Dual Stable Isotopes Carbon (^{13}C) and Oxygen (^{18}O) – An Approach to Identify Desirable Cotton Lines

Ehab AbouKheir* • **Trichy Ganesh Prasad** •
Madavalam Sreeman Sheshshayee • **Makarla Udayakumar**

Department of Crop Physiology, GKVK, University of Agricultural Sciences, Bangalore-560065, Karnataka, India
Corresponding author: * **ehababoukheir@yahoo.com**

ABSTRACT

Enhancement of crop productivity under drought stress can be achieved by maximizing soil water capture by a deep or dense root system and by effective water use by increasing intrinsic ability to fix more carbon per unit water transpired (water use efficiency, WUE). Although the importance of these characters for improving productivity under drought is well known, precise quantification of these traits under field conditions in a large number of genotypes is the major limitation in breeding crop plants for these traits. In recent years, several reports showed the relevance of using stable isotope composition of carbon and oxygen in biomass as a rapid and precise technique to determine the genetic variability in WUE and transpiration rate, respectively in plants. In this study, genetic variability in both carbon and oxygen stable isotopes was examined among 15 upland cotton (*Gossypium hirsutum*) lines and significant genetic variability was noticed. We confirmed the relationship between Δ^{13}C with WUE and Δ^{18}O with transpiration rate among cotton lines. Therefore, measurement of stable isotopes can be used as a surrogate for time-averaged measurement of WUE and transpiration rate. We demonstrate that cotton lines with low Δ^{13}C and high Δ^{18}O accumulated higher biomass due to high WUE coupled with high photosynthetic capacity while maintaining relatively high transpiration. We illustrate the efficiency of selection of cotton lines based on dual stable isotopes (carbon and oxygen) to identify higher biomass.

INTRODUCTION

Cotton (*Gossypium hirsutum*) is usually grown in tropical and subtropical regions where water availability is often limited. In India, around 70% of total area covered by cotton (www.indiastat.com) is rain fed, therefore cotton plant is often exposed to drought which adversely affects biomass thus yield produced.

From an agronomic point of view, drought tolerance must be associated with sustained productivity. Selection of breeding lines for yield *per se* under water-limited conditions helps to achieve substantial yield improvement under water-limited conditions in C3 crops (Araus *et al.* 2002). However, breeding for physiological components of growth and yield sets the stage for target-oriented breeding and helps to pyramid known useful traits to achieve high yield potentials. Reynolds and Tuberosa (2008) explained several secondary traits that deserve exploitation for improving yield under stress conditions. Traits associated with maintaining high tissue water status under drought conditions such as efficient water extraction through a deeper root system, and efficient water use (i.e. water use efficiency or WUE) deserve further attention (Sheshshayee *et al.* 2003, 2010a). Although these traits are important, it is difficult to quantify them accurately, especially in large germplasm accessions and segregating populations. Therefore, there is a need to develop high-throughput techniques to screen large populations rapidly and accurately for water use and WUE traits.

Plants discriminate against the heavy isotope of carbon (^{13}C) during photosynthesis resulting in the depletion of the ^{13}C content in biomass (O'Leary 1981). This deviation of the carbon isotope ratio (^{13}C/^{12}C) of biomass from that of air is called discrimination (Δ^{13}C). In earlier studies, an inverse relationship between WUE and Δ^{13}C was noticed and ^{13}C discrimination is used as a time-integrated surrogate for identifying variation in WUE in several crop species such as wheat (Farquhar and Richards 1984; Condon *et al.* 1990, 2004), groundnut (Nageshwara Rao *et al.* 1993; Write *et al.* 1993, 1994; Nageshwara Rao *et al.* 1995; BinduMadhava *et al.* 2005), rice (Impa *et al.* 2005; Nadaradjan *et al.* 2005), cowpea (Ismail and Hall 1992, 1993; Ashok *et al.* 1999), and soybean (White *et al.* 1996). Similarly, experimental evidence of a correlation between WUE and Δ^{13}C in cotton has previously been provided (Saranga *et al.* 1998; Stiller *et al.* 2005). Saranga *et al.* (1998) showed the genetic variability in WUE and its association with photosynthetic rate and carbon isotope ratio among six commercial and semi-commercial cotton cultivars belonging to upland (*G. hirsutum*) and pima cotton (*G. barbadense*). Considerable genetic variability in WUE and Δ^{13}C among four Australian and three Texas upland cotton cultivars differing in leaf shape was reported (Stiller *et al.* 2005). They also documented a moderately-high broad sense heritability for Δ^{13}C rendering this parameter an useful tool for plant physiologists.

Similarly, the oxygen isotope ratio of plant organic material also provides useful information to plant physicologists. Water vapor molecules containing the lighter isotope of oxygen (^{16}O) diffuse relatively faster during evaporation and/or transpiration compared to molecules with a heavier oxygen isotope (^{18}O). Hence, ^{18}O gets enriched in the leaf water that is left behind and incorporated into leaf biomass. In fact, several reports have demonstrated that leaf water is indeed enriched with ^{18}O compared to the source or

Original Research Paper

xylem water (Flanagan *et al.* 1991; Farquhar and Lloyd 1993; Flanagan 1993; Yakir *et al.* 1993; Yakir 1994, 1998; Barbour and Farquhar 2000). Since the ^{18}O signature of leaf water is progressively imprinted in organic molecules (Sternberg *et al.* 1986), oxygen isotope enrichment in leaf biomass was demonstrated to have a positive correlation with stomatal conductance and thus transpiration rate at a given vapor pressure deficit (VPD) was observed (BinduMadhava *et al.* 1999; Sheshshayee *et al.* 2005). Therefore, $\Delta^{18}O$ in leaf biomass can be used as a surrogate for the time-averaged measurement of stomatal conductance and transpiration rate.

Blum (2005, 2009) opined that selection for high WUE generally leads to varieties with high water conservation and less water use leading to a small biomass and yield. This suggests the need to identify genotypes where WUE is predominantly regulated by maintaining high intrinsic photosynthetic capacity. Thus, high WUE will not be associated with a reduction in biomass. Therefore, selection of genotypes with high water use coupled with high WUE can sustain high biomass and thus yield under well watered and limited water conditions. Although the importance of these traits for improving bio-productivity under irrigated and water limited conditions is well known, lack of efficient techniques for quantification under field conditions in a large number of genotypes is the major limitation to use them in breeding programs. Thus, being rapid and accurate, stable carbon and oxygen isotope ratios provide powerful options in identifying desirable genotypes with superior photosynthetic capacity.

