.

Differences in ABA content

During vegetative growth drought-tolerant genotypes (both the QTL donor parent and the three NIL-QTLs in testcross form) had higher leaf ABA content than the drought-sensitive genotypes under WW conditions. These results suggest a likely constitutive role for ABA in the drought tolerance QTL that plays most of its role when water is still non-limiting. The differences in leaf ABA are not due to a dilution effect at the leaf level since specific leaf area was similar in both pairs of parents (Kholová *et al.*, 2009), and also because genotypes with high ABA had a leaf size similar to or larger than sensitive lines. These data would agree well with the lower Tr in the tolerant genotype, given that ABA is closely involved in the control of stomata aperture (Henson *et al.*, 1983; Morison and Gifford, 1984; Cure and Acock, 1986). Whether the differences in ABA content have a causal effect on Tr and subsequently on yield under drought was not the purpose of the study and would need to be investigated further. The present analysis also showed that genotypic differences in leaf ABA were less marked at the reproductive stage. This might be the consequence of differential sensitivity to ABA between developmental stages (Henson *et al.*, 1983; Winkel *et al.*, 2001). Therefore, overall, the hypothesis is of a role for a constitutive higher production of ABA in tolerant lines to limit leaf water loss under WW conditions, which would save water for the later stage of plant development, hence turning out to be an important aspect of plant adaptation to water-limited conditions, as previously hypothesized (Mortlock and Hammer, 2001; Condon *et al.*, 2002; Serraj *et al.*, 2004; Sinclair *et al.*, 2005; Kholová *et al.*, 2008).

Surprisingly, differences in ABA level between control and stress-treated plants were not found in any of the experiments. Leaf ABA level was even higher in drought-sensitive genotype ICMB 841-P3 during the vegetative growth stage in stress conditions. This is contrary to previous reports in other species where ABA content significantly increased under drought stress conditions (Asch *et al.*, 1995; Chandrasekar *et al.*, 2000; Li and Wang,

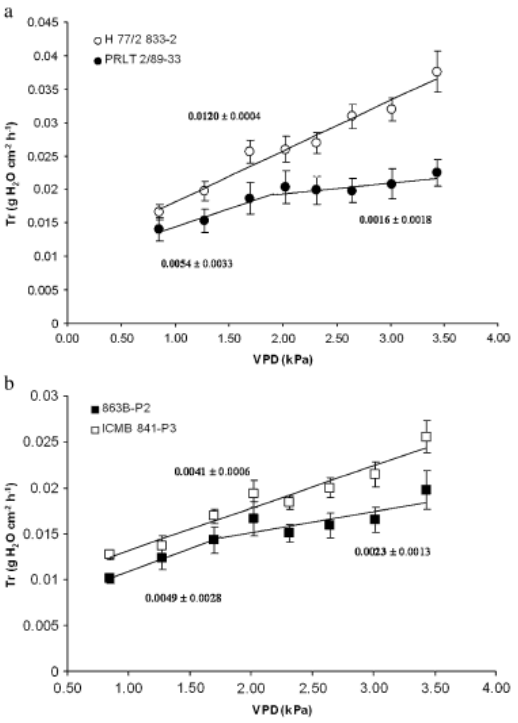

Fig. 5. Transpiration rate (Tr, g cm^{-2} h^{-1}) under well-watered conditions of four pearl millet testcross hybrids [H 77/833-2, ICMB 841-P3 (sensitive), PRLT 2/89-33, 863B-P2 (tolerant)] exposed to increasing VPD regimes. Each point expresses hourly Tr mean values. The experiment used plants at the vegetative stage (Experiment 3, May 2007). Values are the means (±SE) of six plants per treatment and genotype. Numbers above/below the regression curves are the slopes (±SE) of the Tr response. The breakpoint of the slope was 1.91±0.75 kPa for PRLT 2/89-33 (a) and 1.75±0.95 for 863B-P2 (b). The breakpoint of the slope was not significant for H 77/833-2 and ICMB 841-P3.

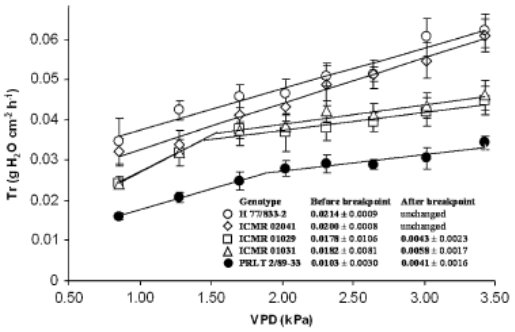

Fig. 6. Transpiration rate (Tr, g cm^{-2} h^{-1}) under well-watered conditions of two pearl millet testcross hybrids of parental lines H 77/833-2 (sensitive), PRLT 2/89-33 (tolerant) and the testcross hybrids of their drought-tolerant NIL-QTLs (ICMR 01029, ICMR 01031, and ICMR 02041) exposed to increasing VPD regimes. Each point expresses hourly Tr mean values of a particular genotype. Plants were tested at the vegetative stage (Experiment 6, July 2007). Values are the means (±SE) of six plants per treatment and genotype. The slope of the Tr response before and after putative breakpoints is indicated on the graph. The breakpoint of the slope was 1.45±0.40 kPa for ICMR 01029, 1.54±0.35 kPa for ICMR 01031, and 1.89±0.41 kPa for PRLT 2/89-33. The breakpoint of the slope was not significant for H 77/833-2 and ICMR 02041.

2003; Yin *et al.*, 2005; Zhang *et al.*, 2005). It could be argued that genotypes may not have been at a similar leaf water potential where WS ABA was assessed, although they were at a similar FTSW level and so experienced the same stress intensity on the basis of soil water content.

Differences in VPD response

Transpiration was sensitive to VPD in drought-tolerant NIL-QTLs and in the drought-tolerant QTL donor parent, suggesting a direct involvement of the QTL in the manifestation of this trait. The lack of breakdown in the response of ICMR 02041 could be explained by a possible recombination in this large QTL region (~30 cM), which would have excluded a putative portion involved in the VPD response of that particular NIL-QTL. This would indicate that only a portion of the QTL may be responsible for this trait. Current efforts to fine-map this QTL should help to generate the material needed to test this hypothesis, i.e. that the QTL region is underlying a cluster of traits contributing to water saving and that are held by different portions of the QTL. The fact that Tr differed between tolerant and sensitive genotypes even at low VPD indicates that the low Tr of tolerant genotypes (Kholová *et al.*, 2010) is not a consequence of the Tr differences only at high VPD. The finding of genotypic differences in the transpiration response to VPD in pearl millet agrees with similar results in soybean (Sinclair *et al.*, 2007), where a 'slow-wilting' genotype of soybean showed a linear increase in transpiration only until ~2 Pa. Above these VPD levels, Tr remained essentially constant, whereas other genotypes maintained a linear increase in transpiration up to VPD values of ~3.5 kPa. The reasons for the rapid change in Tr with a VPD increase are unclear and would probably require rapid control of the stomatal conductivity. Hydraulic signals (Zwieniecky *et al.*, 2001; Sperry *et al.*, 2002; Cochard *et al.*, 2004) are more likely to mediate such a signal than drought signalling cascades (including ABA-dependent and ABA-independent pathway). In fact, previous work (Kholová *et al.*, 2010) showed that Tr could be increased on a short-term basis with defoliation, providing evidence of the likelihood of non-hormone-related signals for the regulation of stomata in pearl millet too. Therefore, these results indicate clearly that in terminal drought-tolerant pearl millet, two distinct water-saving

Table 2. Transpiration efficiency (TE, in g biomass kg^{-1} water transpired) of four pearl millet testcross hybrids [H 77/833-2, ICMB 841-P3 (sensitive), PRLT 2/89-33, 863B-P2 (tolerant)] measured at the vegetative and reproductive stage (Experiments 3 and 4, May 2007), under well-watered (WW) and water stress (WS) conditions, and TE measured across WW and WS treatments

Values are the means (±SE) of six replicate plants per treatment and genotype. Genotypic means followed by the same letter within a treatment are not significantly different. LSD is for treatment differences within each genotype

Genotype	Vegetative stage				Reproductive stage			
	843A×PRLT 2-89/33	843A×H 77/833-2	863B-P2×H 77/833-2	ICMB 841-P3×H 77/833-2	843A×PRLT 2-89/33	843A×H 77/833-2	863-P2×H 77/833-2	ICMB 841-P3×H 77/833-2
TE WW	10.06±0.82 b	12.85±0.96 a	13.80±0.78 a	8.05±0.44 b	9.28±2.77 a	9.24±2.69 a	11.72±2.76 a	8.75±2.74 a
TE WS	13.13±0.81 a	12.87±0.96 a	13.37±0.90 a	10.01±0.81 a	7.41±1.13 a	7.06±1.03 a	9.11±1.04 a	11.30±1.03 a
LSD WW/WS	4.51	4.13	2.84	4.65	5.51	5.01	5.53	4.95
TE WW+WS	11.60±1.15 a	12.86±0.88 a	13.58±0.6 a	9.03±0.53 b	8.35±0.59 a	8.15±0.84 a	10.42±1.38 a	10.03±1.68 a

Table 3. Transpiration efficiency (TE, in g biomass kg^{-1} water transpired) in two parental pearl millet genotypes (H 77/833-2, PRLT 2/89-33) and their three drought-tolerant NIL-QTLs in hybrid form under well-watered (WW) and water stress (WS) conditions, and TE measured across WW and WS treatments, measured at the vegetative stage (Experiment 6, July 2007)

Values are the means (±SE) of six replicate plants per treatment and genotype. Genotypic means followed by same letter within a treatment are not significantly different. LSD is for treatment differences within each genotype.

Genotype	843A×PRLT 2/89-33	843A×H 77/833-2	843A×ICMR 01029	843A×ICMR 01031	843A×ICMR 02041
TE WW	6.19±0.54 a	6.01±0.36 a	7.19±0.37 a	6.92±0.48 a	6.19±0.52 a
TE WS	6.16±0.39 a	6.67±0.21 a	7.31±0.28 a	6.92±0.28 a	6.01±0.25 a
LSD WW/WS	1.16	0.95	1.10	1.45	1.79
TE WW+WS	6.18±0.32 a	6.34±0.24 a	7.24±0.24 a	6.92±0.27 a	6.10±0.28 a

mechanisms operate under WW conditions: (i) a low Tr, which acts across VPD conditions; and (ii) a sensitivity to VPD in tolerant material that further limits the Tr when the VPD is high, >2 kPa. Both these traits would contribute to saving water in the soil profile, even if soil water is not limiting. This water would then be available and crucial for grain filling, as previously indicated (Turner, 2004; Manschadi *et al.*, 2006; Ratnakumar *et al.*, 2009). Thus, both traits are important to consider for the breeding of pearl millet lines with terminal drought tolerance. The data indicate the possibility to phenotype these traits using relatively simple Tr measurements at low and high VPD under natural conditions.

TE analysis

Large differences in TE were not found in different growth conditions and different genotypes. Part of the reason was the higher variability observed in TE values during the reproductive stage and under drought. This is contrary to what was suggested earlier (Sinclair *et al.*, 2005), namely that Tr restriction at high VPD would normally increase TE. A possibility for the lack of differences is that the method used to assess TE was not sensitive enough to pinpoint TE differences arising from differences in sensitivity to VPD. Furthermore, the plants used for TE assessment were grown in the glasshouse at relatively low mean VPD and only rarely faced VPD conditions >1.5–2.0 kPa that would trigger the VPD response of transpiration and the expected transient increase in TE. Another possibility for the lack of TE differences could have been the differences in biomass partitioning to roots, since roots were not included in the TE measurement. Therefore, more work is needed to assess whether gravimetric TE differences could be found in conditions where plants are exposed to higher VPD, or simply whether intrinsic TE increases upon VPD increase in VPD-sensitive materials. In fact, the dry weight increase during the experimental period was lower in some of the tolerant material (data not shown), which suggests that the water-saving mechanisms could simply be reflected in differences in total water use. This lack of TE differences could also be linked to how individual stomata respond to VPD. In work reporting transpiration sensitivity to VPD (Sinclair *et al.*, 2005; Devi *et al.*, 2009; this work) mean stomatal conductance is partially reduced. This is assumed to be a consequence of a reduction of the aperture of all stomata. If that was the case, the intrinsic TE should indeed increase. However, it could be speculated that a decrease in stomatal conductance could be the mean of certain stomata having their conductance unchanged and other stomata that would fully close. Such a situation would normally not modify the intrinsic TE while it would decrease the Tr. Although that explanation may appear speculative, it would fit with reports that stomata are organized in patches (Pospíšilová and Šantrůček, 1994; Mott and Buckley, 2000)

and may not all respond in the same way to external stimuli. In any case, the absence of TE differences stresses that the advantage of the VPD sensitivity trait, along with the lower Tr trait, probably related to ABA, needs to be considered in terms of total water use (lower) rather that in terms of water productivity.

