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

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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 $\Delta^{13}$C with WUE and $\Delta^{18}$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 demonstrated that cotton lines with low $\Delta^{13}$C and high $\Delta^{18}$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.

Keywords: cotton, dual stable isotope ratios, water use efficiency

Abbreviations: A, assimilation rate; $C_{i}$, internal CO$_2$ concentration; CWT, cumulated water transpired; DAS, days after sown; $\Delta^{13}$C, carbon stable isotope discrimination; ET, evapo-transpiration; E, evaporation; $F_{c}$, field capacity; $g_{s}$, stomatal conductance; LAD, leaf area duration; MTR, mean transpiration rate; NAR, net assimilation rate; $\Delta^{18}$O, oxygen stable isotope enrichment; T, transpiration rate; VPD, vapor pressure deficit; WUE, water use efficiency

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 accesses 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 ($\Delta^{13}$C). In earlier studies, an inverse relationship between WUE and $\Delta^{13}$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 $\Delta^{13}$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 $\Delta^{13}$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 $\Delta^{13}$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 physiologists. 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
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 $^{18}\text{O}$ 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. 1985; Sheshshayee et al. 2005). Therefore, $^{18}\text{O}$ 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 belonging to upland cotton (*Gossypium hirsutum*) were used in this study. These consisted of 10 lines (Br5401 to Br5410) 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-291, 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 considered 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 gravimetric 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 = (LA1 + LA2)/2 × 30 days, where LA1 and LA2 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 (gs) and internal CO2 concentration (Ci) 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 (Confo-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 $\delta^{13}\text{C}$ with an analytical uncertainty of < 0.1‰. Carbon isotope discrimination ($\Delta^{13}\text{C}$ expressed in ‰ per mil) was computed as follows, assuming the atmospheric CO2 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.

$$\Delta^{13}\text{C} = \left( \delta^{13}\text{C}_{p} - \delta^{13}\text{C}_{b} \right)/\left( 1 + \left( \delta^{13}\text{C}_{b}/1000 \right) \right)$$

**Oxygen stable isotope in leaf biomass**

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

$$\Delta^{18}\text{O} = \Delta^{18}\text{O}_{b} - \Delta^{18}\text{O}_{w}$$

where $\Delta^{18}\text{O}$ is the isotopic composition in relation to VSMOW (Vienna Standard Mean Ocean Water) and subscripts b and w
refer to leaf biomass and irrigation water, respectively. $\delta^{18}O$ in irrigated water, used during the experiment to irrigate plants, was determined by a CO$_2$-H$_2$O equilibrating device (Gas Bench-III) and has an oxygen isotope signature ($\delta^{18}O_{\text{water}}$) 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 $p \leq 0.05$ 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 $\Delta^{13}C$ and $\Delta^{18}O$ 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 $\Delta^{18}O$ and low $\Delta^{13}C$, high $\Delta^{18}O$ and high $\Delta^{13}C$, low $\Delta^{18}O$ and high $\Delta^{13}C$ and low $\Delta^{18}O$ and low $\Delta^{13}C$, 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 $\Delta^{13}C$ discrimination ($\Delta^{13}C$) varied from 19.12 to 20.96‰ representing significant genetic variability (Table 1). As predicted by theory, $\Delta^{13}C$ was positively correlated with $C_i/C_a$ and negatively with WUE (Fig. 1). Similarly, oxygen isotope enrichment ($\Delta^{18}O$) also showed significant variation among the cotton lines (Table 1). The relationship of $\Delta^{18}O$ with stomatal conductance ($g_s$) and transpiration rate ($T$) (determined by gas exchange) were examined and a significant positive relationship between $\Delta^{18}O$ with $g_s$ and $T$ was noticed (Fig. 2). Also, $\Delta^{18}O$ 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 $\Delta^{13}C$ (high WUE) and high $\Delta^{18}O$ (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 $\Delta^{13}C$ to $\Delta^{18}O$ ratio and the $C_i/g_s$ ratio was observed.