The objective of this study was to assess the genetic variability in carbon and oxygen stable isotopes and to standardize the dual isotope approach as a throughput technique to identify high bio-productivity in cotton lines.

MATERIALS AND METHODS

Plant material

Fifteen cotton lines belong to upland cotton (*Gossypium hirsutum*) were used in this study. These consisted of 10 lines (Br-5401 to Br-5410) obtained from the research station at the University of Agricultural Sciences, Dharward (south India) and the remaining five (LRA-5166, DIS-22, CNH-32, CNH-29I, DIS-380) were obtained from the Central Institute for Cotton Research (CICR) Nagpur (central India). Those lines from different parts of India were being used for developing drought-tolerant lines by conventional breeding. These lines were sown in carbonized rubber containers, with a capacity to hold 20 kg of soil consisting of mixture of red loamy soil and farmyard manure in a 3: 1 proportion (v/v). A single plant was maintained per container. The containers were arranged in a completely randomized design (CRD) with eight replications at a rate of two containers per replication. The experiment was conducted at the field facility of the Department of Crop Physiology, University of Agricultural Sciences, Bangalore, India.

WUE was determined at the whole plant level by gravimetry and at a single leaf level by the gas exchange method.

Gravimetric approach to determine WUE and associated physiological traits

WUE and associated physiological traits were measured as per Udayakumar *et al.* (1998b). Briefly, the method involves weighing containers on a daily basis using a mobile weighing device. The water status of the soil in containers was returned to field capacity every day during morning hours for the experimental duration from 50 days after sowing (DAS) to 80 DAS, which is consider the peak of vegetative growth in cotton; after that reduction in canopy photosynthesis and quantity of root was reported. The amount of water added over the experimental period of 30 days was summed up to arrive at the total evapo-transpiration (ET). Containers were arranged randomly under a mobile rainout shelter (Chauhan *et al.* 1997) which was moved over the experimental area at night and during rain episodes. At the start of the gravi-

metric experiment, the soil water status was brought to field capacity (FC) by adding the appropriate volume of water. The soil surface of all containers was covered with pieces of plastic to minimize evaporation of water from the soil surface. Simultaneously, control containers were filled with the same amount of soil mixture but without plants, which were also maintained at FC through the experimental period, and these were also weighed to quantify the evaporation component (E) of ET. Total water transpired by plants (i.e., cumulative water transpired or CWT) over the experimental period was calculated as the difference between ET and E. At the start (50 DAS) and at the end (80 DAS) of the experiment, roots were carefully removed from soil and dry biomass of leaf, stem and root were determined. The total leaf area was determined form each individual plant by using a leaf area meter (MK-2, Delta-T devices, England). The initial data (at 50 DAS) was recorded in two replications (four plants) whereas the remaining containers (six replications) were used to record the final data (at 80 DAS). WUE was computed from the ratio of biomass produced during the experimental period (30 days) to CWT and expressed in g biomass/liter water transpired. Mean transpiration rate (MTR) was computed from the ratio of CWT to leaf area duration (LAD) over the experimental period. LAD = {LA$_1$+ LA$_2$)/2} × 30 days, where LA$_1$ and LA$_2$ were leaf area at the beginning (50 DAS) and end (80 DAS) of the experiment, respectively. The net assimilation rate (NAR) is a time-averaged measurement of photosynthetic rate which was computed as the ratio of biomass produced during the experimental period to LAD.

Gas exchange measurements

At the single leaf level, WUE is the ratio of assimilation rate (A) to transpiration rate (T) (Caemerrer and Farquhar 1981; Farquhar and Sharkey 1982). The gas exchange parameters were recorded on the third fully expanded leaf from the apex on the main branch during almost at the middle of the experiment, on 60 and 65 DAS. One problem with gas exchange measurements is that they are instantaneous i.e. they are influenced by changes in daily environmental factors making it necessarily to make more measurements and then average the parameters. The observations were recorded when assimilation rate (A), stomatal conductance (g$_s$) and internal CO_2 concentration (C$_i$) reached a steady state value using a portable infrared gas analyzer (IRGA) (LICOR 6400, Lincoln, Nebraska, USA). Data shown is the average of six replications measured on 60 and 65 DAS with two plants for each replication.

Carbon stable isotope in leaf biomass

Carbon isotope ratio was determined with an isotope ratio mass spectrometer (IRMS; Delta-plus, ThermoFisher Scientific, Bremen, Germany) interfaced with an elemental analyzer (NA1112, Carlo-Erba, Italy) via a continuous flow device (Conflo-III, Thermo-Fisher Scientific). A composite leaf sample, comprised of 10 mature leaves representing all positions of the plant canopy, were harvested and oven dried for 3 days at 70°C and homogenized to a fine powder with a ball mill. Six replications from each cotton line were analyzed for $\delta^{13}C_{lb}$ with an analytical uncertainty of < 0.1‰. Carbon isotope discrimination ($\Delta^{13}C$ expressed in ‰ per mil) was computed as follows, assuming the isotopic composition of atmospheric air ($\delta^{13}C_a$) to be -8‰ relative to Pee Dee Belemnite (international standard) (Farquhar *et al.* 1989):

$$\Delta^{13}C = \{\delta^{13}C_a - \delta^{13}C_{lb}\}/ \{1+ (\delta^{13}C_{lb}/1000)\}.$$

Oxygen stable isotope in leaf biomass

The leaf powder samples used for carbon isotope ratio were also used to determine $\Delta^{18}O$ by on-line pyrolysis using TC/EA interfaced with IRMS (Delta-plus) through a continuous flow device (Conflo-III). ^{18}O enrichment in leaf biomass ($\Delta^{18}O$) was computed (Sheshshayee *et al.* 2005) as:

$$\Delta^{18}O (‰) = \delta^{18}O_{lb} - \delta^{18}O_{iw}$$

where $\delta^{18}O$ is the isotopic composition in relation to VSMOW (Vienna Standard Mean Ocean Water) and subscripts lb and iw

refer to leaf biomass and irrigation water, respectively. δ^{18}O in irrigated water, used during the experiment to irrigate plants, was determined by a CO_2-H_2O equilibrating device (Gas Bench-III) and has an oxygen isotope signature ($\delta^{18}O_{iw}$) of -3.75‰.

All stable isotope measurements were made at the National Facility for Stable Isotope Studies, Department of Crop Physiology, University of Agricultural Sciences, Bangalore, India.

Statistical methods

An analysis of variance (ANOVA) was performed for each character in this experiment; subsequently ANOVA was used to determine whether there were differences among cotton lines. Line means were separated by the use of protected critical difference (CD) at p ≤ 0.05 using MSTAT-C software (Anonymous 1998).