Conclusion

It was found that the terminal drought tolerance QTL on pearl millet linkage group 2, previously found to correlate to a lower Tr, also correlated to higher ABA levels in the leaves of WW plants, and to the sensitivity of transpiration to a high VPD level under WW conditions. The low Trs previously found were a consequence not only of genotypic differences in the sensitivity of Tr to high VPD but of two separate water-saving mechanism, i.e. a low Tr at low VPD, which might be related to differences in the leaf ABA content, and a sensitivity to VPD leading to restrained Tr at high VPD. The major trait differences were all found under WW conditions, pointing to constitutive mechanisms underlying the QTL. The rapid response of the Tr to VPD points to a possible role for plant hydraulics in mediating such a rapid response. These traits would contribute to water saving in the soil profile when water is non-limiting. This 'extra' water, available for the later stage of the crop, would become critical to guarantee water supply to the plants at the time of grain filling and therefore for grain yield under terminal drought.

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Conference papers & poster presentations

2.5 Mechanisms underlying drought tolerance in pearl millet (*Pennisetum americanum* L.)

Kholová J, Vadez V, Hash CT (2008). *5th International Crop Science Congress, March 13-18, 2008.* Jeju, South Korea, *Book of abstracts, pp 188.*

In this piece we enclosed the initial effort to characterize a broad spectrum of traits which were commonly referred as having possible link to drought tolerance in other crops. Physiological traits (transpiration efficiency (TE), transpiration response to increased vapor pressure deficit, threshold in volumetric soil moisture where transpiration begins to decline (FTSW threshold), transpiration rate (Tr), stomatal density (SD)), and biochemical traits (chlorophyll (Chl), carotenoids (Car), abscisic acid (ABA), proline (Pro), isozyme analysis of antioxidative enzymes, changes in 2D proteins profiles), were measured in two pairs of pearl millet parental genotypes contrasting in terminal drought tolerance and several QTL-introgression lines in the background of one sensitive parent. As was published further, this work provided the first evidence that the maintenance of low Tr combined with higher levels of ABA and further restriction of Tr in high VPD in optimal water regime could contribute to terminal drought tolerance. Additionally, other investigated traits (stomatal density, photosynthetic pigments content, most of anti-oxidative iso-enzymatic activities) could be hardly related to the differences in drought tolerance between genotypes. Though, there was certain evidence that lower level of proline of tolerant genotypes in certain stage of plant development might contribute to the low Tr maintenance in well watered conditions. Under drought, the only trait diversifying tolerant/sensitive genotypes appeared to be APX5 isoenzyme. The differences in the protein spectrum between tolerant/sensitive genotypes in well-watered and drought conditions were also documented. This latter work is the object of a manuscript that is currently under revision.

Mechanisms underlying drought tolerance in pearl millet (*Pennisetum americanum* L.)

Jana Kholova, V.Vadez, C. T. Hash

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, India.

Introduction

- Pearl millet, an important staple crop for the poor, is well adapted to dry areas. However, there is a need to improve its yield resilience under drought.
- A major quantitative trait loci (QTL) for terminal drought tolerance had been identified, explaining over 30% of the phenotypic variation in grain yield and grain filling. However, there is no clear understanding of the physiological mechanisms underlying the QTL.
- Near-isogenic lines containing the QTL (NIL-QTL) in the background of the recurrent parent H77/833-2 have been developed after 4 rounds of backcrossing.

Objectives:

- It is hypothesized that a better grain filling in pearl millet genotypes holding the QTL could be due to a more controlled use of the available water. Therefore, we compared several traits involved in water use across genotypes contrasting for terminal drought tolerance, including NIL-QTL.

Materials and Methods

- Two pearl millet parental genotypes differing in terminal drought tolerance and originated in different climatic conditions (PRLT2/89-33 - tolerant; H77/833-2 – sensitive) and their three NIL-QTLs (ICMR1029;1031;2041) were grown in well-watered conditions in pots containing 5/9kg soil up to 30/40 days after sowing (DAS, pre-/post-flowering stage of development) in glasshouse conditions with 30/25°C day/night temperature. Growth chambers with set vapor pressure deficit (VPD) changes were used to measure transpiration response.
- Measured traits: - transpiration was measured as water loss after pots were bagged around the plant stem to avoid soil evaporation
  - transpiration rate (Tr) was calculated as: $\frac{\text{water loss (per day or hour)}}{\text{leaf area (cm}^2\text{)}}$
  - stomata were counted on 10mm^2 abaxial leaf surface print using light microscope
  - ABA was estimated by competitive enzyme-linked immuno-sorbent assay (ELISA)
  - proline was estimated spectrophotometrically (Bates *et al.* 1973)

Results

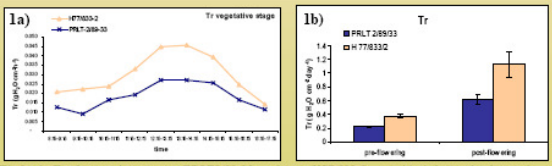

Fig 1a, b Tr of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) during the day circle 30 DAS (a) and final Tr average of 3 day's transpiration in pre-/post-flowering stage (b).

- *Tolerant parent PRLT2/89-33 showed significantly lower Tr than sensitive one (H77/833-2) throughout the course of the day (a) or across phenological stages (b).*

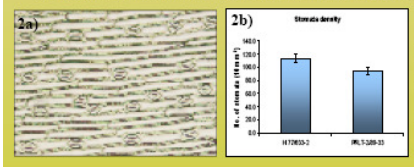

Fig 2a, b Leaf abaxial surface print of first fully developed leaf (a) stomatal density (b) of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive).

- *Stomata density in tolerant genotype PRLT2/89-33 was lower, but was not significantly different due to high variance in measurements.*

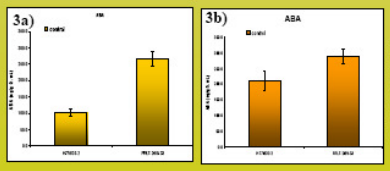

Fig 3a, b ABA content in first fully developed leaf of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) 30 DAS; pre-flowering (a) and 40 DAS; post-flowering stage (b).

- *ABA content in tolerant genotype PRLT2/89-33 was significantly higher than in sensitive genotype H77/833-2 in both pre- and post-flowering stage of development.*
- *The level of significance decreased in post-flowering stage.*

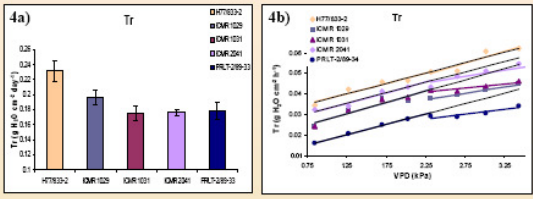

Fig 4a, b Tr average of 3 day's transpiration (a) and Tr response in changing VPD regimes (b) in parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) and their NIL-QTLs (ICMR1029;1031;2041) in pre-flowering stage.

- *Analysis including NIL-QTLs showed that Tr of tolerant parent PRLT2/89-33 did not differ from all the NIL-QTLs, but their Tr values were significantly lower than in sensitive parent (H77/833-2) in well-watered conditions.*
- *Response of Tr in NIL-QTLs to changing VPD followed the trend of tolerant parent – didn't linearly increase transpiration following VPD rising above 2kPa.*

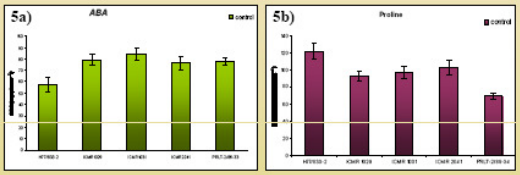

Fig 5a, b ABA content (a) and proline content (b) in the first fully developed leaf of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) and their NIL-QTLs (ICMR1029;1031;2041) in pre-flowering stage.

- *ABA contents in NIL-QTLs did not significantly differ from tolerant parent PRLT2/89-33, but was higher than ABA level in sensitive parent (H77/833-2).*
- *Proline contents of 2 NIL-QTLs (ICMR 1029;1031) were closer to tolerant parent's (PRLT2/89-33) values, but all NIL-QTLs proline contents were lower than in sensitive parent.*

Conclusions

- There were traits (Tr, VPD response, ABA, proline) that differed between pearl millet genotypes contrasting in drought tolerance, furthermore these traits also occurred in NIL-QTLs in a similar manner as in drought tolerant parental genotype.
- High ABA and low proline level (and probably even differences in stomatal density) appears to be involved in mechanisms resulting in lower Tr and finally in water saving of drought tolerant plants even under well-watered conditions.
- VPD response of drought tolerant genotypes seems to be an apparent mechanism participating in utilization of water in conserving manner.
- The major QTL introgressed in hereby examined NILs is probably affecting Tr, VPD response, ABA and proline level and could be used for further screenings in pearl millet breeding for drought tolerance.

References

Bates, L. S., Waldran, R. P. and Teare, I. D. 1973. Plant Soil 39: 205-208.

Current status of work:

Large screening of 108 pearl millet 4th generation NILs progenies derived from the cross between PRLT2/89-33 and H77/833-2 for Tr, ABA and proline for QTL mapping of these traits.

Further detailed analysis of physiological and biochemical traits in these parental genotypes along with selected NIL-QTLs.

2.6 Proline – any prospective for pearl millet (*Pennisetum americanum* L.) drought tolerance improvement?

Kholová J, Vadez V, Hash CT (2008). *Golden Jubilee Conference on challenges and emerging strategies for improving plant productivity Nov. 12-14, 2008,* New Delhi, India, *Book of abstracts, pp 61.*

The work presented was designed to address the persisting doubts about the importance of osmolytes (in this case proline) for pearl millet drought tolerance, since from previous work there was certain evidence that the level of proline may be linked to drought tolerance. In this work, drought tolerant/sensitive parental genotypes along with near isogenic lines (NILs) containing a drought tolerance QTL were used. The level of proline along with plants transpiration and the fraction of transpirable soil water (FTSW) were estimated upon progressive exposure to water deficit at different stages of dry down process. The major finding was that there was no significant increment of proline level due to drought in all genotypes until the FTSW dropped below 20%. By contrast, significant drought induced decline of transpiration occurred at around 35% FTSW in all genotypes. Nevertheless, faster proline accumulation was observed in tolerant genotype compared to sensitive one where most of NILs followed the trend of tolerant parental genotype. Because proline increased only during the last stage of drought stress, and because NILs had almost similar response to the QTL donor parent, it is concluded that proline differences may be influenced by the presence of the QTL but have more likely no direct relation to the yield superiority of tolerant genotypes.

Proline – Any prospective for pearl millet (*Pennisetum americanum* L.) drought tolerance improvement?

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Introduction

Pearl millet, an important staple crop for the poor, is well adapted to dry areas. However, there is a need to improve its yield resilience under drought.

Osmolytes are heavily discussed as possible way for crops yield improvement under water deficit, but their role is still not clear.

A major quantitative trait loci (QTL) for terminal drought tolerance had been identified, explaining over 30% of the phenotypic variation in grain yield and grain filling.

