



## Leaf-level carbon isotope discrimination and its relationship with yield components as a tool for cotton phenotyping in unfavorable conditions

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**ABSTRACT.** The initial goal of this study was to measure the efficiency of carbon isotope discrimination ( $\Delta$ ) in distinguishing between cotton plant genotypes subjected to two water regimes. In addition,  $\Delta$  measurements, leaf water potential and gas exchange ratios were monitored. Using Brazilian breeding lines, this study also tested the usability of  $\Delta$  as a proxy for selecting high-performing yield components in cotton plants grown in unfavorable conditions, particularly water deficiency. For these experiments,  $\Delta$  and yield components were measured and their correlations analyzed. Differences among cotton genotypes for  $\Delta$  ( $p < 0.0001$ ) were verified, and it was found that this variable was significantly correlated with gas exchange. There was a significant positive correlation between  $\Delta$  and seed cotton yield only in the site experiencing severe water deficiency (Santa Helena de Goiás). However,  $\Delta$  had a significant negative correlation with fiber percentage. Our results indicate that  $\Delta$  is a suitable tool for cotton phenotyping, and it may be applied in cotton breeding programs that aim to produce high-performing yield components in unfavorable conditions.

**Keywords:** gas exchange, carbon fraction, cotton, abiotic stress.

### Discriminação isotópica do carbono em nível foliar e sua relação com componentes de rendimento como um ferramenta para fenotipagem de algodoeiro em condições desfavoráveis

**RESUMO.** Inicialmente objetivou-se investigar a possibilidade de uso da discriminação isotópica do carbono ( $\Delta$ ) para distinguir genótipos de algodoeiros submetidos ao déficit hídrico. Adicionalmente à  $\Delta$ , o potencial hídrico foliar e as trocas gasosas foram monitorados durante a progressão do estresse. Em uma segunda fase, buscou-se verificar a utilidade da  $\Delta$  como ferramenta para seleção de plantas com alto desempenho quanto a componentes de rendimento sob condições de campo em ambientes contrastantes, especialmente quanto ao regime hídrico, utilizando linhagens elites brasileiras. Sob deficiência hídrica, verificaram-se diferenças acerca dos genótipos para  $\Delta$  ( $p < 0.0001$ ) a qual também correlacionou-se significativamente com as de trocas gasosas. Em condições de campo,  $\Delta$  também discriminou os genótipos ( $p < 0.0001$  e  $0.0157$  para Santa Helena de Goiás e Montividiu, Estado de Goiás, respectivamente). Verificou-se correlação significativa entre  $\Delta$  e a produção de algodão em caroço onde o déficit hídrico foi mais severo (Santa Helena de Goiás). Todavia,  $\Delta$  correlacionou-se negativamente com a percentagem de fibras. Então, a  $\Delta$  poderá ser utilizada na fenotipagem de algodoeiro sob déficit hídrico sendo incorporado aos procedimentos de seleção de linhagens junto aos programas de melhoramento do algodoeiro visando alto rendimento sob condições desfavoráveis, a exemplo da ocorrência de déficit hídrico.

**Palavras-chave:** trocas gasosas, fracionamento do carbono, algodão, estresse abiótico.

#### Introduction

Water deficiency is an important ecological occurrence that limits crop production and food quality globally, particularly in arid and semi-arid regions of the world. Reductions in productivity are periodically experienced due to drought, and it has been suggested that these losses will increase in the future as a result of global climate change

DEIKMAN et al., 2012; FISCHER; SCHAR, 2010; IPCC, 2007; VERMEULEN et al., 2012).

Approximately 90% (1.2 million hectares) of the cotton-growing area in Brazil is located in the Brazilian savanna biome, where water deficiency occurs regularly from April to May. In this region, historically water deficit begins in April, coinciding with cotton first flowering emission and early cotton

boll development phase, in which the cotton plants demand for water is significantly increased in a second crop season growth (or safrinha). In the last three growth seasons (2009-10, 2010-11 and 2011-12), the lint yield was reduced by approximately 30% due to the occurrence of water deficiency during the reproductive phase in second crop season areas (personal communication). Therefore, the development of tools and approaches for cotton phenotyping is an important first step in developing a cotton breeding program aimed at selecting plants with high-performing yield components when subjected to unfavorable conditions.

Different breeding methods and selection approaches have been used involving progeny rows, individual selection, crossing and backcrossing, physiology and molecular marker approaches (SNP, SSR, AFLP, large-scale sequencing, etc.) for mapping and genetic association studies. However, for selection of cotton with high-performing yield components when grown in unfavorable conditions, including water deficiency, the time-consuming mapping of quantitative traits such as yield and stress tolerance is difficult because the genotype is never unambiguously inferred from the phenotype. Additionally, estimating water use efficiency (WUE) relies either on long-term measurements of plant water consumption or assessments of biomass production, and even then these large-scale approaches are not feasible in individual plant screening efforts (BLUM, 2009; BRITO et al., 2011).