Fifteen cotton lines were classified based on dual stable isotope ratios using standard normal Z-distribution scores. Each geno-type means of Δ^{13}C and Δ^{18}O were converted to a Z score and plotted to categories into the four possible quadrants. The Z scores were obtained as follows (Srikanthbabu *et al.* 2002; Senthilkumar *et al.* 2006):

$$Z = (X - Xi)/SD$$

where X = mean over all genotypes for a character, Xi = mean of individual genotypes (over six replications), SD = standard deviation of error. However, genotypes that fell into quadrants (groups) I, II, III, and IV had high Δ^{18}O and low Δ^{13}C, high Δ^{18}O and high Δ^{13}C, low Δ^{18}O and high Δ^{13}C and low Δ^{18}O and low Δ^{13}C, respectively.

The relationship between different parameters was analyzed via simple linear regression. Regression was supported by Pearson's correlation analysis. The analysis was conducted based on genotype mean. One line, which turned out to be an outlier, was omitted in the regression analysis in **Fig. 1**.

RESULTS

Genetic variability in both carbon and oxygen stable isotope ratios was assessed amongst 15 cotton (*Gossypium hirsutum*) lines. The ^{13}C discrimination (Δ^{13}C) varied from 19.12 to 20.96‰ representing significant genetic variability (**Table 1**). As predicted by theory, Δ^{13}C was positively correlated with C_i/C_a and negatively with WUE (**Fig. 1**). Similarly, oxygen isotope enrichment (Δ^{18}O) also showed significant variation among the cotton lines (**Table 1**). The relationship of Δ^{18}O with stomatal conductance (g_s) and transpiration rate (T) (determined by gas exchange) were examined and a significant positive relationship between Δ^{18}O with g_s and T was noticed (**Fig. 2**). Also, Δ^{18}O and MTR (determined by gravimetric approach) was significantly associated (**Fig. 3**).

Total water transpired by a plant is a function of transpiration rate and the area of the plant canopy. There was a strong positive relationship between total water transpired by cotton plants during the experimental period and predicted total transpiration (**Fig. 4A**), which was computed by multiplying stable oxygen enrichment (as transpiration rate) with LAD (defined as functional leaf area during the experimental period). A significant relationship between root biomass and predicted total water transpired was also observed (**Fig. 4B**).

Cotton lines used in this investigation have been classified using the normal Z distribution method based on dual isotope ratios. Lines were distributed into four different quadrants (groups). The average biomass produced by the lines in group I with low Δ^{13}C (high WUE) and high Δ^{18}O (high transpiration rate) was significantly higher than that produced by lines in other groups (**Table 2**). In addition, mean photosynthetic rate (A), NAR as well as root biomass of lines in group I were also high (**Table 2**). The mesophyll capacity was examined for all different categories by assessing the C_i/g_s ratio in gas exchange (**Table 2**) hence, high mesophyll capacity. A significant positive relationship between Δ^{13}C to Δ^{18}O ratio and the C_i/g_s ratio was observed.

Table 1 Carbon isotope discrimination (Δ^{13}C) and oxygen isotope enrichment (Δ^{18}O) among fifteen cotton lines (*Gossypium hirsutum*).

Variety	Δ^{13}C(‰)	Δ^{18}O(‰)
Br-5401	20.29	26.39
Br-5402	19.12	25.87
Br-5403	20.62	26.31
Br-5404	20.56	26.06
Br-5405	20.25	26.56
Br-5406	20.11	26.06
Br-5407	20.72	25.77
Br-5408	20.17	25.79
Br-5409	20.17	25.81
Br-5410	20.75	26.78
LRA-5166	20.39	25.32
DIS -22	20.13	25.53
CNH-32	20.22	25.81
CNH-29 I	20.96	26.98
DIS-380	20.57	25.74
Mean	20.4	26.1
CD = 5%	0.46	1.43
CV %	4.45	5.1
p-value	0.008	0.0003

Data shown was average of 6 replicates, each replicate had two plants. CD: critical difference at 5% and CV: co-efficient of variance.
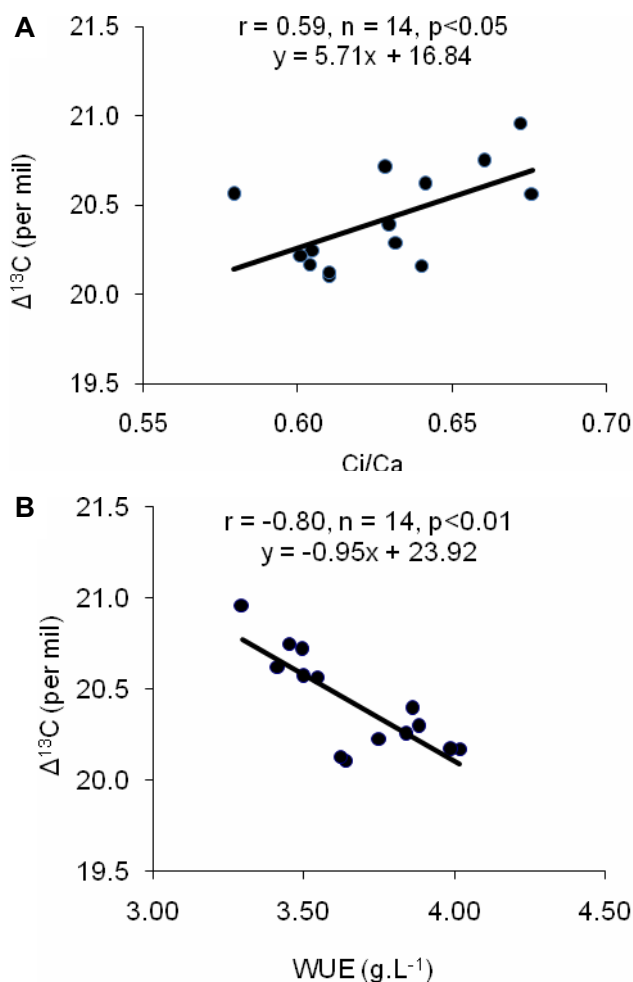
Fig. 1 Relationship between carbon isotope discrimination (Δ^{13}C) with (**A**) C_i/C_a and (**B**) water use efficiency (WUE) amongst 15 cotton lines (*G. hirsutum*).
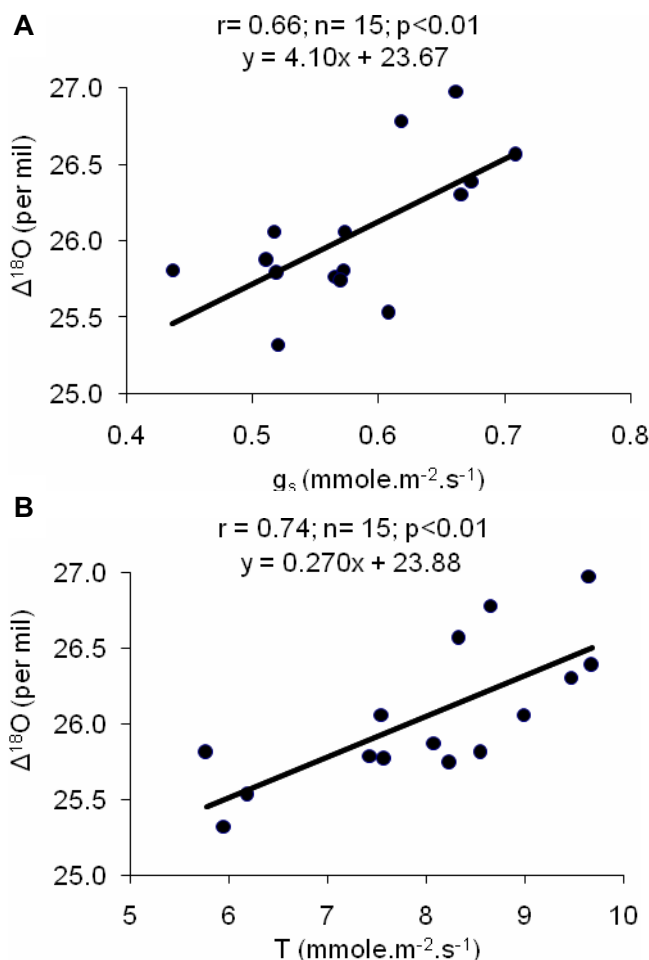
DISCUSSION