Near-isogenic lines containing the QTL (NIL-QTL) in the background of the recurrent parent H77 have been developed after 4 rounds of backcrossing.

Objectives:

It is hypothesized that better crops yield may depend upon the ability to accumulate osmolytes (like proline) which are supposed to contribute to growth maintenance and yield under drought; contrarily recent studies show the effect of osmolytes accumulation in shoots may cause faster soil water depletion and so more rapid progress of drought to such crops. To test these hypotheses we compared proline levels during several stages of controlled dry down process in genotypes contrasting for terminal drought tolerance, including NIL-QTL differing in drought tolerance as well.

Materials and Methods

• Two pearl millet parental genotypes differing in drought tolerance, based on yield assesment in the field (PRLT – tolerant; H77 – sensitive) along with four NILs (ICMR1029;1031;2042; 2044) were analyzed. ICMR1029; 1031 were found superior to ICMR 2042; 2044.

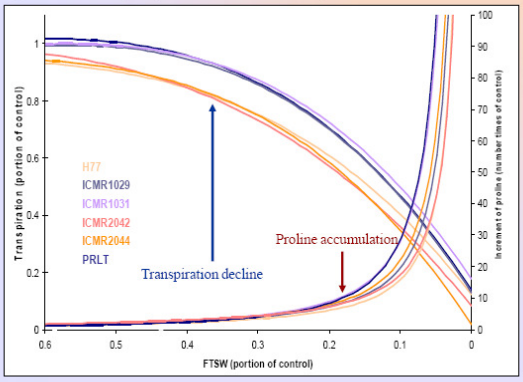
•Plants were grown 25 days (pre-flowering developmental stage) in pot cultures in glasshouse conditions with 30/25ºC day/night temperature in pots filled with 8 kg alfisol.

•Pots were bagged with foil to avoid soil transpiration. Drought was imposed gradually by setting a maximum daily water loss of 100g/day to all plants. Sampling was done 6 times during all the phases of dry down process.

• Transpiration was estimated gravimetrically, fraction of transpirable soil water (FTSW) was calculated as a fraction of total water remaining in soil during the dry down process, proline was estimated spectrophotometricaly (Bates et al. 1973) in the first fully developed leaves.

Results

Figure 1: Succession in transpiration decline and proportional proline accumulation during controlled dry down process in drought tolerant (PRLT) and sensitive (H77) parental genotypes along with their NIL-QTLs (ICMR1029;1031;2042; 2044).



❖ **Drought induced transpiration restriction occurred around 35% (0.35 on x-axis) of FTSW wherever proline started to accumulate only in the latest phase of drought when FTSW dropped below 20% (0.2 on x-axis).**

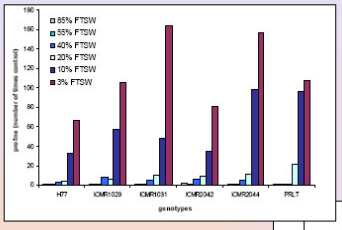
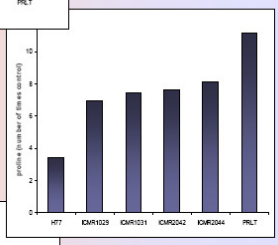


Figure2: Proportional increment of proline in drought tolerant (PRLT) and sensitive (H77) parental genotypes along with their NIL-QTLs (ICMR1029; 1031; 2042; 2044) in different phases of the dry down process.

Figure 3: Average of proportional proline increment in drought tolerant (PRLT) and sensitive (H77) parental genotypes along with their NIL-QTLs (ICMR1029; 1031; 2042; 2044) between 35-15% FTSW.



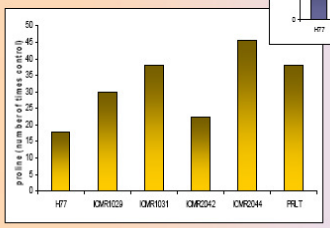


Figure 4: Average of proportional proline increment in drought tolerant (PRLT) and sensitive (H77) parental genotypes along with their NIL-QTLs (ICMR1029; 1031; 2042; 2044) throughout full dry down process.

❖ **Investigated genotypes showed diversity in dynamics of proline accumulation as well as in total proportional proline accumulation; Sensitive genotype (H77) accumulated less proline and less rapidly than tolerant genotype (PRLT). NIL-QTLs (ICMR1029; 1031; 2044) mostly shared the pattern of proline accumulation with tolerant parent. ICMR2042 had mixed character of drought response in terms of proline accumulation- accumulated proline faster during initial proline shoot up, but total proline increment was comparable with H77.**

Conclusions

• Proline accumulated in the latest stage of drought only after plants' transpiration declined due to drought.

•Total proportional increment of proline and rapidity in proline accumulation under drought discriminated between parents and NILs; ICMR1029,1031,2044 showed more similarities with PRLT whereas ICMR2042 showed a response comparable to H77.

Differences found in proline accumulation may be due to presence of QTL but has probably no direct impact on yield parameters of investigated genotypes

References

Bates L. S., Waldran R. P and Teare I.D. 1973. Rapid determination of free proline for water stress studies. Plant Soil 39:205-208

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2.7 Pearl millet genotypes differing for a terminal drought tolerance QTL contrast for traits related to the control of leaf water loss

Vadez V, Kholová J, Kakkera A, Hash CT, Yadav R, Kočová M. Interdrought III, October 11-16, 2009, Shanghai, China. Book of abstracts 5.20

This work sums up the till-date progress in understanding the mechanism of drought tolerance in pearl millet as described above but is extended with the initial efforts to map previously identified key traits in the RIL population. In addition, it report on some data collected in lysimeters (long and large tubes mimicking a real soil profile and where plants can be grown up to maturity). For that, transpiration rate (Tr) was examined in 106 recombinant inbred lines (RILs) derived from the cross between PRLT-2/89-33 and H77/833-2. Parental lines have shown a good segregation with both parents at each end of the distribution. Furthermore, the patterns of water extraction were assesed in lysimeters under terminal drought conditions. Overall the total water extracted from the tubes hardly differed between tolerant and sensitive NIL lines, indicating that the terminal drought tolerance QTL have very likely no bearing on the rooting characteristics of genotypes. By contrast, tolerant and sensitive NILs differed in their kinetics of water extraction. Tolerant NILs and tolerant parents sustained substantial water uptake in the late stages of terminal stress as opposed to sensitive NILs and sensitive parent, which took up more water before and during anthesis. Data suggest that indeed, tolerant genotypes appears to maintain water extraction for longer periods of time under terminal dcourgh stress as suggested in the previous work. This work is a first eloquent confirmation of previous work presented here (Kholová et al. 2010a, b) that the lower leaf conductance and other traits related to plant water use differences in the tolerance materials contribute indeed to a pattern of water use, measured under „real“ conditions, that leaves water available during the grain filling period.

Control of leaf water losses and terminal drought tolerance in pearl millet

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[2] Institute of Biological, Environmental and Rural Sciences, Gogerddan, Aberystwyth University, SY23 3EB, United Kingdom,
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[4] Charles University in Prague, Dept. of Genetics and Microbiology, Viničná 5, Prague 2, 128 43, The Czech Republic

Introduction

A major QTL for terminal drought tolerance (yield) has been identified but the physiological mechanisms underlying the QTL remain unclear

Near-isogenic lines containing the QTL (NIL-QTL) in the background of the recurrent parent H77/833-2 have been developed after 4 rounds of backcrossing.

Here we test whether terminal drought tolerant lines have: (i) a better control of water loss even before stress appears; (ii) differences in kinetics/amounts of water extraction.

Material and Methods

Plant material

- Two pearl millet parental genotypes differing in terminal drought tolerance (PRLT2/89-33 - tolerant; H77/833-2 – sensitive), three NIL-QTLs (ICMR1029;1031;2041), and testcross hybrids of 106 RILs (F6) from the cross between parental lines were used.

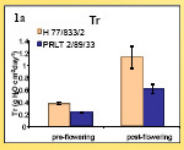
Assessment of leaf water loss

- Plants were grown in well-watered conditions in pots containing 5/9kg soil up to 30/40 days after sowing (DAS, pre-/ post-flowering stage) in glasshouse conditions with 30/25°C day/night temperature. 106 RILs were grown outdoors in pots (5Kg) up to 30 DAS to characterize segregation pattern of transpiration rate (Tr g d^{-1} cm^{-2}). Growth chambers were used to measure the Tr response to step increases in vapor pressure deficit (VPD).
- Transpiration was measured gravimetrically after pots were bagged around the plant stem to avoid soil evaporation
- Tr was calculated as g water loss cm^{-2} per unit of time (d or h)
- ABA was estimated by competitive enzyme-linked immuno-sorbent assay (ELISA)

Assessment of water extraction

- Plants were grown in 2.0 m long and 25 cm diameter PVC tubes, filled with 140 kg of sieved and fertilized (DAP and muriated potash at 200ppm, 1% manure) Alfisol, at a bulk density of 1.4 (Vadez et al, 2008). These tubes mimicked closely the field conditions for the soil volume available and aerial plant spacing. Three seeds were planted in each tube, thinned to one plant/tube at 2 weeks after sowing.
- Weighing was done every week from flag leaf stage (similar across lines) until maturity. A 2 cm layer of plastic bead controlled soil evaporation by 90%.
- Plants were grown under well-watered (WW) conditions until flag leaf. Last irrigation (2 L/plant equivalent to 50 mm in field conditions) was applied to water stress (WS) plants at flowering stage.

Results – Control of leaf water loss



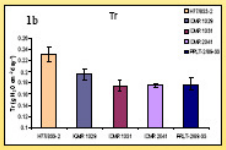


Fig 1a, b Tr of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) in pre-/post-flowering stage (1a) and Tr of parentals and NIL-QTL lines at pre-flowering stage (1b), all under WW

- Tolerant PRLT2/89-33 had lower Tr than sensitive H77/833-2 across stages (1a)
- NILs followed trend of tolerant parent (1b) in well-watered conditions

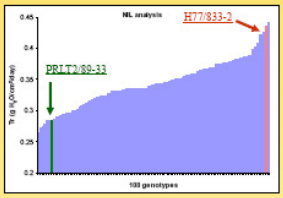


Fig 2 Tr (g water cm^{-2} d^{-1}) in RILs population and parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) at 30 DAS, under well-watered conditions

- About two-fold variation – QTL mapping in progress
- PRLT2/89-33 and H77/833-2 are at the tail of the distribution

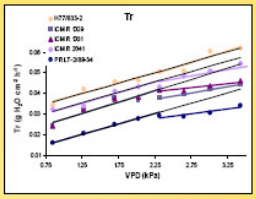
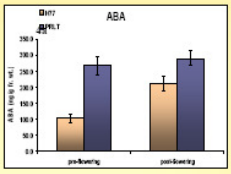


Fig 3 Tr response to changing VPD regimes in parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) and NIL-QTLs (ICMR1029;1031;2041) in pre-flowering stage, under well-watered conditions

- Response of Tr to changing VPD is linear in all genotypes up to 2.0 kPa.
- Above 2.0 kPa, Tr follows a similar slope in H77/833-2, whereas that slope is decreased in tolerant parent and NIL-QTLs.



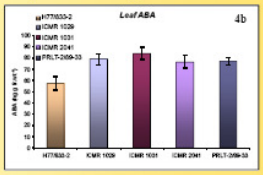
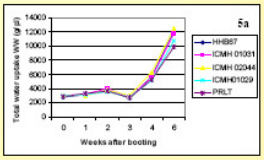


Fig 4 a, b ABA content in the first fully developed leaf of parental genotypes (PRLT2/89-33 – tolerant; H77/833-2 – sensitive) in both pre- and post-flowering stages (1a) and NIL-QTLs (ICMR1029;1031;2041) in pre-flowering stage (1b), under well-watered conditions

- ABA content was higher in tolerant PRLT2/89-33 than in sensitive genotype H77/833-2 in both pre- and post-flowering stage
- NIL-QTLs lines exhibited similar values to tolerant parent.