As a complementary selection strategy, the use of  $\Delta$  could reduce the amount of time required by traditional approaches used in cotton breeding programs that select for high-performing yield components in water-deficient plants.  $\Delta$  could help cotton breeders rapidly identify these lines using an integrative plant response.  $\Delta$  has been used as an indirect indicator for WUE (BRITO et al., 2011; ELAZAB et al., 2012; FARQUHAR et al., 1989) and correlates closely with water deficiency tolerance (CENTRITTO et al., 2009). Measurements of leaf and grain  $\Delta$  have been successfully used extensively as a proxy for WUE evaluation in many plant species (FARQUHAR; RICHARDS, 1984). The link between  $\Delta$  and WUE is predicated on the concept that both are functionally dependent on intercellular  $\text{CO}_2$  concentration ( $c_i$ ) (FARQUHAR et al., 1989). It has been established that the extent to which  $C_3$  plants discriminate against  $\Delta$  during carbon assimilation can determine their WUE. Therefore, because of the correlation between  $\Delta$  and gas exchange values, we hypothesize that  $\Delta$  can be used as a proxy for cotton gas exchange ratios and yield

components (FARQUHAR et al., 1989; BRITO et al., 2011). The performance as measured by  $\Delta$  among genotypes at a particular site reflects their performance in  $\text{CO}_2$  assimilation and transpiration efficiency. With this knowledge, cotton breeders could select for genotypes with high-performing yield components using  $\Delta$  as a proxy without the need for many years of laborious and expensive field testing.

The initial aim of the present study was to determine the efficiency of  $\Delta$  in distinguishing between cotton plant genotypes grown under different water regimes, employing genotypes with similar phenotypes grown in a greenhouse. The second phase of this study was conducted under field conditions to determine the usability of  $\Delta$  as a proxy for selecting cotton genotypes with high-performing yield components when grown in unfavorable conditions. A proper understanding of the relationships between cotton yield components and  $\Delta$  may further define the use of  $\Delta$  in cotton breeding programs and may allow for its incorporation into the phenotyping procedures that aim to select plants with high-performing yield components in unfavorable conditions.

## Material and methods

### Greenhouse experiments

Greenhouse experiments were conducted at Embrapa experimental station, located in the city of Campina Grande, Paraíba State, Brazil. Air temperature and relative humidity were measured with a HOBO Micro Station Data Logger (Onset Computer, Bourne, Massachusetts, USA). At time of gas exchange measurements, the photosynthetically active radiation (PAR) was measured using a Quantum Sensor LI-COR (Q-45556) attached to the LI-COR 6400 (LICOR-6400, LI-COR Inc., Lincoln, NE, USA). Photosynthetic photon flux density (PPFD) varied from 900 to 1,641  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The experiment was conducted in a completely randomized design in a factorial combination that analyzed twelve genotypes and two water regimes (watered/always irrigated and stressed/with water deficiency imposed at first flower emission) with five replications. Genotypes were chosen to represent a range of yield responses to water deficiency observed in a preliminary greenhouse study. These genotypes had similar phenotypes, with only five days between the earliest and latest flowering times. Genotypes Guazuncho 2 and Acala SJ4 were included to check for higher and lower  $\Delta$ , respectively (unpublished data).

For this study, the moisture percentage in the substrate (washed sand and peat - 1:1, v/v) was measured by oven drying at 105°C to a constant weight. For field capacity procedures, each pot was filled with 22.0 kg of the mixture described above. Before filling, small holes were cut into the bottom of the pots to facilitate initial drainage. Subsequently, water was added to each pot until saturation. After filling, pots were covered with plastic and put aside to drain for 24 hours, after which the holes were then sealed and all pots were weighed to obtain the field capacity. The experimental unit consisted of one cotton plant sowed in a polyethylene pot (30 L) filled with a mixture of washed sand and peat (1:1, v/v). The seeds used in the experiment were obtained from Embrapa Germplasm Bank. The plants were irrigated regularly, maintaining 70% of field capacity until 44 days after emergence (DAE) but before the first flower emission.

At the emergence of the first flower at 45 DAE, plants of each cultivar were separated into two groups: one continued to receive regular irrigation (well-watered plants) while the other was subjected to water deficiency (water deficiency-stressed plants). For all genotypes, the water deficiency was allowed to progress until LWP reached approximately -1.50 and -3.0 MPa, considered here to be moderate and severe stress levels, respectively, at predawn (between 4:30 and 5:00 am). An Oregon Corvallis pressure chamber 97330 (PMS Instrument Company, Albany, OR, USA) was used to monitor the progression of water deficiency. For each treatment, a leaf from the upper portion of the middle third of each plant was used; leaves were always collected from the same position on the plant on each measurement date, and five replicates were used for each treatment.

When the LWP average of five plants reached -1.50 or -3.0 MPa (BRITO et al., 2011), leaves from the second youngest fully expanded leaves of all genotypes were used for gas exchange analyses. On average, the LWP took nine days after withholding irrigation to reach -3.0 MPa. Once this point was reached, samples were taken from the first youngest fully expanded leaves for carbon isotope fraction analyses.

The net assimilation rate ( $P_n$ ), stomatal conductance ( $g_s$ ), and transpiration rate ( $E$ ) were measured for 9-11h under artificial saturating photosynthetic photon flux (PPF) (1200 mol m<sup>-2</sup> s<sup>-1</sup>) using a portable photosynthesis system infrared gas analyzer (LI-COR 6400XTR, Nebraska, USA) (ULLAH et al., 2008).