Breeding to improve crop productivity under water-limited conditions is a formidable challenge, owing mainly to the complexity of drought stress and equally complex crop responses. Although significant progress has been achieved in

Table 2 Classification of cotton lines (*Gossypium hirsutum*) based on dual stable isotope composition.

Groups	n	CWT	TDM	WUE	NAR	Root wt.	A	g_s	A/g_s	C_i/g_s
		L.plant^{-1}	g.plant^{-1}	g.L^{-1}	g.m^{-2}.d^{-1}	g.plant^{-1}	µmol.m^{-2}.s^{-1}	mmol.m^{-2}.s^{-1}		
I	3	14.54	55.77	3.86	6.76	7.25	41.89	0.60	69.74	348.55
II	5	13.10	50.63	3.85	6.66	5.58	38.14	0.53	72.49	424.11
III	3	13.76	50.48	3.68	6.47	4.93	35.98	0.55	67.75	435.63
IV	4	15.59	53.31	3.43	6.47	6.59	38.88	0.59	66.82	410.89
CD = 5%		1.00	2.80	0.14	0.19	0.50	2.00	0.02	1.79	39.25
p-value		0.001	0.007	0.001	0.01	0.0001	0.001	0.003	0.02	0.0014

Groups: I: High Δ^{18}O Low Δ^{13}C, II: Low Δ^{18}O low Δ^{13}C, III: Low Δ^{18}O high Δ^{13}C, IV: High Δ^{18}O high Δ^{13}C, n: Number of genotypes belong to each group. CD: critical difference at 5%

Fig. 2 Relationship between oxygen isotope enrichment (Δ^{18}O) with (**A**) stomatal conductance (gs) and (**B**) transpiration rate (T) amongst 15 cotton lines (*G. hirsutum*).

breeding for drought tolerance through selection for high absolute yields under stress, its application in breeding programs catering for a wide range of environments is becoming increasingly limited (Branch and Hildebrand 1989; Cooper and Hammer 1996; Jackson *et al.* 1996; Araus *et al.* 2002). Hence, introgression of a few important and relevant drought tolerance traits into a single genetic background through a "trait-based" breeding approach is being suggested as the most plausible strategy.

WUE and root traits as important traits

Several plant traits relevant to improving drought tolerance have been identified (Reynolds and Tuberosa 2008). However, any drought tolerance trait would have relevance only if associated with superior growth rates under water limited conditions (Udayakumar and Prasad 1994; Blum 2005). Hence, bio-productivity under stress conditions depends on the ability of a plant to harness water from deeper soil profiles associated with good root system and use the extracted

water more efficiently (WUE) to produce more biomass (Richard *et al.* 2002; Condon *et al.* 2004). Significant genetic variability in root traits and WUE have been reported which raises the hopes for exploiting these traits (Udayakumar *et al.* 1998a; Li *et al.* 2005; AbouKheir *et al.* 2006, 2008). Despite the existence of exploitable variability in WUE and root traits, progress in breeding for these traits has been rather slow. The lack of reliable techniques to assess the variability in large germplasm accessions and segregating population were perhaps the most notable constraints in breeding for drought stress.
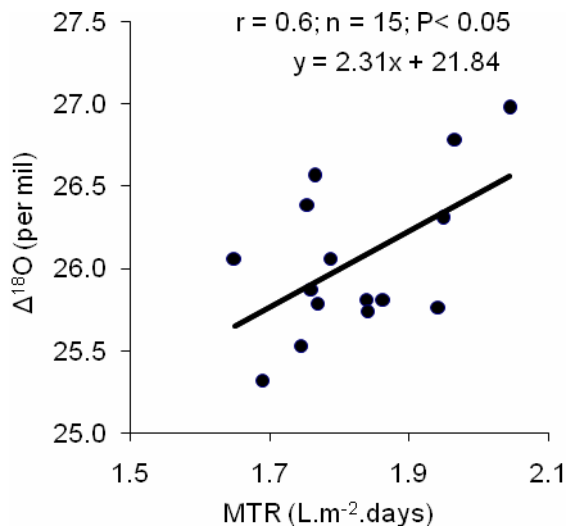
Δ^{13}C as a surrogate for WUE

The discovery that plants discriminate against the heavy carbon isotope (O'Leary 1981) and establishment of the theory linking Δ^{13}C with WUE through correlation with the C_i/C_a ratio (Farquhar *et al.* 1989) significantly removed the lacuna in assessing variability in WUE. Our results reconfirmed the relationship between Δ^{13}C with the C_i/C_a ratio thus with WUE in cotton (**Fig. 1**). ^{13}C discrimination is used as a time-integrated surrogate for WUE in cotton (Stiller *et al.* 2005; AbouKheir *et al.* 2008). Many studies have shown significant genetic variability in Δ^{13}C in many species (Farquhar *et al.* 1989; Hall *et al.* 1994; Condon and Hall 1997; Saranga *et al.* 1998; Sheshshayee *et al.* 2003; Impa *et al.* 2005; Stiller *et al.* 2005) and moderately high heritability in Δ^{13}C (Stiller *et al.* 2005). In this study, we demonstrated that genetic variability in Δ^{13}C exists amongst cotton lines (**Table 1**).