Results – Water extraction



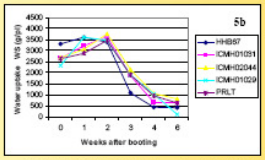


Fig 5 a, b Water uptake profile in H77/833-2 (=HHB67, sensitive), PRLT2/89-33 (tolerant), and NIL-QTLs under WW (5a) and WS conditions (5b)

- No major genotypic differences in the profile except one week before maturity in WW plants
- Water uptake of HHB67 higher around flowering (Wk 0-2), and lower during grain filling (Wk 3-5) than in tolerant PRLT2/89-33 and NILs
- Total water uptake under WS not different between lines (12.2-12.7 kg) except 13.5 kg in ICMR 02044

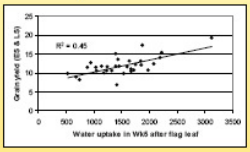


Fig 6 Relationship between water extraction at week 5 after flag leaf and the grain yield across parentals and NIL-QTLs.

Higher grain yield under terminal drought clear related to higher water uptake during grain filling

Conclusion

- Lower transpiration rate when water is available likely helps tolerant lines to keep soil water and make it available for grain filling
- Further restriction of Tr occurred at high VPD in tolerant lines
- There might be a link between a low Tr and a high leaf ABA content but the causal relations are still unclear
- Total water uptake under stress showed little variation – Rather, the pattern of water uptake varied in a way that tolerant plant used less water soon after stress imposition, and more during the grain filling period

2.8 Genotypes contrasting for terminal drought tolerance also contrast for the developmental pattern of water use in varying environmental conditions.

Kholová J, Zindy P, Hash CT, Kočová M, Vadez V. Australian Summer Grain Conference, 21-24. July, Gold Coast, Australia. 2010, Book of abstracts 7 (peer-reviewed and accepted conference publication).

In previous work we focused on the transpiration rate as one factor responsible for limiting the overall plant transpiration. In addition to that, traits related to the leaf canopy development are also critical factors with regards to plant water use. Indeed, a more vigorous genotype would also use more water initially than a genotype having low vigor. In addition to that, how leaf development gets affected by environmental cues such as VPD is also very important to investigate. Similar work indicate, indeed, that the leaf expansion rate of maize genotypes varies with VPD.
This conference paper investigates the variability of water saving mechanisms in relation to environmental conditions in which the plant canopy development takes place. Two genotypes contrasting for terminal drought tolerance PRLT-2/89-33 and H77/833-2 and differing in the water use dynamics were exposed to glasshouse conditions (VPD between 3.6-0.4kPa) and growth chambers set at vapor pressure deficit (VPD) of 1.13 and 2.55kPa. In all conditions listed, we measured leaf expansion, and evaluated how differences in leaf expansion affect the overall water use under well-watered conditions under high and low VPD and relate to transpiration rate (Tr) and transpiration efficiency (TE).
We found, that tolerant genotype exhibited lower Tr compared to sensitive in all growing conditions, however lower Tr was linked to higher TE only in VPD 1.13kPa. Tolerant genotype also produced higher LA than sensitive in VPD 1.13kPa and in glasshouse conditions. Also, leaves appeared with delay in tolerant genotype, but its leaves grew longer and more rapidly in VPD 1.13kPa and in glasshouse conditions. In VPD 2.55kPa the tolerant genotype grew shorter leaves and utilized less water compared to H77/833-2 from around 14 DAS (when leaf No.6 was expanding) and this pattern of water use was similar in glasshouse conditions. Under lower VPD there was no difference in the amount of water utilized between both genotypes during observed developmental stages (up to 21 DAS, when leaf No. 9 just emerged). The pattern of water use of H77/833-2 was quite similar in different VPD conditions. We conclude that leaf area development, in relation with water use efficiency and leaf conductance aspects, lead to pattern of water use of genotypes that are highly environment-specific. These differences measured under well-watered conditions could have important consequences on water available for grain filling under terminal water stress. Water saving mechanisms need to be seen both from the angle of an increased water productivity when development takes place at low VPD, and from a lower water use linked to lesser leaf area development but no water productivity advantage when plant development takes place under high VPD.

Genotypes contrasting for terminal drought tolerance also contrast for the developmental pattern of water use in varying environmental conditions

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Abstract:
Current scientific efforts have sharpened the discussion about various water use strategies and their importance for crops drought tolerance. It was previously found that tolerant pearl millet (*Pennisetum glaucum* (L.) R. Br.) genotypes had lower vegetative stage transpiration rate (Tr, g cm^{-2} d^{-1}) in well-watered conditions, which would confer a yield advantage under terminal drought. A set of experiments in glasshouse conditions (VPD between 3.6-0.4kPa) and growth chambers set at vapor pressure deficit (VPD) of 1.13 and 2.55kPa were developed to test how leaf area development affect the overall water use under well-watered conditions under high and low VPD and relate to transpiration rate (Tr) and transpiration efficiency (TE). Tolerant genotype (PRLT 2/89-33) exhibited lower Tr compared to sensitive (H77/833-2) in all growing conditions, however lower Tr was linked to higher TE only in VPD 1.13kPa. Tolerant genotype (PRLT 2/89-33) produced higher LA than sensitive H77/833-2 in VPD 1.13kPa and in glasshouse conditions. Also, leaves appeared with delay in tolerant genotype, but its leaves grew longer and more rapidly in VPD 1.13kPa and in glasshouse conditions. In VPD 2.55kPa the tolerant genotype grew shorter leaves and utilized less water compared to H77/833-2 from around 14 DAS (when leaf No.6 was expanding) and this pattern of water use was similar in glasshouse conditions. Under lower VPD there was no difference in the amount of water utilized between both genotypes during observed developmental stages (up to 21 DAS, when leaf No. 9 just emerged). The pattern of water use of H77/833-2 was quite similar in different VPD conditions. We conclude that leaf area development, in relation with water use efficiency and leaf conductance aspects, lead to pattern of water use of genotypes that are highly environment-specific. These differences measured under well-watered conditions could have important consequences on water available for grain filling under terminal water stress. Water saving mechanisms need to be seen both from the angle of an increased water productivity when development takes place at low VPD, and from a lower water use linked to lesser leaf area development but no water productivity advantage when plant development takes place under high VPD.

Introduction:
Efforts to identify the different component of the adaptation of crops to water limitation have often used the simple concept where Yield = T x TE x HI (T-amount of water transpired, TE – transpiration efficiency, HI – harvesting index). According to this formula yield basically depends on: (i) the amount of water that

plant can make available for transpiration (T), (ii) efficiency of transpired water in terms of carbon fixation through photosynthesis (iii) the conversion of biomass into grain (HI). However, this formula overlooks possible interactions effects between the terms of the equation. In particular, it overlooks the fact that there may be stages where water utilization (T) might be critical for some other component of the equation (e.g. HI). Therefore, it appears clearer that, at least for certain crops and conditions, the timing of water utilization throughout plants development might be a component of drought adaptation even more important to consider than the components of the Passioura's equation (Sinclair *et al*. 2005, Blum 2009, Kholová *et al*. 2010a, b). For instance pearl millet genotypes tolerant to terminal stress were able to restrict transpiration rate (TR) before stress conditions occurred and in so doing could save water in the soil profile that would be available for grain filling (Kholová *et al.* 2010a, b).

In these previous studies, the focus was put on understanding the control of leaf water losses under well-watered conditions from the angle of the regulation of stomata opening. Tolerant genotypes were also shown to restrict TR to greater extend when VPD crossed 2kPa. In addition to stomatal conductance aspect, control of leaf water loss depends on the extent of leaf area development. Similar experiments in maize (Reymond *et al*. 2003) shown that leaf area development is influenced by the vapor pressure deficit (VPD) and that there was a genotypic variation in this VPD leaf development interaction. As such, genotypes having leaf area development sensitive to VPD would restrict their water use. However, part of that effect could be counterbalanced by the fact that transpiration efficiency is lower at high VPD, TE being in an inverse relationship to VPD (Bierhuizen and Slatyer 1965, Tanner and Sinclair 2003). So, looking at how plant leaf area development and transpiration efficiency combine to determine plant water use and how this varies under low and high VPD conditions is an important complement to previous work on stomatal conductance aspects (Kholová *et al*. 2010a, b).

Therefore, the aim of this study was to compare the leaf area development characteristics of genotypes known to contrast in the TR restriction in VPD regimes above 2kPa, to assess this putative variation under different VPD regimes, to test transpiration efficiency differences, and assess how these presumed differences could result into different plant water use.

Materials and Methods:

Genetic material: Two pearl millet (*Pennisetum glaucum* (L.) R. Br.) genotypes contrasting in tolerance under drought stress; (PRLT 2/89-33 (tolerant) and H77/833-2 (sensitive)) were selected for the study based on our previous experiments (Serraj *et al.* 2005; Yadav *et al.* 2004), where tolerance/sensitivity was assessed on test-cross hybrids of these inbred parental lines, developed by crossing the inbred parental lines to the most common male sterile line tester 843A (Stegmeier *et al.* 1998). Tolerance of these hybrids was based on yield under terminal drought stress in several years of field trials, and on the panicle harvest index (PNHI), an index that proxies for the success of spikelet fertility and the degree of grain filling (Bidinger *et al.* 1987). Tolerant genotype PRLT 2/89-33 derive from the ICRISAT Bold Seeded Early Composite, which is an elite breeding population based on Iniadi landrace germplasm from West Africa. PRLT 2/89-33 was shown to tightly restrict water loss especially in VPD crossing 2kPa which was presumed to be a part of its water conservation mechanism and may suggest certain

advantage under terminal drought conditions. Sensitive genotype H77/833-2 has North Indian origin and is heat resistant parental genotype of many commercially used hybrids of this area. H77/833-2 was shown to use the maximum available soil water at any point of its growth and development and lacking any tight control of transpiration as in the case of tolerant genotype.

Plant growth conditions: Plants were grown individually in 6" and 10" diameter pots for growth chamber/glasshouse experiments in the mixture of Alfisol, sand and manure (5:2:1) and kept well-watered during all developmental stages. Smaller pots were used for early growth stage sequential harvests (see below).

For examination of plant growth and development in optimal conditions (Exp. 1) plants were grown in glasshouse conditions where VPD fluctuated between 3.6/0.4kPa during day/night up to 38 DAS (approximately 14 days after flag leaf appeared; during early grain filling stage). For each genotype, there were 25 replicated pots divided in 5 separate sets (5 replications for each genotype randomized in each set) which were harvested sequentially every week starting at 17 DAS.

For investigation of VPD effect on plant growth and development (Exp. 2) plants were germinated in glasshouse and 5 days after sowing (DAS; in 3 leaf stage) pots were transferred into growth chambers (GCh) under different VPD conditions and same light intensity (800 µmol photon m^2 s^{-1}) during the 12.5 h day cycle. Five replicated pots of each genotype were randomized in a GCh set to low day VPD (1.13 kPa) with combination of 28ºC and 70 % RH during day (22ºC and 70 % RH were set for night cycle), whereas five replicated pots of each genotype were randomized in another GCh set to high VPD (2.55kPa) with 30ºC and 40 % RH during day (25ºC and 70 % RH during night cycle).

Analysis of growth and development:

i. Transpiration was monitored daily from the beginning of the experiment under well-watered conditions. Plants were watered to excess and left draining overnight on the first day of experiments. Next day morning pots were bagged with plastic bag tightly around the plants stem (Exp. 1) or a thick layer of plastic beads was applied to the soil surface (Exp. 2) to avoid soil evaporation. Therefore any water losses would be mostly due to plants transpiration. Pots were weighed and this first value was considered as "saturated weight" i.e. 100% soil capacity. Pots were then weighted every morning and re-watered up to 80% of saturated weight. Transpiration was monitored throughout the experiment. In Exp. 1, the transpiration rate (TR; g cm^{-2} d^{-1}) was assessed with the set of plants that was harvested weekly and calculated as the average of three days transpiration before plants were harvested divided by the leaf area (LA) at harvest. Similar procedure was use to assess TR in Exp. 2.
ii. Transpiration efficiency (TE) was assessed each week in Exp. 1, using the sequential harvests (at 17, 24, 31, 38 DAS) to monitor the biomass increases and the daily transpiration assessment to compute weekly water use. TE was assessed over the entire growth period in Exp. 2. For TE estimation in Exp. 2, the biomass at the beginning of the transpiration monitoring was estimated from an extra plant grown in each pot, which was thinned and its dry weights determined at 5 DAS.