At -3.0 MPa at predawn, the first fully expanded leaf per pot was collected from each stressed or irrigated plant, oven-dried, and ground to a fine

powder for the analysis of carbon isotope composition ( $\delta^{13}C$ ) using a mass spectrometer (DELTA-S Finnigan Mat, Bremen, Germany) at Biosciences Institute in the Universidade Estadual de São Paulo (UNESP), Botucatu, São Paulo State, Brazil. The atmospheric isotopic composition was considered to be -8‰ in relation to the international standard, Pee Dee Belemnite (PDB). A second standard calibrated against a fossil belemnite from the Pee Dee formation was used for comparison. The  $\Delta$  values were calculated (FARQUHAR et al., 1989) as described in Equation 1 (FARQUHAR et al., 1989), where  $\delta a$  refers to the atmospheric isotopic composition and  $\delta p$  refers to the plant isotopic composition.

$$\Delta^{13}C = \frac{\delta a - \delta p}{1 + \frac{\delta p}{1000}} \quad (1)$$

### Field experiments

In this second step, our objective was to investigate whether  $\Delta$  could distinguish between Brazilian cotton lines and to assess its relationship with yield components under field conditions. For this purpose, experiments were conducted in two sites termed Santa Helena de Goiás, Goiás State (17°48'49"S, 50°35'49"W, at 563 m altitude - sowed on January 27, 2010) and Montividiu, Goiás State (17°26'39"S, 51°10'29"W, at 940 m altitude - sowed on January 23, 2010), which represent contrasting environments of the Brazilian savanna with respect to altitude, temperature, rain accumulation and distribution (Table 1). Before cotton planting, these areas were cultivated with *Sorghum bicolor* (L.) Moench, whose soil chemical data are shown in Table 2. In these two sites, the first flower emerged at 57 (on March 25, 2010) and 62 (on March 26, 2010) days after sowing in Santa Helena de Goiás and Montividiu, respectively. After first flower emission, 72 and 137 mm of rainfall accumulated until cutout (cessation of new vegetative and reproductive growth), respectively.

Eighteen cotton breeding lines were sown during the second crop season (safrinha corn) in a randomized complete block design with four replications for both locations. The plot size was 4 rows by 5 m, with a row spacing of 0.45 m and plant density of 9 plants m<sup>-1</sup>. Starter fertilizer (04-30-16 of N-P-K plus 0.4% boron and zinc) at a rate of 300 kg ha<sup>-1</sup> was band applied adjacent to each row at planting. Cotton was side dressed with 20-00-30 of N-P-K plus 0.3% boron at 200 kg ha<sup>-1</sup> at square initiation. Weed and insect control were performed according to cotton crop recommendations for the Brazilian savanna (FREIRE, 2011).

**Table 1.** Monthly mean maximum and minimum temperature and cumulative rainfall that occurred in the 2010 cotton growing season for the two locations. Historical climatic data are also shown.

Month	Santa Helena de Goiás - GO						Montividiu - GO					
	Tmax		Tmin		Rainfall		Tmax		Tmin		Rainfall	
February	30.31	30.10†	20.07	19.40†	187	208†	27.13	30.60†	18.91	20.10†	378	186†
March	32.00	30.10	20.92	19.40	185	213	28.61	30.60	17.5	20	180	210
April	30.54	29.20	17.47	17.90	51	71	27.95	29.80	14.77	18.33	48	119
May	29.46	28.40	13.99	16.40	00	28	27.08	29.20	12.25	16.80	37	38
June	29.78	27.80	11.57	14.70	05	13	26.78	28.60	11.0	14.90	32	19
July	30.88	28.00	12.54	14.10	01	07	28.00	28.80	11.00	14.10	27	08
August	32.08	30.70	10.98	15.70	00	13	29.85	31.40	19.4	15.70	00	22

†At least twenty-year average for two locations.

**Table 2.** Soil chemical characteristics from 0-20 cm and 21-40 cm of soil profile in Santa Helena de Goiás (SHEGO) and Montividiu (MONT).

Localities	M.O	pH	P <sup>+</sup>	K <sup>+</sup>	S	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Al <sup>3+</sup>	CTC	V	B	Cu	Mn	Zn
SHEGO	mg dm <sup>-3</sup>	CaCl <sub>2</sub>	-----mg dm <sup>-3</sup> -----				-----cmol. dm <sup>-3</sup> -----		%			-----mg dm <sup>-3</sup> -----		
0-20 cm depth	32	5.2	6.8	64	29	3.5	1	0	8	58.5	0.19	1.5	46.7	1.2
21-40 cm depth	24	5.1	0.5	33	54	1.7	0.4	0	6.3	34.7	0.18	2.1	28.5	0.1
MONT														
0-20 cm depth	44	5.1	16.3	162	8	4.1	1	0.1	10.7	51.4	0.47	2.9	26.4	10.3
21-40 cm depth	35	5.1	2.1	132	37	2.9	0.7	0	8.3	47.2	0.44	1.5	13.9	3.9

P was extracted using Melliish solution and pH was measured in water. SHEGO and MONT are abbreviations of Santa Helena de Goiás and Montividiu trials sites, respectively.

To determine whether  $\Delta$  could be used as a proxy for selecting cotton genotypes with high-performing yield components when subjected to water deficiency, we performed  $\Delta$  analysis on the bulk of five plants randomly chosen per plot. The first youngest fully expanded leaves were sampled 95 days after sowing (at the boll development phase), with the two sites sampled at 23 and 16 days after the occurrence of the last rainfall in Santa Helena de Goiás and Montividiu, respectively. Subsequently, the leaves were oven-dried and ground to a fine powder for analysis of carbon isotope composition ( $\delta^{13}\text{C}$ ) and  $\Delta$  (FARQUHAR et al., 1989), as described above. At maturity, seed cotton yield (SCY), lint yield (LY), fiber percentage (Fiber) and boll weight (Boll) were analyzed.