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

<table>
<thead>
<tr>
<th>Variety</th>
<th>$\Delta^{13}C$(‰)</th>
<th>$\Delta^{18}O$(‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br-5401</td>
<td>20.29</td>
<td>26.39</td>
</tr>
<tr>
<td>Br-5402</td>
<td>19.12</td>
<td>25.87</td>
</tr>
<tr>
<td>Br-5403</td>
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</tr>
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</tr>
<tr>
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<td>26.06</td>
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<tr>
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<td>25.79</td>
</tr>
<tr>
<td>Br-5409</td>
<td>20.17</td>
<td>25.81</td>
</tr>
<tr>
<td>Br-5410</td>
<td>20.75</td>
<td>26.78</td>
</tr>
<tr>
<td>LRA-5166</td>
<td>20.39</td>
<td>25.32</td>
</tr>
<tr>
<td>CNH-32</td>
<td>20.22</td>
<td>25.81</td>
</tr>
<tr>
<td>CNH-291</td>
<td>20.96</td>
<td>26.98</td>
</tr>
<tr>
<td>DIS-380</td>
<td>20.57</td>
<td>25.74</td>
</tr>
<tr>
<td>Mean</td>
<td>20.4</td>
<td>26.1</td>
</tr>
<tr>
<td>CD = 5%</td>
<td>0.46</td>
<td>1.43</td>
</tr>
<tr>
<td>CV %</td>
<td>4.45</td>
<td>5.1</td>
</tr>
</tbody>
</table>

$p$-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.
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 Tuberousa 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.

### Δ13C as a surrogate for WUE

The discovery that plants discriminate against the heavy carbon isotope (O’Leary 1981) and establishment of the theory linking Δ13C with WUE through correlation with the C/12C ratio (Farquhar et al. 1989) significantly removed the lacuna in assessing variability in WUE. Our results confirmed the relationship between Δ13C with the C/12C ratio thus with WUE in cotton (Fig. 1). 13C 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 Δ13C 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 Δ13C (Stiller et al. 2005). In this study, we demonstrated that genetic variability in Δ13C exists amongst cotton lines (Table 1).

### Δ18O 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 material 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 18O in leaf material. The transfer of oxygen isotope signature into biomass through carboxyl displacement is well elucidated (Shenberberg et al. 1986). Hence, the Δ18O of leaf biomass serves as a powerful time-averaged surrogate for stomatal conductance at a given VPD (Udayakumar et al. 1998a; BinuduMadhava et al. 2005; Sheshshayee et al. 2005, 2010b). In this study, a significant relationship between Δ18O with g, and Δ18O with T were seen amongst 15 cotton lines (Fig. 2). Δ18O 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 conduc-
tance 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-

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<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>CWT L.plant1</th>
<th>TDM g.plant1</th>
<th>WUE g.L-1</th>
<th>NAR g.m-2.d-1</th>
<th>Root wt. g.g.plant1</th>
<th>A μmol.m-2.s-1</th>
<th>g. mmol.m-2.s-1</th>
<th>A/g.</th>
<th>C/g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
<td>14.54</td>
<td>55.77</td>
<td>3.86</td>
<td>6.76</td>
<td>7.25</td>
<td>41.89</td>
<td>0.60</td>
<td>69.74</td>
<td>348.55</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>13.10</td>
<td>50.63</td>
<td>3.85</td>
<td>6.66</td>
<td>5.58</td>
<td>38.14</td>
<td>0.53</td>
<td>72.49</td>
<td>424.11</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>13.76</td>
<td>50.48</td>
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<td>IV</td>
<td>4</td>
<td>15.59</td>
<td>53.31</td>
<td>3.43</td>
<td>6.47</td>
<td>6.59</td>
<td>38.14</td>
<td>0.53</td>
<td>72.49</td>
<td>424.11</td>
</tr>
</tbody>
</table>

CD = 5% 1.00 2.80 0.14 0.19 0.50 2.00 0.02 1.79 39.25

**Fig. 2** Relationship between oxygen isotope enrichment (Δ18O) with (A) stomatal conductance (g.s) and (B) transpiration rate (T) amongst 15 cotton lines (*G. hirsutum*).

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2006, 2010b). In this study, a significant relationship between Δ18O with g, and Δ18O with T were seen amongst 15 cotton lines (Fig. 2). Δ18O 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-

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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 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).

\[ \Delta^{13}C \text{ to } \Delta^{18}O \text{ ratio a good estimate of photosynthetic capacity} \]

The amount of CO₂ fixed per unit of water transpired is an interesting physiological yardstick of WUE but our interest here is in maximizing CO₂ 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 CO₂ and stomatal conductance (Ci/gs). Thus, at a given stomatal conductance, variations in intercellular CO₂ should be dependent on the efficiency of mesophyll to fix the available substrate CO₂ (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 Ci and gs, 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 (\(\Delta^{13}C/\Delta^{18}O\)) 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 CO₂ response curve as well as Ci/gs 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-
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; Scheidegger 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.

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