In Exp. 2 plants were harvested only at 21 DAS.

iii. During both experiments, increase in length of all leaves (Exp. 1) and 5-7th leaf (Exp. 2) was measured every morning (in Exp. 1) and every morning and evening (in Exp. 2) with a ruler and at the time of harvest, total plant leaf area (LA) was measured with LA meter (model LI-3100 Licor, Lincoln, NE). For expressing leaves growth, time scale in degree days (^{o}day) was used (according to Singh *et al.* 1998), with a temperature range 10-45^{o}C.

iv. Amount of water utilized in particular time periods (daily in Exp. 1 and every few days in Exp. 2) was monitored and the slopes of increase in transpiration expressed in Exp. 1.

Results:

In Exp. 1 & 2 TR of tolerant genotypes was almost always significantly lower in tolerant genotype (PRLT 2/89-33) across all environmental conditions (Fig. 1a, b). This confirmed similar results of previous studies (Kholova *et al*. 2010 a, b); Interestingly in Exp. 2 the TR difference between tolerant and sensitive genotype was far smaller in high VPD regime (2.55kPa) than in low VPD regime (1.13kPa; Fig. 1b). Also, differences in TR between contrasting genotypes were not reflected in differences in TE in glasshouse conditions (Exp. 1; Fig. 2a) and in high VPD regime (2.55 kPa; Exp 2; Fig. 2 b), confirming similar results in previous studies (Kholova *et al*. 2010b). By contrast, TE of tolerant genotype (PRLT 2/89-33) was significantly higher in low VPD regime (1.13 kPa) compared to sensitive one (H77/833-2) in Exp. 2 (Fig. 2b). TR of PRLT 2/89-33 was also much lower than in H77/833-2 in those low VPD conditions.

This TE variation might be related to the leaf appearance and development pattern as shown in Exp. 2. There we found that leaves of tolerant genotype (PRLT 2/89-33) emerged approx. 5^{o}days later compared to sensitive H77/833-2. However, leaves of tolerant genotype (PRLT 2/89-33) grew longer and expanded for longer time period (for approx. 7^{o}days more) compared to sensitive genotype (H77/833-2) under low VPD (1.13 kPa; Fig. 3 b). Contrarily, in high VPD regime (2.55 kPa) the leaves expanded at similar speed for comparable time period in both investigated genotypes (Exp. 2, Fig. 3 b). As such, the leaf length of PRLT 2/89-33 was reduced under high VPD whereas that of H77/833-2 was not and was even slightly increased.

Leaves of tolerant genotype PRLT 2/89-33 in Exp. 1 emerged with around 10^{o}days delay (similarly as in low VPD conditions in Exp. 2), but the differences in leaf elongation rate between genotypes were not large (similarly as in high VPD conditions in Exp. 2). Leaves growth pattern related well to the total LA as shown on Fig. 4 a, b. There were constant differences between genotypes in total LA measured at most of the sequential harvests but these differences diminished with plants age in Exp. 1.; i.e. PRLT 2/89-33 (tolerant) attained significantly higher LA at most of the points of harvest compared to H77/833-2 (sensitive). However, total LA in Exp. 2 differed significantly only in low VPD (1.13 kPa) regime and was not statistically distinguishable in high VPD (2.55 kPa) regime (Fig. 4 b).

Finally, the total quantity of water used during plant growth and development was similar in the glasshouse and the high VPD regime between genotypes of Exp. 2 (Fig 5a, b). However, there were genotypic differences in the dynamics in utilization of this available water conditioned by environment, which reflected the leaf area development pattern: In glasshouse environment (Exp. 1) and high VPD

regime (Exp. 2) PRLT 2/89-33 (tolerant) utilized more water until around 14/18 DAS in GCh/GH in average compared to H77/833-2 (sensitive; data not shown). However, after this breakpoint onwards PRLT 2/89-33 used less water compared to H77/833-2. Contrarily, in low VPD regime in Exp. 2 tolerant genotype (PRLT 2/89-33) utilized similar amount of water during its growth up to 21 DAS (Exp. 2).

Discussion:
Here we confirmed observations of our previous studies (Kholova *et al*. 2010a, b) that TR of terminal drought tolerant genotype (PRLT 2/89-33) was invariably lower compared to its sensitive counterpart (H77/833-2) across VPD conditions tested. However, the genotypic difference in TR appeared to be smaller under high VPD of the growth environment. This is different from the previous studies where TR differences were higher under high VPD in PRLT 2/89-33 than in H77/833-2. This may be explained by the fact that plants were grown under high VPD conditions. By contrast, plants in the previous study were grown under low VPD and then were transferred to a growth chamber to test the response to VPD. Differences in root hydraulics were hypothesized to be responsible for the differences in the TR response to VPD (Kholova *et al*. 2010b). The results here suggest that the environment affect plant's development in a way that affects plant hydraulics. Also, as shown before (Kholova *et al*. 2010b), the differences in TR were not reflected in differences in TE (biomass production per amount of water transpired) in environments where VPD was above 2kPa which was also the case for a substantial part of the day in the glasshouse environment. However, a lower TR in the low VPD environment related to higher TE for tolerant PRLT 2/89-33. This difference may be explained by the higher leaf growth of PRLT 2/89-33 and the similar water use. By contrast in high VPD environment the leaf expansion rate was comparably decreased in PRLT 2/89-33 while it was unchanged in H77/833-2, leading to less water use in PRLT 2/89-33, despite the smaller differences in TR under high VPD.

Conclusion:
The overall aim of this study was to show the importance of environmental conditions for plant development and what could be its further consequences in terms of water use. We conclude that the pattern of water use depends both on the control of stomata opening and on the leaf area development pattern. Both these traits are highly environment-specific and results in differences in the overall plant water use before stress occurs, with direct consequences on plant adaptation to terminal drought stress. Therefore, previously proposed water saving mechanisms need to be seen both from the angle of an increased water productivity when plant development takes place at low VPD, and from a lower water use when development takes place under high VPD, which relates to a restricted leaf area development.

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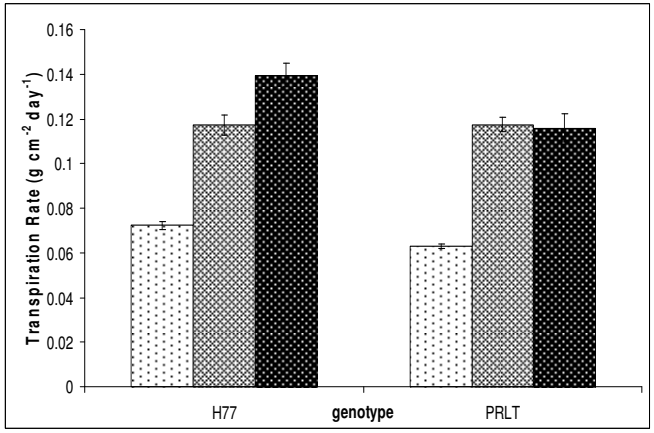
Figure 1 a, b: Transpiration rate of terminal drought tolerant (PRLT) and sensitive (H77) genotypes in glasshouse conditions in three different developmental stages (24, 31, 38DAS) (a) and in growth chamber conditions with day cycle VPD 2.55 and VPD 1.13 at 21 DAS (b).



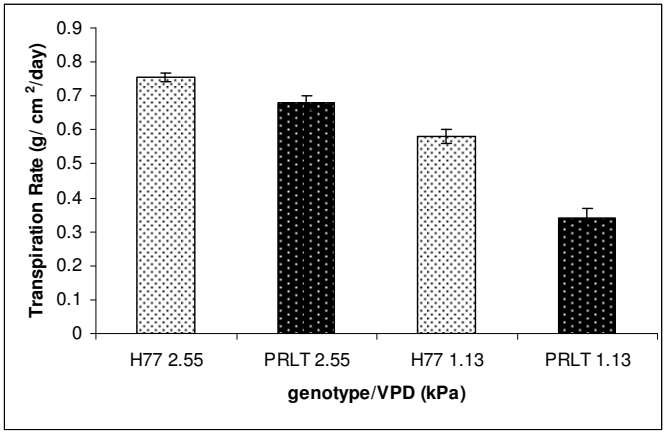
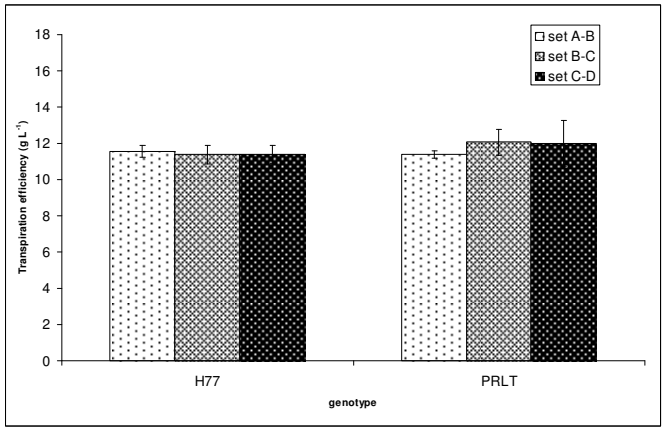


Figure 2 a, b: Transpiration efficiency of terminal drought tolerant (PRLT) and sensitive (H77) genotypes in glasshouse conditions between three different developmental stages (17-24, 24-31, 31-38DAS) (a) and in growth chamber conditions with day cycle VPD 2.55 and VPD 1.13 at 21 DAS (b).



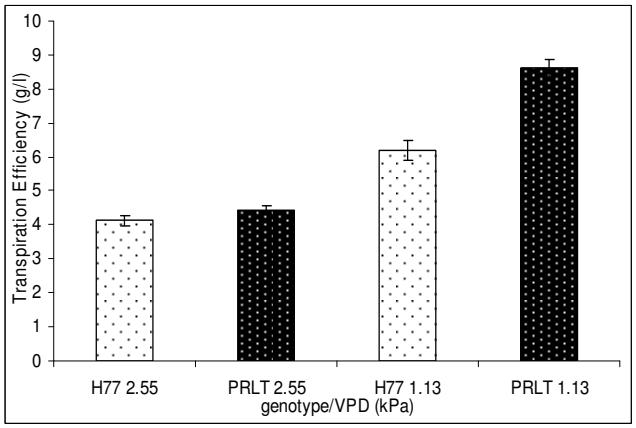
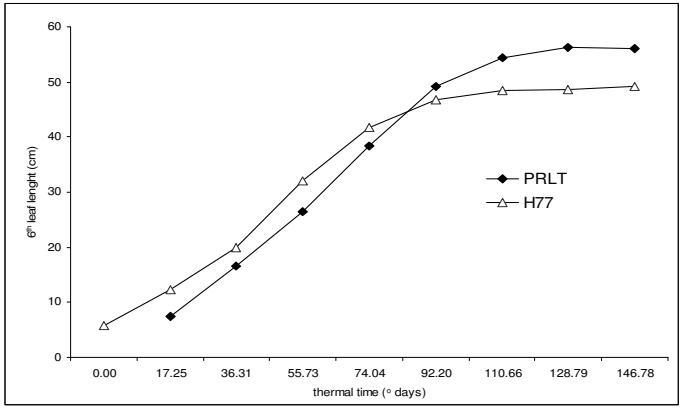


Figure 3 a, b: Length of 6th leaf of terminal drought tolerant (PRLT) and sensitive (H77) genotypes in glasshouse conditions (a) and in growth chamber conditions with day cycle VPD 2.55 (full line) and VPD 1.13 (dotted line, b).