### Statistical analysis

Homogeneity of variances was tested by Bartlett's test. Pearson's correlation coefficient analyses were estimated for all variables in greenhouse and field trial conditions. The data were subjected to analysis of variance (ANOVA) using the SAS procedure PROC GLM. For the greenhouse trial, when the interaction was significant, each factor was tested at each level of the other factor using the LSMEANS (least-square means) statement with the option SLICED (LITTELL et al., 2006). In this experiment, a multiple comparisons test was applied to the mean (Tukey-Kramer alpha = 0.05). When only the main effects were significant, the variables were analyzed without consideration of the other effects. Data from field experiments were subjected to ANOVA and means were grouped using the Scott-Knott grouping method (SCOTT; KNOTT, 1974). Analyses were

done using the SAS System ver. 9.1.3 (SAS INSTITUTE, 1982).

## Results and discussion

### Greenhouse trial

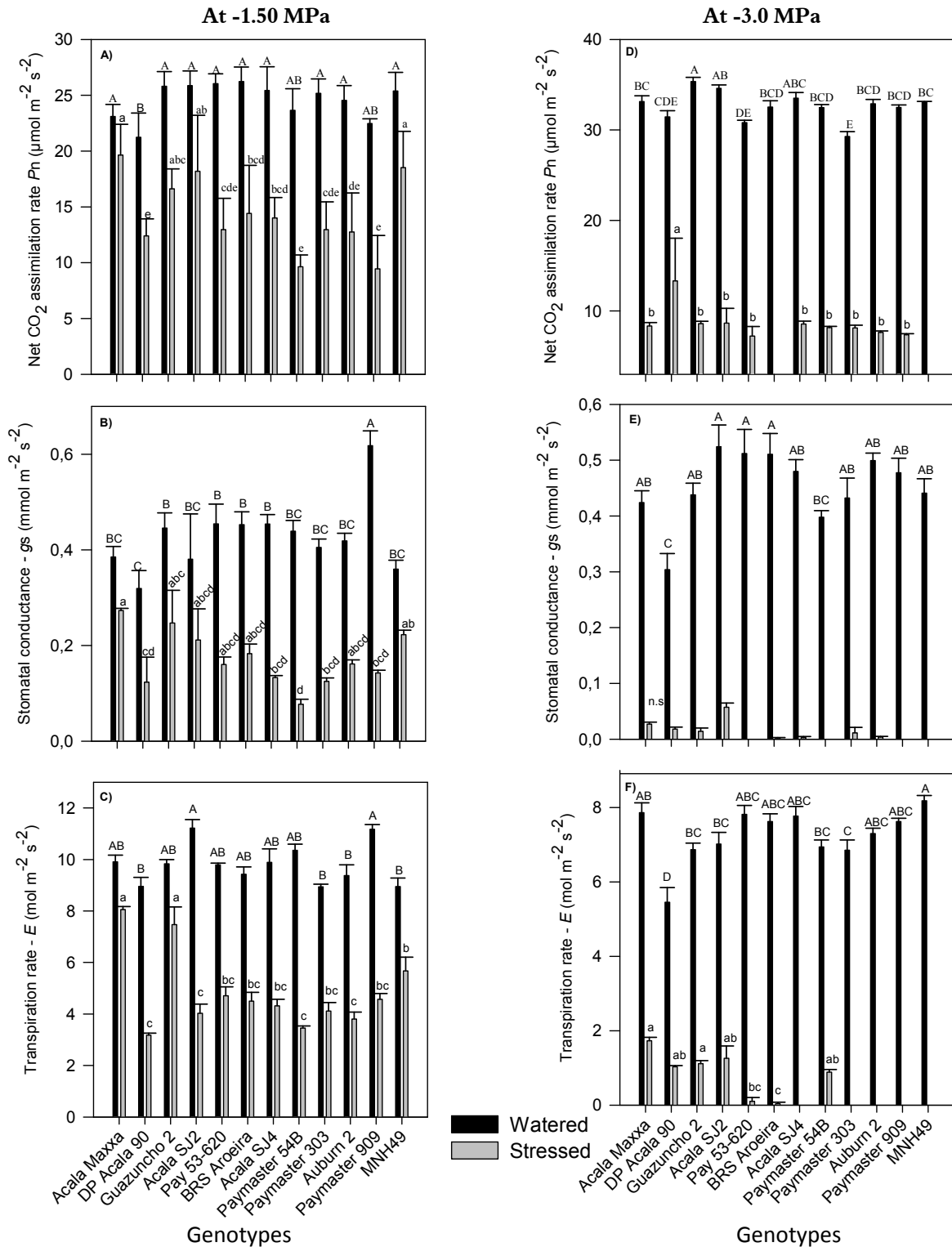
The LWP of those leaves maintained under well-watered conditions were always kept at -0.5 MPa or greater (less negative) at predawn (data not shown). At nine days after the suspension of irrigation, Acala SJ4 and Guazuncho 2 reached -2.70 and -3.57 MPa LWP at predawn, respectively. Although these two genotypes are considered to be tolerant of water deficiency, the performance presented in this report clearly indicates the variation in their ability to maintain higher LWP when subjected to water deficiency. Additionally, it is also necessary to determine the ability of each cultivar to use available water, especially when studies are conducted in containers where the water available is more limited by container dimensions than substrate characteristics.