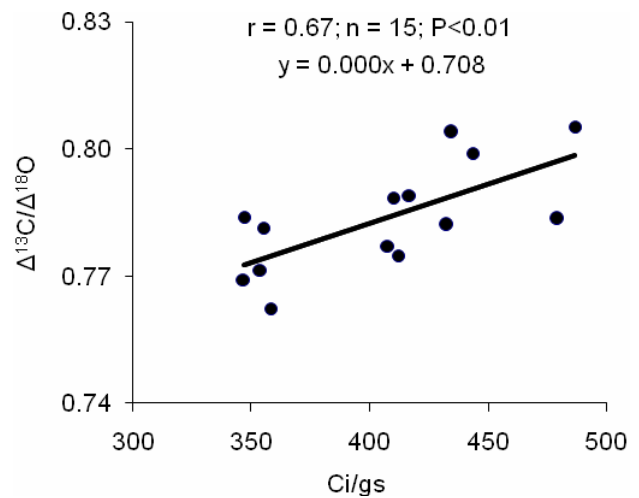**Δ^{18}O as a surrogate for transpiration rate**

Besides WUE, the total biomass accumulated is a function of the total water used through transpiration (Passioura 1986). During transpiration, the enrichment of leaf mater with the heavy isotope of oxygen is well known (Flanagan *et al.* 1991; Farquhar and Lloyd 1993; Flanagan 1993; Yakir *et al.* 1993; Yakir 1994, 1998; Barbour and Farquhar 2000). This is because a lower vapor pressure of the heavy isotope coupled with its slower diffusivity. Thus increased transpiration either due to higher stomatal conductance or high VPD results in enrichment ^{18}O in leaf mater. The transfer of oxygen isotope signature into biomass through carboxyl displacement is well elucidated (Sternberg *et al.* 1986). Hence, the Δ^{18}O of leaf biomass serves as a powerful time-averaged surrogate for stomatal conductance at a given VPD (Udayakumar *et al.* 1998a; BinduMadhava *et al.* 2005; Sheshshayee *et al.* 2005, 2010b). In this study, a significant relationship between Δ^{18}O with g_s and Δ^{18}O with T were seen amongst 15 cotton lines (**Fig. 2**), Δ^{18}O and MTR was also related significantly (**Fig. 3**). Since all cotton lines examined in this investigation were kept under the same environment condition, the oxygen isotope ratio hence would strongly represent the variations in stomatal conductance and transpiration.
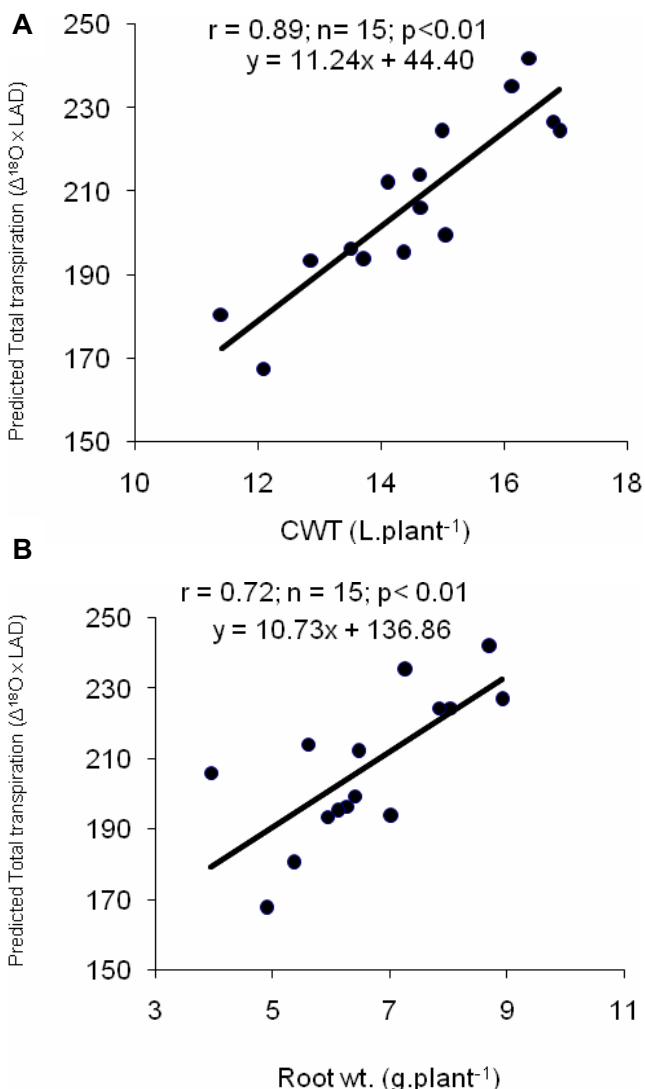
Although water loss by transpiration is influenced by leaf orientation on the canopy, surface characters of the leaf, stomatal sensitivity, etc., total transpiration is predominantly influenced by evaporating surface area of the canopy and transpiration rate that is strongly related with the extent of root development to supply water to match the transpi-

Fig. 3 Relationship between oxygen isotope enrichment (Δ^{18}O) and mean transpiration rate (MTR) amongst 15 cotton lines (*G. hirsutum*).

Fig. 4 Relationship between predicted total water transpired (Δ^{18}O × LAD) with (**A**) cumulative water transpired (CWT) and (**B**) root biomass (g) amongst 15 cotton lines (*G. hirsutum*).

Fig. 5 Relationship between C_i/g_s ratio with stable isotope ratio (Δ^{13}C/Δ^{18}O) amongst 15 cotton lines (*G. hirsutum*).

shown). However, measuring a root system accurately is quite difficult and tedious work thus, our results demonstrate an ability to estimate root biomass with the help of oxygen isotope enrichment technique (**Fig. 4B**).