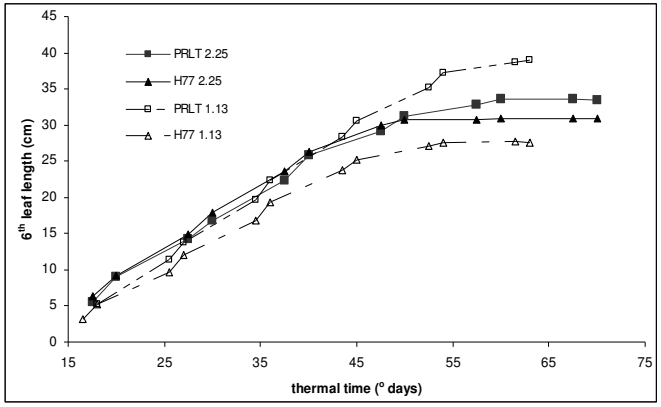
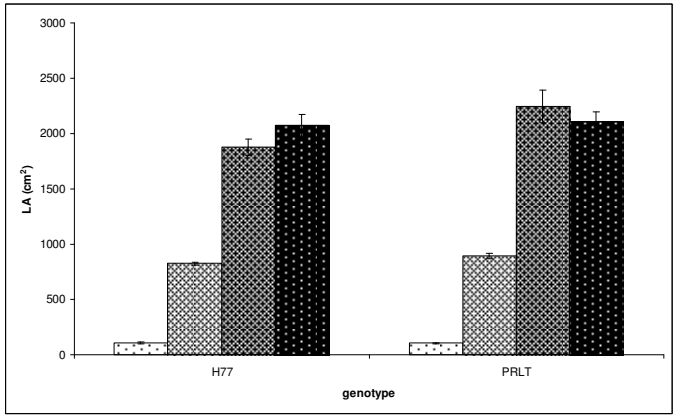


Figure 4a, b: Total leaf area of terminal drought tolerant (PRLT) and sensitive (H77) genotypes in glasshouse conditions during four different developmental stages (17 (pre-harvested plants) 24, 31, 38DAS) (a) and in growth chamber conditions with day cycle VPD 2.55 and VPD 1.13 at 21 DAS (b).



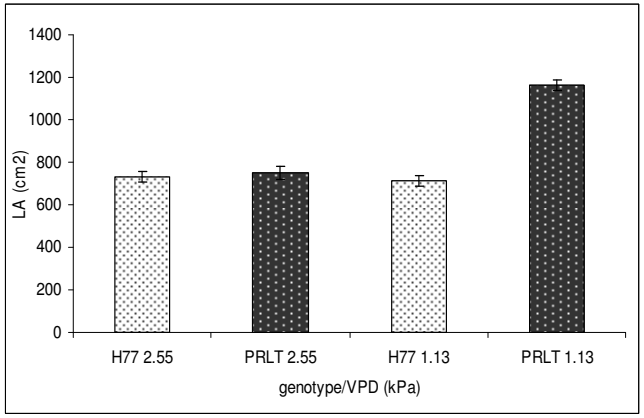
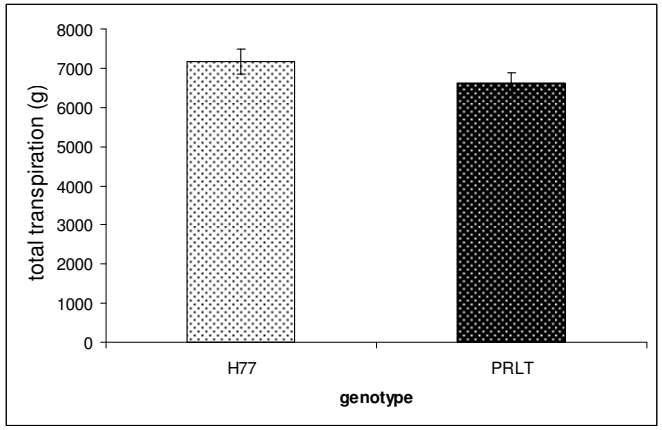
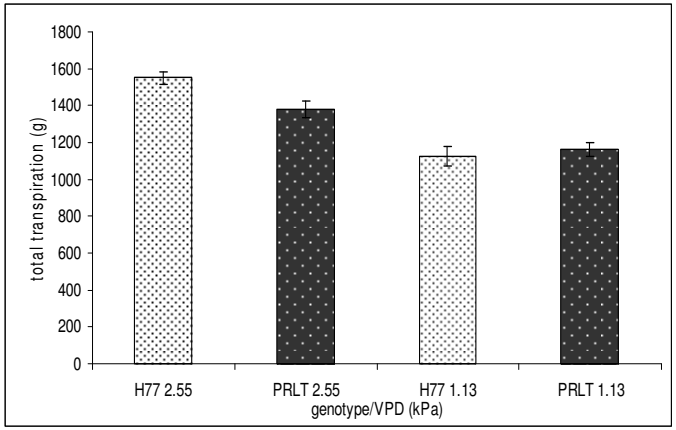


Figure 5a, b: Total amount of water utilized during the plants development by terminal drought tolerant (PRLT) and sensitive (H77) genotypes in glasshouse conditions up to 38DAS (a) and in growth chamber conditions with day cycle VPD 2.55 and VPD 1.13 up to 21DAS (b).





2.9 List of other publications

1) Kholová J, Sairam RK, Meena RC, Srivastava GC (2009): **Response of maize genotypes to salinity stress in relation to osmolytes and metal-ions, oxidative stress and antioxidant enzymes activity.** Biologia Plantarum 53(2): 249-256.
2) Kholová J, Sairam RK, Meena RC (2010): **Osmolytes and metal ions accumulation, oxidative stress and antioxidant enzymes activity as determinants of salinity stress tolerance in maize genotypes**. Acta Physiologiae Plantarum. 32 (3)477-486.
3) Kočová M, Holá D, Rothová O, Kholová J, Johnová E and Bartáková J (2007): **Response of physiological and morphological parameters of maize to hypoxia and posthypoxia growing conditions.** *The Influence of Abiotic and Biotic Stressors on Plants 2007*, Prague, Czech Republic, March 21-22, 2007. *Proceedings* (ISBN 978-80-87011-00-3; 978-80-213-1621-8), 332-339.
4) Holá D, Kočová M, Rothová O, Kholová J, Benešová M and Červinková H (2007): **Changes in physiological and morphological traits of three generations of maize (*Zea mays* L.) due to water deficit.** *The Influence of Abiotic and Biotic Stressors on Plants 2007*, Prague, Czech Republic, March 21-22, 2007. *Proceedings* (ISBN 978-80-87011-00-3; 978-80-213-1621-8), 324-331.

2.10 List of other posters presented at international conferences

5) Zindy P, Kholová J, Vadez V. (2008): **Does exogenous ABA affect transpiration differentially in pearl millet lines contrasting for terminal drought tolerance?** Golden Jubilee Conference on "Challenges and Emerging Strategies for Improving Plant Productivity" 12-14 November, IARI, New Delhi, India, Book of abstracts 61.
6) Kholová J, Vadez V, Hash C T, Yadav R and Kočová M (2009): **Elucidating of Link Between Physiological And Biochemical Traits Influencing Drought Tolerance In Pearl Millet.** *Plant and Animal Genome VXII Conference*, January 9-14 2009, San Diego, US pp. 320
7) Vadez V, Kholová J, Hash C T, Yadav R and Kočová M (2009): **Contrast In Physiological Traits In Pearl Millet Genotypes Differing For A Terminal Drought Tolerance QTL.** *Plant and Animal Genome VXII Conference*, January 9-14, San Diego US pp. 319
8) Kočová M., Holá D., Rothová O., Kholová J., Benešová M., Bartáková J., Červinková H. (2007): **The response of inbred and hybrid maize genotypes to water deficiency.** *Plant Experimental Biology Conference (The 11. Days of Plant Physiology)*, Olomouc, Czech Republic, July 9-12, 2007. *Book of Abstracts*, p. 113.
9) Kočová M., Holá D., Rothová O., Kholová J., Červinková H., Fridrichová L. (2008): **The inheritance of photosynthetic and growth parameters in drought.stressed plants of two inbred lines of maize and their F1 and F2 hybrids.** *The XVI. Congress of the Federation of European Societies of Plant Biology*, Tampere, Finsko, 17.-22.8.2008. *Physiol. Plant.., Suppl. 133*, P09-088.
10) Kočová M., Holá D., Rothová O., Ždánská H., Kholová J. (2004): **Recovery of photosynthetic apparatus from drought or waterlogging is different in inbred and hybrid maize.** *The XIV. Congress of the Federation of European Societies of Plant Physiology*, Krakow, Polsko, 23.-27.8.2004. *Acta Physiol. Plant., Suppl. 26*, str. 192.

3. DISCUSSION

To elucidate the mechanism of terminal drought tolerance of pearl millet, several physiological and biochemical traits were evaluated and their importance for drought tolerance mechanisms drafted.

Transpiration rate

Crucial results for understanding millet drought tolerance strategy were brought out from the comparison of Tr of contrasting genotypes under well-watered conditions. Tolerant genotypes (PRLT 2/89-33, 863B-P2) showed lower Tr compared to their sensitive oppositions (H77/833-2, ICMB 841-P3) across developmental stages. In addition, similar Tr variability was also found on detached leaves of same genotypes sampled from the field. Suggestion, that Tr could be part of the terminal drought tolerance QTL was confirmed with all tolerant NILs exhibiting Tr similar to tolerant parent and lower than sensitive one. These results show a relationship between the terminal drought tolerance of PRLT 289/33 and NILs and their lower rate of water loss per unit of leaf area under well-watered conditions. This trait, as we hypothesized, would allow relatively more water to remain available in the soil profile for later developmental stages, in particular during the grain filling period, and would have great value under terminal drought conditions and in environments where soil evaporation is limited. This interpretation would fit well the fact that the terminal drought tolerance QTL is responsible for a better panicle harvest index (PNHI), i.e. a proxy for grain filling. Such data have not been reported so far despite the importance of plants water management in well-watered conditions was previously few times discussed (Mortlock and Hammer 2001, Condon et al. 2002, Sinclair et al. 2005, 2007, 2010).

Stomata density

Our aim to at least partially explain differences in Tr (as discussed above) by examining the number of stomata didn't confirm our initial hypothesis. We expected variability in stomata density may provide an explanation for previously observed differences in Tr as was shown in Masle et al. (2005) who provided evidences that gene *ERECTA* influenced the stomata density and consequently even plants TE and has considerable impact on drought tolerance. This seems not to be the case of pearl millet. So, if stomata number may play a role in drought tolerance (Muchow and Sinclair 1989), our results agree with previous assertion that stomatal regulation rather than density is more important for regulating water loss in pearl millet (Henson al. 1981, Liu et al. 2003, Zhang et al. 2005).

The relation between transpiration rate and leaf area ratio (LAR)

The Tr may not be only dependent upon internal biochemical regulation of plants but may be also influenced by physical characteristics of plants' internal architecture, e.g. xylem vessels parameters, endo- and exo- dermis root structure (Sperry et al. 2002, Cochard et al. 2004, Zwieniecky et al. 2001). From our overall results, it was apparent that drought tolerance contrasting genotypes in well-watered conditions exhibited comparatively similar values of total dry matter (TDM), but differed more in leaf area (LA). Therefore, we considered a possible tuning of Tr by the relative proportion of TDM supported by each unit of leaf area, as in previous work (Black and Squire 1979). We showed that both tolerant genotypes (PRLT 2/89-33 and 863B-P2) had more LA supporting each unit of total biomass and

simultaneously lower values of Tr compared to the sensitive ones (H77/833-2, ICMB 841-P3). However, the Tr of tolerant genotypes remained lower than in sensitive ones, even after normalizing for LAR. Such results suggest that the larger LAR of tolerant genotypes may naturally confer them lower values of Tr and consequently allowing them more efficient control of water use. When the LAR was experimentally altered to test the possibility of short-term adjustment, the Tr of remaining LA adjusted very quickly to the exponential function. This would suggest that a hydraulic control of the change in Tr could be involved in such a rapid change of the stomata opening.