Results from a general linear model (GLM) analysis indicated significant cotton genotypes x water regime interactions for dependent variables such as  $\Delta$ , net  $\text{CO}_2$  assimilation rate, stomatal conductance and transpiration rate, highlighting the interdependence between these factors. The simple main effect of both factors was compared separately. Interaction effects sliced by cotton genotype showed a significant effect for all dependent variables, except for Acala SJ2 and Paymaster 303 genotypes, which showed similar  $\Delta$  under stressed and non-stressed conditions ( $p = 0.4285$  and  $p = 0.9578$ , respectively).

Significant genotype variation was observed for all gas exchange variables ( $P_n$ ,  $E$ , and  $g_s$ ) under well-

watered conditions. However, in these conditions there was no substantial variation between genotypes for  $P_n$  (well-watered plants were evaluated when stressed plants reached  $-1.5$  MPa)

(mean values of  $24.58 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), with the highest ( $26.24 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and lowest ( $21.24 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) values for BRS Aroeira and Deltapine Acala 90, respectively (Figure 1A).



**Figure 1.** Net CO<sub>2</sub> assimilation rate ( $P_n - \mu\text{mol m}^{-2} \text{s}^{-1}$ ) (A and D), stomatal conductance ( $g_s - \text{mmol m}^{-2} \text{s}^{-1}$ ) (B and E) and transpiration rate ( $E - \text{mmol m}^{-2} \text{s}^{-1}$ ) (C and F) of twelve cotton genotypes grown under stressed (S) and watered (W) conditions at  $-1.50$  and  $-3.0$  MPa LWP at predawn.

Water deficiency induced a substantial reduction in  $P_n$  for all genotypes. When compared to well-watered conditions, the stressed genotypes presented an average reduction of 42.40% in  $P_n$ , with maximum ( $19.64 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) and minimum ( $9.44 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) mean values for Acala Maxxa and Paymaster 909, respectively (Figure 1A). The  $g_s$  varied among cotton genotypes and water regimes when evaluated at LWP of -1.50 MPa at predawn, ranging from  $0.320$  to  $0.62 \text{ mol m}^{-2} \text{s}^{-1}$  and from  $0.08$  to  $0.28 \text{ mol m}^{-2} \text{s}^{-1}$  in well-watered and water-deficient conditions, respectively (Figure 1B). Under water deficiency, cotton genotypes did not differ in  $g_s$  at -3.0 MPa (Figure 1E). Differences on  $E$  were verified for cotton genotypes under the water regimes imposed at -1.50 and -3.0 MPa LWP measured at predawn (Figure 1C and 1F). Under water-deficient conditions, there were reductions in the transpiration rate of 51.11 and 93.18% at -1.50 and -3.0 MPa, respectively, when compared to the well-watered regime. Under the well-watered regime, the MNH 49 cultivar had the highest value for  $E$  (-3.0 MPa), whereas the lowest value was verified in DP Acala 90. Acala Maxxa, Guazuncho 2, Acala SJ 2, Paymaster 54B, and DP Acala 90 had the highest values for  $E$  (-3.0 MPa) under water-deficient conditions (Figure 1F). At -1.5 MPa, cultivars Acala Maxxa and Guazuncho 2 had the highest values for  $E$  (Figure 1C).

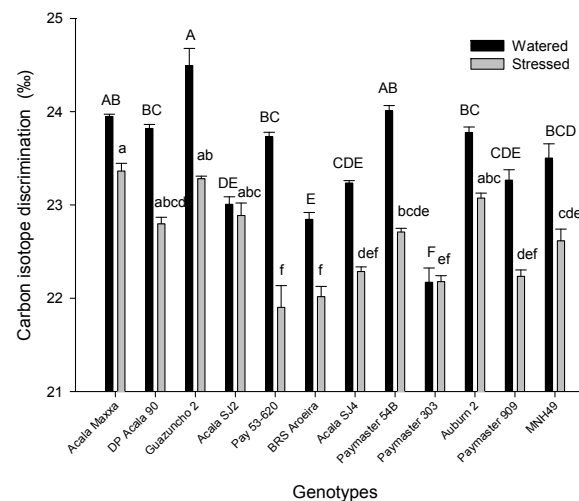
A significant interaction was observed between genotype and water regime ( $p < 0.0001$ ) for  $\Delta$ , indicating distinct cotton genotypes in each water regime. Under constant irrigation,  $\Delta$  ranged from 22.17 for Paymaster 303 to 24.49 for Guazuncho 2, a standard for water deficiency tolerance in our study (Figure 2). Under water-deficient conditions, Acala Maxxa had the highest  $\Delta$  (23.36), while Paymaster 53-620 (Pay. 53-620) had the lowest mean (22.02).

**Table 3.** Correlation analysis based on carbon isotope discrimination ( $\Delta$ ), net  $\text{CO}_2$  assimilation rate ( $P_n$  – at -1.50 and -3.0 MPa LWP), transpiration rate ( $E$  – at -1.50 and -3.0 MPa LWP), and stomatal conductance ( $g_s$  at -1.50 and -3.0 MPa LWP) of twelve cotton genotypes grown under stressed (S) and watered (W) conditions. Twelve genotypes with five replications for each water regime ( $N = 60$ ) were used for the analysis. The upper diagonal line refers to the coefficient correlation under water-deficient conditions, while the lower diagonal line refers to well-watered plants.