Δ^{13}C to Δ^{18}O ratio a good estimate of photosynthetic capacity

The amount of CO_2 fixed per unit of water transpired is an interesting physiological yardstick of WUE but our interest here is in maximizing CO_2 fixation under drought stress per unit leaf area. Hence, plants may need more photosynthetic capacity to produce a relatively equivalent amount of dry matter compared to the situation under non-stress conditions (Songsri *et al.* 2009). An earlier report demonstrated a rapid and efficient way to estimate mesophyll capacity by the ratio of intercellular CO_2 and stomatal conductance (C_i/g_s). Thus, at a given stomatal conductance, variations in intercellular CO_2 should be dependent on the efficiency of mesophyll to fix the available substrate CO_2 (Sheshshayee *et al.* 1996). Though rapid, gas exchange measurements are instantaneous and hence not reliable especially in highly changing environments and in a large number of genotypes. Carbon and oxygen isotope ratios of plant organic matter integrate the diurnal as well as seasonal variations in C_i and g_s, respectively (Hubick *et al.* 1988; Farquhar *et al.* 1989; BinduMadhava *et al.* 1999; Sheshshayee *et al.* 2003, 2005). Demonstration of carbon and oxygen stable isotope ratio (Δ^{13}C/Δ^{18}O) as a good time-averaged estimate of *in vivo* photosynthetic capacity was reported by BinduMadhava *et al.* (2005). They estimated the photosynthetic capacity by measuring the initial slope of CO_2 response curve as well as C_i/g_s amongst contrasting genotypes of cowpea and groundnut that differed in transpiration rate and WUE. A similar relationship was noticed in our study (**Fig. 5**) that clearly indicated the relevance of the dual isotope ratio in identifying a high photosynthetic capacity type.

Dual stable isotopes as an efficient tool to identify "Capacity Types"

Despite the establishment of the relevance of WUE and development of a powerful high throughput screening option, many breeding attempts to improve WUE were not successful. The major reason is naturally plants tend to increase WUE by decreasing transpiration rate through a reduction in stomatal conductance thus, a decrease in biomass produced (Richards and Condon 1993; Richards 1996; Condon *et al.* 2002, 2004; Blum 2009). In the improvement of crop plants, it is extremely important to combine high WUE with relatively high water use so that there is no re-

ration demand. Thus, transpiration for a given leaf area must be related to root biomass and hence could be a good indicator of root traits. A significant relationship was noticed between root biomass and cumulative water transpired during the experimental period among cotton lines (data not

duction in biomass produced. Towards identifying such lines, 15 cotton lines were classified into four categories based on their differences in $\Delta^{13}C$ and $\Delta^{18}O$. The $\Delta^{13}C$ and $\Delta^{18}O$ values were transformed to normal Z distribution therefore; the lines segregated into four groups differ in the composition of two stable isotopes. Group II showed high WUE similar to group I but with less biomass produced (**Table 2**); this group belongs to conductance types where WUE increased through a decrease in transpiration primarily coupled with a reduction in g_s (**Table 2**). It is therefore, not surprising that selection for those lines (group II) resulted in smaller plants that use less water over the season (Blum 2005). However, high productivity is associated with greater water use (Blum 2009) and such lines belonging to group IV showed highest water transpired but low WUE (**Table 2**). Thus, increasing water use would be more rewarding under conditions where water availability is not a limitation. Several reports showed that high yielding cotton, wheat and rice genotypes have greater stomatal conductance and transpiration under drought stress (Blum *et al.* 1982; Sanguneti *et al.* 1999; Araus *et al.* 2002; Izanloo *et al.* 2008; Blum 2009). Although lines in group IV produced a good biomass but they showed high CWT and thus are water spenders. Since around 70% of cotton areas in India is under rain fed such water spenders genotypes may not be very appropriate. Therefore, WUE can be a potential physiological trait provided the photosynthetic carbon assimilatory capacity determines the difference in WUE. Such a type, called the "Capacity type" (Udayakumar *et al.* 1998; Scheidegge *et al.* 2000), like group I, has higher WUE without any substantial reduction in water use because of a good root system capable to harnessing water from a deeper soil profile to sustain the transpiration demand (**Table 2**). At any given water regime, lines in group I will produce higher biomass and are hence highly suited for both irrigated and intermittent stress situations.

In summary, this study revealed the importance of using dual (carbon and oxygen) stable isotope ratios as time-average surrogates for WUE and transpiration rate, respectively. The two stable isotopes can provide an excellent tool to identify the "Capacity type" capable of producing high bio-productivity under both irrigated and rain-fed conditions in cotton.

ACKNOWLEDGEMENTS

REFERENCES

AbouKheir E, BinduMadhava H, Sheshshayee MS, Prasad TG, Udaya-kumar M (2006) Genetic variability in WUE and its physiological control among cotton hybrids and cultivars. In: *The First International Conference on the Theory and Practices in Biological Water Saving (ICTPB)*, 21-25 May 2006, Beijing, China, p 134 (Abstract)

AbouKheir E, Sheshshayee MS, Udayakumar M, Prasad TG (2008) Assessing the genetic variability in whole plant WUE and associated physiological traits in cotton. In AAB International Conference on "Resource Capture by Crops: Integrated Approach", 14-16 September 2008, University of Nottingham at Sutton Bonington Campus, UK (Abstract)

Anonymous (1998) Compendium of environment statistics: Central statistical organization. Department of Statistics, Ministry of Planning and Program Implementation, GOI, New Delhi (Abstract)

Araus JL, Slafer GA, Reynolds MP, Royo C (2002) Plant breeding and drought in C3 cereals: What should we breed for? *Annals of Botany* **89**, 925-940

Ashok IS, Hussein A, Prasad TG, Udayakumar M, Nageswara Rao RC, Wright GC (1999) Variation in transpiration efficiency and carbon isotope discrimination in cowpea (*Vigna unguiculata* (L.) Walp) genotypes. *Australian Journal of Plant Physiology* **21**, 507-516

Barbour MM, Farquhar GD (2000) Relative humidity and ABA induced variation in carbon and oxygen isotope ratio of cotton leaves. *Plant Cell and Environment* **23**, 473-483

BinduMadhava H, Sheshshayee MS, Devendra R, Prasad TG, Udayakumar M (1999) Oxygen ($\delta^{18}O$) isotope enrichment in the leaves as a potential surrogate for transpiration and stomatal conductance. *Current Science* **76 (11)**, 1427-1428

BinduMadhava H, Sheshshayee MS, Shashidhar G, Prasad TG, Udaya-kumar M (2005) Ratio of stable carbon and oxygen isotope discrimination ($\Delta^{13}C/ \delta^{18}O$) reflects variability in leaf intrinsic carboxylation efficiency in plants. *Current Science* **189 (7)**, 1256-1258