Threshold of fraction of transpirable soil water (FTSW threshold)

We found that the FTSW threshold of tolerant parental genotypes was lower compared to sensitive ones in vegetative developmental stage. This meant that the transpiration dropped upon progressive soil drying in relatively dryer soil in the tolerant lines than in the sensitive one. This finding was initially puzzling us, since we had expected that a conservative use of water in tolerant lines would have led them to initiate a transpiration decline at higher FTSW thresholds (wetter soil). However, this genotypic variability in transpiration response to soil drying was in agreement with data obtained in groundnut (Bhatnagar-Mathur et al. 2007), and is in agreement with similar finding comparing terminal drought tolerant lines of chickpea (Zaman et al. personal communication) and cowpea (Belko et al. personal communication). In addition, the response measured in one contrasting parental pair and their NILs-QTL gave evidence that the FTSW threshold obtained for superior NILs-QTL was similar to that of tolerant PRLT 2/89-33 and QTL donor parent. By contrast, the FTSW threshold obtained for NILs-QTL that did not yield better than H77/833-2 in the field was indeed similar to that of sensitive H77/833-2. These data provide evidence for a role of the QTL in explaining the differences in these thresholds and hence the role of these thresholds differences in understanding the variability between lines for their terminal drought tolerance (Kholová et al. 2010 a). The reasons for these differences are intriguing, given that: (i) tolerant genotypes have a lower Tr under well-watered conditions, which would denote a more "conservative" water use (see above); (ii) tolerant genotypes have lower FTSW threshold for transpiration decline under drought that indicate they attempt to maximize water use. Both could in fact be related. Indeed, a lower Tr in tolerant lines under well-watered (WW) conditions would lead to lower daily transpiration, which would logically drive the transpiration ratio (TR) of drought-exposed plants upwards, and consequently the normalized transpiration ratio (NTR). Therefore, the maintenance of a NTR under drought conditions at level close to that of well-watered plants, which leads to having lower FTSW threshold for the beginning of the transpiration drop, might simply be a consequence of the lower rate of water loss per unit of leaf area (Tr) in the well-watered plants of tolerant genotypes. In fact, this agrees well with the fact that the presence/absence of the QTL appeared to discriminate well for both a lower/higher Tr, and lower/higher FTSW threshold. Same interpretation could be drawn from similar data in transgenic groundnut (Bhatnagar-Mathur et al. 2007). The only unexplained issue is the fact that the FTSW thresholds were not significantly different at reproductive stage whereas Tr was still different.

Leaf abscisic acid (ABA) content

During vegetative growth drought tolerant genotypes (both the QTL-donor parent and three NIL-QTLs in testcross form) had higher leaf ABA content than the drought sensitive genotype under well-watered conditions. These results suggest a likely constitutive role of ABA in the drought tolerance QTL that plays most of its role when water is still non-limiting. The differences in leaf ABA are not due to dilution effect at the leaf level since SLA was similar in both pair of parents (Kholová et al. 2009), and also because genotypes with high ABA had similar to larger leaf size than sensitive lines. These data would agree well with the lower transpiration rate (Tr) in the tolerant genotype, given that ABA is closely involved in the control of stomata aperture (Henson et al. 1983, Morison and Gifford 1984, Cure and Acock 1986). Whether the differences in ABA content have a causal effect on Tr and subsequently on yield under drought was not the purpose of the study and would need to be further investigated. Our analysis also showed that genotypic differences in leaf ABA were less marked at reproductive stage. This might be the consequence of differential sensitivity to ABA between developmental stages (Henson et al. 1983, Winkel et al. 2001). So, overall, our hypothesis is of a role of a constitutive higher production of ABA in tolerant lines to limit leaf water loss under well-watered conditions, which would save water for the later stage of plant development, hence turning out to be an important aspect of plant adaptation to water-limited conditions as previously hypothesized (Mortlock and Hammer 2001, Condon et al. 2002, Serraj et al. 2004, Sinclair et al. 2005, Kholová et al. 2008, 2010a, b).
Surprisingly, we did not find larger differences in ABA level between control and stress treated plants in any of the experiments conducted. Leaf ABA level was higher only in drought sensitive genotype ICMB 841-P3 during vegetative growth stage in stress conditions. This is contrary to previous reports in other species where ABA content significantly increased under drought stress conditions (Asch et al. 1995, Chandrasekar et al. 2000, Li and Wang 2003, Yin et al. 2005, Zhang et al. 2005). It could be argued that genotypes may not have been at similar leaf water potential where WS ABA was assessed, although they were at similar FTSW level and so same stress intensity on the basis of soil water content. However, it was shown that ABA effect on stomata may not be dependent only on all over ABA concentration in plant tissues, but strongly depends on e.g. pH difference between xylem and leaves cells (Wilkinson and Davies 1999). So, more than the absolute amounts, it is rather the distribution of ABA that appears to matter and so, having no increase in ABA under stress does not preclude its role. In any case, the traits that matter to contribute to terminal drought tolerance appear to be constitutive and this fits well with the fact that the differences in ABA were found mostly under well watered conditions. More work remains to be done to elucidate the exact role of ABA on the control of leaf water losses.

VPD response

Our further investigations showed that transpiration was sensitive to VPD in most of the drought tolerant NIL-QTLs and in the drought tolerant QTL-donor parent, suggesting a direct involvement of the QTL in the manifestation of this trait. The lack of breakdown in the response of one of the tested NIL-QTLs (ICMR 02041)

could be explained by a possible recombination in this large QTL region (about 30 cM), which would have excluded a putative portion involved in the VPD response of that particular NIL-QTL. This would indicate that only a portion of the QTL may be responsible for this trait. Our further efforts to fine-map this QTL using the "high resolution cross" population (see in 3.1.x) should help generate the material needed to test this hypothesis, i.e. that the QTL regions is underlying a cluster of traits contributing to water saving and that are held by different portions of the QTL. The fact that Tr differed between tolerant and sensitive genotypes even at low VPD indicate that the low Tr of tolerant genotypes (Kholová et al. 2010) is not a consequence of the Tr differences at high VPD only. The finding of genotypic differences in the transpiration response to VPD in pearl millet agrees with similar results in soybean (Sinclair et al. 2007), where a "slow-wilting" genotype of soybean showed a linear increase in transpiration only until about 2 kPa. Above these VPD levels, transpiration rates remained essentially constant, whereas other genotypes maintained a linear increase in transpiration up to VPD values of about 3.5 kPa. The reasons for that rapid change in Tr upon a VPD increase are unclear and would probably require rapid control of the stomata conductivity. Hydraulic signals (Zwieniecky et al. 2001, Sperry et al. 2002, Cochard et al. 2004) are more likely to mediate such signal than drought signaling cascades (including ABA dependent and ABA independent pathway). In fact, our previous work (Kholová et al. 2010) showed that Tr could be increased on a short term basis with defoliation (see above), giving evidence of the likelihood of non-hormone related signals for the regulation of stomata in pearl millet too. So, these results indicate clearly that in terminal drought tolerant pearl millet, two distinct water saving mechanisms operate under well-watered conditions: (i) a low transpiration rate, which acts across VPD conditions; (ii) a sensitivity to VPD in tolerant material that further limits the transpiration rate when VPD is high, above 2 kPa. Both these traits would contribute to saving water in the soil profile, even if soil water is not limiting. This water would then be available and crucial for the grain filling as previously indicated (Manschadi et al. 2006; Turner 2004, Ratnakumar et al. 2009, Sinclair et al. 2010). So, both traits are important to consider for the breeding of pearl millet lines having terminal drought tolerance. The data indicate the possibility to phenotype these traits using relatively simple Tr measurements at low and high VPD conditions under natural conditions.

TE analysis

We did not find large genotypic differences in TE both across growth conditions and across investigated genotypes. This is contrary to what was earlier suggested (Sinclair et al. 2005), and then also contrary to our own expectations. Indeed a restriction of Tr would mean a decreased stomatal conductance, which „mechanically" would increase TE. A possibility for the lack of differences is that the gravimetric method used to assess TE was not sensitive enough to pinpoint TE differences arising from differences in sensitivity to VPD (see Kholová et al. 2010b). Furthermore, the plants used for TE assessment were grown in the glasshouse at relatively low mean VPD and only rarely faced VPD conditions above 1.5-2.0 kPa that would trigger the VPD response of transpiration and the expected transient increase in TE. Another possibility for the lack of TE differences could have been the differences in biomass partitioning to roots, since roots were not included in the TE measurement. So, more work is needed to assess whether

gravimetric TE differences could be found in conditions where plants are exposed to higher VPD, or simply whether intrinsic TE increases upon VPD increase in VPD-sensitive materials. In fact, the dry weight increase during the experimental period was lower in some of tolerant material, which suggests that the water saving mechanisms could simply reflect in differences in total water use. This lack of TE differences could also be linked to how individual stomata respond to VPD. In the work that reported transpiration sensitivity to VPD (Sinclair et al. 2009, Devi et al. 2009, Kholová et al. 2010b) mean stomatal conductance was partially reduced. Somehow, we assume it is a consequence of a reduction of the aperture of *all* stomata. If that was the case, indeed the intrinsic TE should increase. However, we could speculate that stomatal conductance decrease could be the *mean* of *certain* stomata having conductance unchanged and *other* stomata that would fully close. Such situation would normally not modify intrinsic TE while it would decrease Tr. Although that explanation may look speculative, it would fit with reports that stomata are organized in patches (Pospíšilová and Santrůček 1994, Mott and Buckley 2000) and may not all respond the same way to external stimuli. In any case, the absence of TE differences in given environmental conditions stresses that the advantage of the VPD sensitivity trait, along with the lower Tr trait, likely related to ABA, needs to be considered in term of total water use (lesser) rather that in term of water productivity.

Sensitivity of leaf expansion to VPD

As mentioned above, there was evidence that plants in the natural cycles of VPD changes (fluctuating around the 2kPa threshold; see above) tend to save water in the profile through tight regulation of Tr. This water conservation mechanism didn't reflect in any remarkable differences in TE, as would be expected. Therefore, it is desirable to dissect further the relation between plants growth and their TE, Tr and water use when exposed to constant "low VPD" (VPD below 2kPa) or "high VPD" (VPD above 2kPa) conditions. Investigation of plants exposed to these constant VPD regimes confirmed observations of our previous studies (Kholová et al. 2010a, b) that Tr of terminal drought tolerant genotype (PRLT 2/89-33) was invariably lower compared to its sensitive counterpart (H77/833-2) across VPD conditions tested. However, the genotypic difference in Tr appeared to be smaller under high VPD of the growth environment. This may be explained by the fact that plants were grown under high VPD conditions and would have been affected in their development (especially the canopy). By contrast, plants in the previous study were grown under low VPD and then were transferred to a growth chamber to test the response to increasing VPD and did show sensitivity to VPD. Differences in root hydraulics were hypothesized to be responsible for the differences in the Tr response to VPD (Kholová et al. 2010b). It was also found that the differences in Tr were not reflected in differences in TE (biomass production per amount of water transpired) in environments where VPD was above 2kPa which was also the case for a substantial part of the day in the glasshouse environment (Kholová et al. 2010b). However, a lower Tr in the low VPD environment related well to a higher TE for tolerant PRLT 2/89-33. This difference may be explained by the higher leaf growth of PRLT 2/89-33 at the level of water use that remains similar to the high VPD conditions. By contrast, in high VPD environment the leaf expansion rate was comparably decreased in PRLT 2/89-33 while it was unchanged in H77/833-2, leading to less water use in PRLT 2/89-33, despite the smaller differences in Tr

under high VPD. Therefore, previously proposed water saving mechanisms need to be seen both from the angle of an increased water productivity when plant development takes place at low VPD, and from a lower water use when development takes place under high VPD, which relates to a restricted leaf area development.