	$\Delta^{13}\text{C}$	$P_n$ -1.50	$E$ -1.50	$g_s$ -1.50	$P_n$ -3.0	$E$ -3.0	$g_s$ -3.0
$\Delta^{13}\text{C}$	1	0.3710**	0.3780**	0.2870*	0.2590*	0.6730**	0.2270 <sup>n.s.</sup>
$P_n$ -1.50	-0.2540 <sup>n.s.</sup>	1	0.5800**	0.4780**	-0.1260 <sup>n.s.</sup>	0.4360**	0.4430**
$E$ -1.50	0.1010 <sup>n.s.</sup>	-0.0385 <sup>n.s.</sup>	1	0.6340**	-0.0950 <sup>n.s.</sup>	0.4050**	0.0288 <sup>n.s.</sup>
$g_s$ -1.50	-0.1700 <sup>n.s.</sup>	0.1910 <sup>n.s.</sup>	0.6810**	1	0.0639 <sup>n.s.</sup>	0.2480 <sup>n.s.</sup>	0.0097 <sup>n.s.</sup>
$P_n$ -3.0	0.2200 <sup>n.s.</sup>	0.1890 <sup>n.s.</sup>	0.1890 <sup>n.s.</sup>	0.1890 <sup>n.s.</sup>	1	0.4000**	0.2460 <sup>n.s.</sup>
$E$ -3.0	-0.1280 <sup>n.s.</sup>	0.2220 <sup>n.s.</sup>	0.1320 <sup>n.s.</sup>	0.4210**	-0.0033 <sup>n.s.</sup>	1	0.5320**
$g_s$ -3.0	-0.2200 <sup>n.s.</sup>	0.4520**	0.3360**	0.2700*	0.2490 <sup>n.s.</sup>	0.5000**	1

\*( $p < 0.05$ ), \*\*( $p < 0.01$ ) and n.s.- non-significant.

Our results clearly demonstrate a reduction in  $\Delta$  in plants grown in water-deficient conditions. In these conditions, decreases were also observed in  $P_n$ ,  $g_s$ , and  $E$  (Figures 1A, B, C, D, E and F) and a positive significant correlation was found between  $\Delta$  and all gas exchange variables ( $P_n$ ,  $g_s$ , and  $E$ ) evaluated at an LWP of -1.50 or -3.0 MPa at predawn, except for  $g_s$  at -3.0 MPa ( $p < 0.084$ ) (Table 3). This performance highlights the possible role of stomatal closure regulation, coordinated by abscisic acid (ABA), on gas exchange and  $\Delta$  variables found in this report. As stomatal closure during drought is mainly regulated by an ABA signaling pathway, this hormone can influence the WUE and therefore the leaf  $\Delta$  ratio, given that stomatal closure will decrease  $\text{CO}_2$  concentration in the leaf ( $c_i$ ). The decreased  $\text{CO}_2$  concentration will decrease the ratio between internal and ambient  $\text{CO}_2$  concentration ( $c_i/c_a$ ), which affects the discrimination against the carbon isotope  $^{13}\text{C}$  during  $\text{CO}_2$  fixation by rubisco. Therefore, ABA-induced drought stress is reflected in leaf carbon isotope composition.



**Figure 2.** Mean carbon isotope discrimination ( $\Delta$ ) of twelve cotton genotypes grown under stressed (S) and watered (W) conditions at the emission of the first flowers, sampled at -3.0 MPa LWP at predawn.

As shown in this report, a positive correlation between  $\Delta$  and gas exchange variables has also been found for cotton growing in field conditions (LEIDI et al., 1999). As previously reported, genotypes showing higher photosynthesis and conductance rates are also generally more productive across the entire soil moisture gradient (CENTRITTO et al., 2009; LIU et al., 2012; ZHU et al., 2010) and often show a positive correlation with yield when subjected to water-deficient conditions (SINCLAIR et al., 2004). As cited by Boyer (1965), there are numerous investigations showing that, for cotton, as water deficiency increases, stomata close, resulting in reduced photosynthesis. He also noted that leaf growth is affected by falling LWP at the first sign of turgor loss, but that a measurable reduction in photosynthesis begins later, at about -1.2 MPa when measured at predawn.

In the first step of this study, where the main objective was to check the efficiency of  $\Delta$  at distinguishing between cotton genotypes grown in different water regimes and determine the relationship between  $\Delta$  and gas exchange, our data support the view that WUE, based on  $\Delta$ , is an integrative parameter with  $P_n$ ,  $g_s$ , and  $E$ . Cotton genetic variation for photosynthesis or gas exchange has not been deeply studied, which is surprising due to its importance as a genetic resource for crop improvement. Differences in the responses found between genotypes are indicative of an underlying variation in photosynthesis and/or conductance related to the diffusion of  $\text{CO}_2$  to the carboxylation sites of rubisco, considering the role of stomata in this complex process (FARQUHAR et al., 1989). Many reports have shown genetic variation in photosynthetic traits for several crop species, including cotton (FLOOD et al., 2011; ULLAH et al., 2008). In fact, lower  $\Delta$  can arise as a function of low  $g_s$  or high carbon assimilation, both leading to a high WUE (FARQUHAR et al., 1989; SETTER, 2012). In our study, the MT 04-1540 genotype maintained low values for  $\Delta$  associated with high lint yield values in the Santa Helena de Goiás site, where the water deficiency was more severe. In Montividiu, where the water deficiency was moderate, this genotype also maintained low  $\Delta$  values and showed higher lint yield, indicating its high WUE when submitted to unfavorable conditions.