Blum A (2005) Drought resistance, water-use efficiency and yield potential – are they compatible, dissonant or mutually exclusive? *Australian Journal of Agricultural Research* **56**, 1159-1168

Blum A (2009) Effective use of water (EUW) and not water use efficiency (WUE) is the target of crop yield improvement under drought stress. *Field Crops Research* **112**, 119-123

Blum A, Mayer J, Gozlan G (1982) Infrared thermal sensing of plant canopies as a screening technique for dehydration avoidance in wheat. *Field Crops Research* **5**, 137-146

Branch WD, Hildebrand GL (1989) Pod yield comparison of pure line groundnut selections simultaneously developed from Georgia and Zimbabwe breeding programs. *Plant Breeding* **102**, 260-263

Caemerrer SV, Farquhar GD (1981) Some relationship between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153**, 378-383

Cernusak LA, Winter K, Aranda J, Turner BL (2008) Conifers, angiosperm trees, and lianas: growth, whole-plant water and nitrogen use efficiency, and stable isotope composition ($\Delta^{13}C$ and $\Delta^{18}O$) of seedlings grown in a tropical environment. *Plant Physiology* **148**, 642-659

Chauhan YS, Saxena NP, Nageswara Rao RC, Johansen C, Ravindranath K (1997) Portable rainout shelter, a useful tool in drought research. *ACIAR Food Legume Newsletter* **25**, 9

Condon AG, Farquhar GD, Richards RA (1990) Genotypic variation in carbon isotope discrimination and transpiration efficiency in wheat: Leaf gas exchange and whole plant studies. *Australian Journal of Plant Physiology* **17**, 9-22

Condon AG, Hall KT (1997) Adaptation to adverse environments; variation in WUE within crop species. In: Jackson LE (Ed) *Ecology in Agriculture*, Academic Press, San Diego, USA, pp 89-116

Condon AG, Richards RA, Rebetzke GJ, Farquhar GD (2004) Breeding for high water use efficiency. *Journal of Experimental Botany* **55**, 2447-2460

Condon, AG, Richards RA, Rebetzke GJ, Farquhar GD (2002) Improving intrinsic water use efficiency and crop yield. *Crop Science* **42**, 122-131

Cooper M, Hammer GL (1996) *Plant Adaptation and Crop Improvement*, CAB International, Wallingford, UK, 656 pp

Farquhar GD, Hubick KT, Condon AG, Richards RA (1989) Carbon isotope fractionation and plant water use efficiency. In: Rundel PW, Ehleringer JR, Nagy KA (Eds) *Stable Isotope in Ecological Research*, Springer-Verlag, New York, pp 21-40

Farquhar GD, Lloyd J (1993) Carbon and oxygen isotope effects in the exchange of carbon dioxide between terrestrial plants and the atmosphere. In: Ehleringer JR, Hall AE, Farquhar GD (Eds) *Stable Isotopes and Plant Carbon–Water Relations*, Academic Press, San Diego, USA, pp 47-70

Farquhar GD, Richards RA (1984) Isotopic composition of plant carbon correlates with water use efficiency of wheat cultivars. *Australian Journal of Plant Physiology* **11**, 539-552

Farquhar GD, Sharkey TD (1982) Stomatal conductance and photosynthesis. *Annual Review of Plant Physiology* **7**, 315-345

Flanagan LB (1993) Environmental and biological influence on the stable oxygen and hydrogen isotopic composition of leaf water. In: Ehleringer JR, Hall AE, Farquhar GD (Eds) *Stable Isotopes and Plant Carbon–Water Relations*, Academic Press, San Diego, pp 71-90

Flanagan LB, Brain JF, Ehleringer JR (1991) Stable oxygen and hydrogen isotope composition of leaf water in C₃ and C₄ plant species under field conditions. *Oecologia* **88**, 394-400

Hall AE, Richards RA, Condon AG, Wright GC, Farquhar GD (1994) Carbon isotope discrimination and plant breeding. *Plant Breeding Review* **12**, 81-113

Hubick KT, Shorter R, Farquhar GD (1988) Heritability and genotype × environment interaction of carbon isotope discrimination and transpiration efficiency in peanut. *Australian Journal of Plant Physiology* **15**, 799-813

Impa SM, Nadaradjan S, Boominathan P, Shashidhar G, Bindhumadhava H, Sheshshayee MS (2005) Carbon isotope discrimination accurately reflects variability in WUE measured at a whole plant level in rice. *Crop Science* **45**, 2517-2522

Ismail AM, Hall AE (1992) Correlation between water use efficiency and carbon isotope discrimination in diverse cowpea genotypes and isogenic lines cowpea. *Crop Science* **32**, 7-12

Ismail AM, Hall AE (1993) Inheritance of carbon isotope discrimination and water use efficiency in cowpea. *Crop Science* **33**, 498-503

Izanloo A, Condon AG, Langridge P, Tester M, Schnurbusch T (2008) Different mechanisms of adaptation to cyclic water stress in two South Australian bread wheat cultivars. *Journal of Experimental Botany* **59**, 3327-3346

Jackson P, Robertson M, Cooper M, Hammer G (1996) The role of physio-

logical understanding in plant breeding; from a breeding perspective. *Field Crops Research* **49**, 11-39

Li Z, Mu P, Li C, Zhang H, Li Z, Gao Y, Wang X (2005) QTL mapping of root traits in a doubled haploid population from a cross between upland and lowland *japonica* rice in three environments. *Theoretical and Applied Genetics* **110**, 1244-1252

Nadaradjan S, Impa SM, Shashidhar GP, Sheshshayee MS, Udayakumar M, Prasad TG (2005) Overlapping QTLs for WUE and carbon isotope discrimination in DHLs of rice. *Journal of Plant Biology* **32**, 1-8

Nageswara Rao RC, Udayakumar M, Farquhar GD, Talwar HS, Prasad TG (1995) Variation in carbon isotope discrimination and its relationship to specific leaf area and ribulose-1,5-bisphosphate carboxylase content in groundnut genotypes. *Australian Journal of Plant Physiology* **22**, 545-551

Nageswara Rao RC, Williams JH, Wadia KDR, Hubuck KT, Farquhar GD (1993) Crop growth, WUE and CID in groundnut (*Arachis hypogaea L.*) genotypes under end season drought conditions. *Annuls of Applied Biology* **122**, 357-367

O'Leary MH (1981) Carbon isotope fractionation in plants. *Phytochemistry* **20**, 553-567