Anti-oxidative enzymes and photosynthetic pigments

Ascorbic peroxidase (APX)

Usually, APX is found increased with drought treatment in various plant species; e.g. wheat, beans, rice, alfalfa (Kele and Oncel 2002, Torres-Franklin et al. 2007, Sharma and Dubey 2005 Rubio et al. 2002). APX is referred as an enzyme with strong affinity to its substrate H_2O_2 and it was suggested that even slight increase in APX activity may play crucial role in allowing ROS scavenging capacity (Mittler and Zilinskas 1994). Here we found higher total APX5 activity under water stress conditions compared to control conditions, moreover there was a notable difference in APX5 activity between sensitive and tolerant genotypes. Lower proportional APX5 activity under drought was found in H 77/833-2 (sensitive genotype) than in PRLT 2/89-33 and QTL-NILs. It is unlikely that the lower APX-5 activity in H 77/833-2 could be explained by a delayed stimulation by water stress since this genotype also showed an earlier decline in transpiration upon progressive exposure to water stress treatment compared to the tolerant genotypes (Kholová et al. 2010). We interpret that this isozyme may simply not respond to the stress treatment in this genotype. In any case, APX5 isoenzymatic bands were more intense in drought tolerant compared to drought sensitive genotypes, therefore APX5 expression might be linked to the introgressed QTL genome portions involved in terminal drought tolerance. Other APX isoenzymatic activities didn‘t discriminate between tolerant/sensitive genotypes. Differential roles of various isozymes are well documented (e.g. Foyer et al. 1994, Hernandez et al. 1995, Gomez et al. 1999, Fadzilla et al. 1997), although we are not aware of any work emphasizing the importance of particular APX isozymes for the adaptation to drought stress conditions.

Catalase (CAT)

Contrary to APX, CAT has low affinity to H_2O_2 which suggests its restricted role in counteracting the oxidative damage to cells (Cruz de Carvalho 2008). Even reports on CAT activity under drought are very heterogeneous. CAT was shown increased in e.g *Prunus*, tomato, sesame, alfalfa or wheat (Sofo et al. 2005, Űnayayar 2005, Fazeli et al. 2007, Rubio et al. 2002, Luna et al. 2004), but decreased or unchanged in sunflower, pea and some grasses (Zhang and Kirkham 1994, Iturbe-Ormaetxe et al. 1998, Fu and Huang 2001). In our experiments, a new CAT isozyme was induced under drought conditions, but the total CAT activity did not increase significantly under drought stress. This was in part because the new CAT isoform accounted for only 6.5% of the total CAT activity. Similar induction of CAT isozyme was documented in rice exposed to severe drought stress (Srivalli et al. 2003). Furthermore, the proportional isozyme activities were very similar under drought conditions. Therefore, our results suggest that based on CAT activity we could not discriminate genotypes on the basis of the absence or presence of a

drought tolerance QTL.

Superoxid dismutase (SOD)

Our data obtained on SOD isoenzymatic activities are in contrast with the previous study made on pearl millet by Patil et al. (2005). They reported increased SOD activities during the late stages of drought imposition, although well after activities of APX and CAT had increased. Unfortunately, this field study did not document the soil water content that would permit a rigorous comparison with our findings. Our findings are, however, similar to studies on alfalfa, *Arabidopsis thaliana*, wheat, pea, *Ctenante setosa,* tomato and maize where no SOD activity increment was documented in leaves tissues under severe water stress (Bartoli et al. 1999; Borsani et al. 2001; Irigoyen et al. 1992, Iturbe-Ormaetxe et al. 1998, Ünyayar et al. 2005, Terzi and Kadioglu 2006, Bai et al. 2006). In any case, none of the SOD measurements could discriminate QTL-NILs lines from H77/833-2, suggesting that SOD activity and isoenzymatic composition are probably not causally related to the presence/absence of QTL in pearl millet genotypes included in the present study.

Photosynthetic pigments contents

Our analysis of photosynthetic pigments content generally agreed with most of the previous studies. In our experiments, drought stress caused a significant decline in total chlorophyll and carotenoids content in the magnitude usually described as "non-lethal" (roughly 10-30%) under harsh drought stress. Similar decline in photosynthetic pigments content was previously observed in pearl millet (Ashraf et al. 2001) and other species (Kyparissis et al. 1995; Terzi and Kadioglu 2006). Together, the increase of Chl/Car ratio due to drought conditions was reported (MunneÂ-Bosch and Alegre 2000). In contrast to these results we found a decreased Chl/Car ratio suggesting the involvement of other strong ROS scavenging mechanisms additional to carotenoids (Richardson et al. 2004, Zhang et al. 2008). Although a significant increase in the Chl *a/b* ratio was previously reported (Ashraf et al. 2001), we found only an insignificant increment in Chl *a/b* ratio in all genotypes under stress treatment.

The major finding was that none of these changes could clearly discriminate QTL holding genotypes from H77/833-2. Usually, no significant differences were found between parental genotypes. In several cases QTL-NILs showed even higher trait values (Chl *a*, total Chl and Car) compared to both parental genotypes. Although a relationship between photosynthetic pigments stability and drought tolerance has been proposed in other species like peanut, wheat or maize (Pastori and Trippi 1992; Kraus et al. 1995; Arunyanark et al. 2008) our data suggest there is no evident relationship between the maintenance of photosynthetic pigments or their ratios, or their changes under drought, with presence/absence of terminal drought tolerance QTL in the pearl millet genotypes tested.

Relation between photosynthetic pigments contents and anti-oxidative enzymes activities

We found that the two CAT, two SOD, and three APX isozymes correlated

positively with the Chl/Car ratio under drought conditions, whereas two APX isozymes had negative associations with the Chl/Car ratio under well-watered conditions. This agrees with the hypotheses presented by Farrant et al. (2003) who described, that chlorophyll maintenance under drought should be compensated by other mechanisms. Indeed, we found that both total chlorophyll and carotenoids decreased under drought stress conditions. Furthermore, the increase in the ratio of chlorophyll content (potential source of ROS)/carotenoids (ROS scavengers) – indicated that the carotenoids content decreased relatively more than the chlorophyll content. Hypothetically, disrupted photosynthetic pigment ratios could lead to higher production of harmful ROS, and in such case, the ROS may exceed the scavenging capacity of carotenoids some of which act as direct scavengers of ROS produced via chlorophyll as described previously (e.g. Chow 1994, McKersie and Leshem 1994, Richardson et al. 2004). The significant negative correlations between the Chl/Car ratio and several isozymes of CAT, SOD and APX then suggest that these isozymes may play this additional ROS scavenging role to maintain the Chl/Car within non-lethal range.

Proline content

To analyze the putative proline contribution to drought tolerance contrasting genotypes along with NIL-QTL genotypes were analyzed as in previous studies. The major finding was that there was no significant increment of proline level due to drought in all genotypes until the FTSW dropped below 20%. By contrast, significant drought induced decline of transpiration occurred at around 35% FTSW in all genotypes. Nevertheless, faster proline accumulation was observed in tolerant genotype compared to sensitive one where most of NILs followed the trend of tolerant parental genotype. Because proline increased only during the last stage of drought stress, and because NILs had almost similar response to the QTL donor parent, it is concluded that proline differences may be influenced by the presence of the QTL but have more likely no direct relation to the yield superiority of tolerant genotypes.

Initial RIL mapping trials

As drafted above (see in 2.7.), trials were conducted to map several candidate traits (Tr, biomass related traits), which seems to play a role in the terminal drought tolerance mechanism. According to the expectations, several positive alleles increasing the Tr value from the terminal drought sensitive parent were located in the same genomic region where the major terminal drought tolerance QTL was identified previously (Yadav et al. 2002, Bidinger et al. 2007). Initial analysis shows, these „Tr alleles“ interact with each other as well as with the biomass characteristics. The strenght of these interactions probably depends on the environment in which plants development takes place (as discussed above). Therefore, the work is in progress to characterize and understand these inter-allelic and genotype × environment interactions and its contribution to drought tolerance.

4. CONCLUSION

We have studied a number of physiological, morphological and biochemical traits in orphaned crop of semi-arid agricultural regions – pearl millet (*Pennisetum glaucum* (L.) R. Br.) in connection to water limiting environment. The overall aim of this study was to elucidate the mechanisms of pearl millet terminal drought tolerance with a particular focus on plants water use and plants developmental characteristics in varying environmental conditions. We obtained several most important and novel results:

1. Genotypes contrasting for terminal drought tolerance, based on seed yield in the field conditions, also contrasted in the control of leaf water loss, in particular under well watered conditions, indicating these traits were constitutive. This trend was directly related to the presence or absence of a terminal drought tolerance QTL. The tolerant/QTL-holder genotypes had a lower rate of water loss per unit leaf area (Tr, g water cm^{-2} d^{-1}). We hypothesize that this characteristic would contribute to a more conservative water use in the field conditions, making more water available for the grain filling stage, which would be very important for terminal drought conditions. This hypothesis remains to be tested. A lower Tr would also lead to having lower FTSW threshold where transpiration declines upon progressive exposure to water deficit, making that drought stressed plant would behave like well watered plants until the soil has become dryer than for sensitive lines. Since Tr was measurable on whole plants but also on single detached leaves that could be collected from the field Tr may be a very convenient trait to phenotype across a range of experimental conditions. Although more work is needed to understand better how Tr is regulated, Tr may be further considered as an insightful tool for selection screening in pearl millet breeding programs.

2. The terminal drought tolerance QTL on pearl millet linkage group 2, previously found to correlate to a lower transpiration rate, also correlated to higher ABA levels in the leaves of well-watered plants, and to the sensitivity of transpiration to high VPD level under well-watered conditions. The low transpiration rate previously found were not only a consequence of genotypic differences in the sensitivity of Tr to high VPD but to two separate water saving mechanism, i.e. a low Tr at low VPD, which might be related to differences in the leaf ABA content, and a sensitivity to VPD leading to a further restriction of Tr at high VPD. The major trait differences were all found under well-watered conditions, pointing at constitutive mechanisms underlying the QTL. The rapid response of transpiration rate to VPD points to a possible role of plant hydraulics in mediating such a rapid response. These traits would contribute to water saving in the soil profile when water is non-limiting. This "extra" water, available for the later stage of the crop would become critical to guaranty water supply to the plants at the time of grain filling and therefore for grain yield under terminal drought.

3. The pattern of water use depends both on the control of stomata opening and on the leaf area development pattern. Both these traits are highly environment-specific and result in differences in the overall plant water use before stress occurs, with direct consequences on plant adaptation to terminal drought stress. Therefore, previously proposed water saving mechanisms need to be seen both from the angle

of an increased water productivity when plant development takes place at low VPD, and from a lower water use when development takes place under high VPD, which relates to a restricted leaf area development.

4. Although the APX5 isozyme activity increased under water stress and showed large qualitative differences between the sensitive H 77/833-2 and the group of genotypes holding a drought tolerance QTL, most anti-oxidant isozyme activities showed no change under water stress and band intensities were similar in all genotypes. Similar findings were obtained for the photosynthetic pigment concentration and its changes under drought. This absence of relationship between the presence/absence of the QTL and a differential response in the ROS scavenging and the content of photosynthetic pigment was likely not related to the experimental conditions, which were previously successfully used to discriminate genotypes for water-conserving mechanisms in a clear relation to the presence/absence of that QTL in the very same materials (Kholová et al. 2010a, b). These results suggest that the anti-oxidant machinery or the response of photosynthetic pigments to water stress may not play a direct causal role on the terminal drought tolerance of pearl millet that is conferred by the QTL. However, the anti-oxidative machinery appeared to be closely linked to the balance between carotenoids and chlorophyll, proxied by the Chl/Car ratio.

5. Leaf proline accumulation pattern in stress conditions showed certain link to the presence/absence of terminal drought tolerance QTL and therefore the proline content might be influenced by the QTL genomic region. However, this trait has probably little effect on yield superiority of tolerant genotypes as the FTSW threshold for proline accumulation under progressing water stress was far lower than that for the significant decline in transpiration. Therefore it is concluded that increased levels of proline in the latest stage of drought exposure might contribute rather to the plants survival than to the yield enhancement.

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