Drought tolerance can occur if plants maintain a favorable water balance through the expression of morphological and/or physiological traits that reduce water loss through transpiration by mechanisms that regulate stomatal conductance and/or increase water absorption capacity through the growth of a deeper

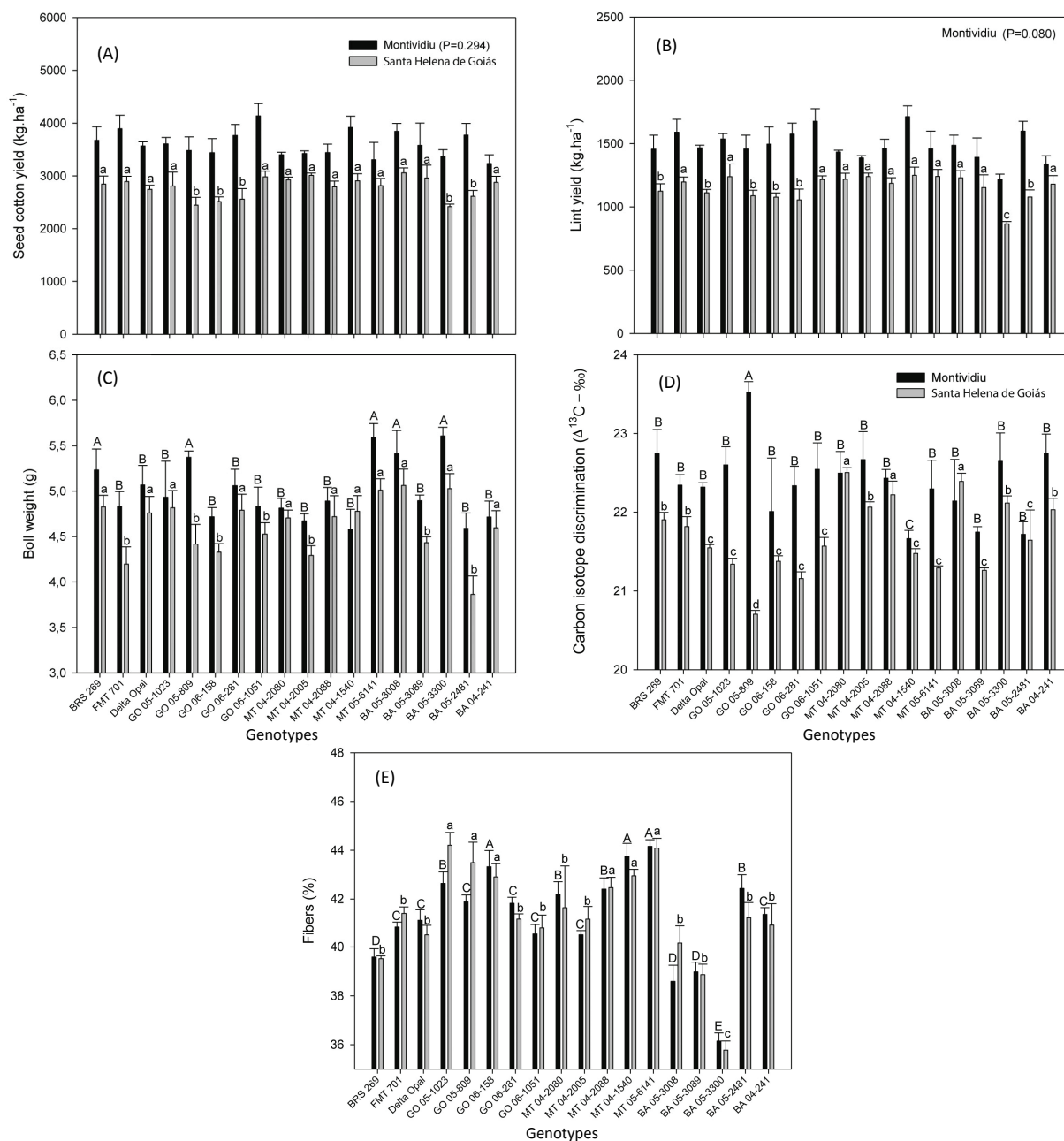
and more extensive root system or via osmotic adjustment. In the first case, stomatal closure reduces  $\text{CO}_2$  diffusion, leading to a reduction in the rate of photosynthesis, consequently reducing growth and economic yield, depending on the development phase of stress imposition, stress intensity and its duration. This strategy is commonly found as an adaptive response in plants surviving under extremely stressful conditions, allowing them to redirect absorption and energy, which are usually used in metabolic routes of growth and production, to then be used in the synthesis of protective molecules (ZHU, 2002). This strategy is used by certain crop species growing in areas where droughts are severe and persist for the greater part of the plant life cycle. Well-established for cotton, strategies using stomatal conductance regulation, osmotic adjustment capability and the expression of a larger and deeper root mass may help the plant to maintain its water status at a relatively higher level favoring high fiber yield when cotton is submitted to water deficiency. The expression of these characteristics could allow the plant to maintain greater stomatal conductance and a higher rate of  $\text{CO}_2$  diffusion, increasing the rate of photosynthesis, growth, and yield. However, under field conditions, measurements of these variables are not practical and use destructive methods, limiting their feasibility in a breeding program. Thus, the adoption of direct screening methods that use integrative variables that are easy to measure, such as  $\Delta$ , may lead the selecting for cotton plants with high-performing yield components under water deficiency or for cotton germplasms with more robust root systems feasible on a large scale. Additionally, currently available tools for refined genotyping and phenotyping, along with the associated increase in detailed information concerning the interaction of photosynthetic phenotypes with the environment, will provide new insights into the genetic regulation of this complex trait (BRITO; BELTRÃO, 2011; REBETZKE et al., 2006).