Passioura JB (1986) Resistance to drought and salinity: Avenues for improvement. *Australian Journal of Plant Physiology* **13**, 191-201

Reynolds M, Tuberosa R (2008) Translational research impacting on crop productivity in drought-prone environments. *Current Opinion in Plant Biology* **11**, 171-179

Richards RA (1996) Defining selection criteria to improve yield under drought. *Plant Growth Regulation* **20**, 157-166

Richards RA, Condon AG (1993) Challenges ahead in using carbon isotope discrimination in plant-breeding programs. In: Ehleringer JR, Hall AE, Farquhar GD (Eds) *Stable Isotopes and Plant Carbon–Water Relations*, Academic Press, New York, pp 451-462

Richards RA, Rebetzke GJ, Condon AG, Van Herwaarden AF (2002) Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. *Crop Science* **42**, 111-121

Sanguineti MC, Tuberosa R, Landi P, Salvi S, Maccaferri M, Casarini E, Conti S (1999) QTL analysis of drought related traits and grain yield in relation to genetic variation for leaf abscisic acid concentration in field-grown maize. *Journal of Experimental Botany* **50**, 1289-1297

Saraga Y, Flash I, Yakir D (1998) Variation in water use efficiency and its relation to carbon isotope ratio in cotton. *Crop Science* **38**, 782-787

Scheindegger Y, Saurer M, Bahn M, Siewolf RTW (2000) Linking stable oxygen and carbon isotopes with stomatal conductance and photosynthetic capacity: A conceptual model. *Oecologia* **125**, 350-357

Senthilkumar M, Kumar G, Srikanthbabu V, Udayakumar M (2006) Assessment of variability in acquired thermotolerance: Potential option to study genotypic response and the relevance of stress genes. *Journal of Plant Physiology* **164**, 111-125

Sheshshayee MS, Abou Kheir E, Sreevathsa R, Srivastava N, Mohanraju B, Karaba NN, Prasad TG, Udayakumar M (2010a) Sustaining crop productivity through improvement in root traits: Phenotyping and breeding for relevant traits. In: de Oliveira AD (Ed) *Root Genomics*, Springer, the Netherlands (in press)

Sheshshayee MS, BinduMadhava H, Ramesh R, Prasad TG, Lakshminarayana MR, Udayakumar M (2005) Oxygen isotope enrichment (Δ^{18}O) as a measure of time averaged transpiration rate. *Journal of Experimental Botany* **422 (56)**, 3033-3039

Sheshshayee MS, BinduMadhava H, Shankar AG, Prasad TG, Udaya-

kumar M (2003) Breeding strategies to exploit water use efficiency for crop improvement. *Journal of Plant Biology* **30 (2)**, 253-268

Sheshshayee MS, Krishnaprasad BT, Natraj KN, Prasad TG, Shankar AG, Udayakumar M (1996) Ratio of intercellular CO_2 concentration to stomatal conductance is a reflection of mesophyll efficiency. *Current Science* **70**, 671-674

Sheshshayee MS, BinduMadhava H, Ramesh R, Prasad TG, Udayakumar M (2010b) Relationship between oxygen isotope enrichment (Δ^{18}O) in leaf water, biomass stomatal conductance. *Isotopes in Environment and Health Studies* **46 (1)**, 122-129

Songsri P, Jogloy S, Holbrook CC, Kesmala T, Vorasoot N, Akkasaeng C, Patanothai A (2009) Association of root, specific leaf area and SPAD chlorophyll meter reading to water use efficiency of peanut under different available soil water. *Agriculture Water Management* **96**, 790-798

Srikanthbabu V, Kumar G, Krishnaprasad BT, Gopalakrishna R, Savitha M, Udayakumar M (2002) Identification of pea genotypes with enhanced thermotolerance using temperature induction response (TIR) technique. *Journal of Plant Physiology* **159**, 535-545

Sternberg LSL, DeNiro MJ, Savidge RA (1986) Oxygen isotope exchange between metabolites and water during biochemical reactions leading to cellulose synthesis. *Plant Physiology* **82**, 423-427

Stiller WN, Read JJ, Constable GA, Reid PE (2005) Selection for water use efficiency traits in cotton breeding program cultivar difference. *Crop Science* **45**, 1107-1113

Udayakumar M, Prasad TG (1994) ^{13}C isotope discrimination in plants – A potential technique to determine WUE. In: Nageswara Rao RC, Wright GC (Eds) *Selection for WUE in Grain Legumes*, Report of Workshop held at ICRISAT. Andhra Pradesh, India, pp 42-45

Udayakumar M, Rao RCN, Wright GC, Ramaswamy GC, Stephan AR, Gangadhar GC, Aftab Hussain IS (1998a) Measurement of transpiration efficiency in field condition. *Journal of Plant Physiology and Biochemistry (New Delhi)* **1**, 69-75

Udayakumar M, Sheshshayee MS, Nataraj KN, BinduMadhava H, Devendra R, Aftab Hussain IS, Prasad TG (1998b) Why breeding for water use efficiency has not been successful. An analysis and alternate approach to exploit this trait for crop improvement. *Current Science* **74**, 994-1000

White DS, Bell MJ, Wright GC (1996) The potential to use carbon isotope discrimination as a selection tool to improve water use efficiency in soybean. In: *Agronomy – Science with its Sleeves Rolled Up. Proceeding of the 8th Australian Agronomy Conference*, Toowoomba, Queensland, p X (Abstract)

Wright GC, Hubick KT, Farquhar GD, Nageswara Rao RC (1993) Genetic and environmental variation in transpiration efficiency and its correlation with carbon isotope discrimination and specific leaf area in peanut. In: Ehleringer JR, Hall AE, Farquhar GD (Eds) *Stable Isotopes and Plant Carbon–Water Relations*, Academic Press, San Diego, USA, pp 22-67

Wright GC, Nageswara Rao RC, Farquhar GD (1994) Water use efficiency and carbon isotope discrimination in peanut under water deficit conditions. *Crop Science* **34**, 92-97

Yakir D (1994) Variation in the natural abundance of oxygen-18 enrichment in leaf water of cotton plants grown under wet and dry conditions: evidence for water compartmentation and its dynamics. *Plant, Cell and Environment* **13**, 49-56

Yakir D, Berry JA, Giles L, Osmond CB (1993) The ^{18}O of water in the metabolic compartment of transpiring leaves. In: Ehleringer JR, Hall AE, Farquhar GD (Eds) *Stable Isotopes and Plant Carbon–Water Relations*, Academic Press, San Diego, USA, pp 528-540