#### Field trials

In both locations,  $\Delta$  also discriminated between cotton genotypes ( $p < 0.0001$  and  $0.0157$  for Santa Helena de Goiás and Montividiu, respectively). Higher  $\Delta$ , seed cotton yield (SCY), lint yield (LY) and boll weight means were obtained in the Montividiu location (Figure 3), where the environment was more suitable (lower mean night temperatures and higher rainfall accumulation) for cotton growth and development compared with the Santa Helena de Goiás site (Table 1).

For the trial conducted in Santa Helena de Goiás, where the water deficiency was more severe, there was a significant and positive correlation

between  $\Delta$  and SCY (Table 4). Conversely,  $\Delta$  had a significant but negative correlation with fiber percentage.



**Figure 3.** Seed cotton yield (SCY  $\text{kg ha}^{-1}$ ), lint yield (lint  $\text{kg ha}^{-1}$ ), boll weight ( $\text{g boll}^{-1}$ ), mean carbon isotope discrimination ( $\Delta$ ) and fiber percentage (%) for eighteen cotton elite lines grown in the Brazilian savanna regions of Santa Helena de Goiás and Montividiu.

**Table 4.** Phenotypic correlation among carbon isotope discrimination ( $\Delta^{13}\text{C} \text{‰}$ ), seed cotton yield (SCY –  $\text{kg ha}^{-1}$ ), fiber percentage (%), lint yield (LY –  $\text{kg ha}^{-1}$ ) and boll weight (g) of 18 cotton genotypes grown in Santa Helena de Goiás (upper diagonal of table) and Montividiu (lower diagonal) during the year 2010.

	$\Delta^{13}\text{C}$		SCY		Fiber		LY		Boll
$\Delta^{13}\text{C}$	-		0.255	*	-0.328	**	0.090	n.s.	0.090
SCY	-0.087	n.s.	-		-0.050	n.s.	0.888	**	0.064
Fiber	-0.131	n.s.	-0.042	n.s.	-		0.403	**	-0.209
LY	-0.134	n.s.	0.926	n.s.	0.335	**	-		-0.028
Boll	0.171	n.s.	0.192	n.s.	-0.318	**	0.056	n.s.	-

\*( $p < 0.05$ ), \*\*( $p < 0.01$ ) and n.s.- non-significant.



Although the root variables were not measured in this study, the positive correlation between  $\Delta$  and SCY strongly suggests that genetic variation in root characteristics, such as greater and deeper root mass, may help plants maintain relatively higher water status levels due to an increased capacity for absorbing moisture from this deep soil profile, even under dry conditions. This greater capacity to absorb moisture allows the plant to maintain greater stomatal conductance and a higher rate of  $\text{CO}_2$  diffusion, increasing the rate of photosynthesis, growth, and, consequently, yield of the cotton plants growing in this site. As has been shown in wheat, water deficiency decreased the stomatal conductance, plant accumulated transpiration and aerial biomass accumulation, resulting in an increase in carbon isotope composition (ELAZAB et al., 2012). On the other hand, the negative correlation between  $\Delta$  and fiber percentage indicates that there is an increase in sink power redirecting carbohydrates to seed formation, which is detrimental to fiber formation. In fact, immature seeds and developing fiber are components of cotton ovules and share concurrent and similar phases of their developmental events. By the coincidence of their proximity, fiber and seed formation are competing sinks fed through a common funiculus. During this phase, cellulose microfibrils are added to the wall of the fiber, while oils, proteins, and carbohydrates are deposited in the embryo (KLOTH; TURLEY, 2010). As showed in other reports, transgenic cotton plants with increased hormone indole-3-acetic acid levels in the ovule epidermis have increased numbers of lint fibers. According to these authors, because there was no difference in seed cotton yield between transgenic lines and the nontransgenic control, the greater lint yield is mainly due to the enhanced lint percentage. The lower seed weight of transgenic cotton might result from a changed ratio in the distribution of carbohydrates in the developing seed (ZHANG et al., 2011).

Most breeding programs aimed at increasing productivity in the cotton species *G. hirsutum* and *G. barbadense* in the last 50 years have focused on increases in the net rate of photosynthesis and tolerance for elevated temperatures via increased stomatal conductance (RADIN et al., 1994). In our report, the positive correlations found between  $\Delta$   $^{13}\text{C}$  and gas exchange and  $\Delta$   $^{13}\text{C}$  and yield components strongly suggest an integrative effect of gas exchange on the  $\Delta$  and its association with yield components. These results also clearly show the role of stomatal conductance on the rate of  $\text{CO}_2$  diffusion, which results in an increased rate of photosynthesis and yield.

In this environment, the goal is to develop a cultivar that maintains a higher stomatal conductance as a result of a favorable water balance achieved through morphological changes, such as increased water absorption capacity through the growth of a deeper and more extensive root system. A larger and deeper root mass may help the plant to maintain its water status at a relatively high level due to its increased capacity for exploration for water in the soil profile. This feature allows the plant to maintain greater stomatal conductance and a higher rate of  $\text{CO}_2$  diffusion, increasing the rate of photosynthesis, growth, and yield. Under field conditions, measurements of root characteristics are not practical and use destructive methods, limiting their use in breeding programs (ULLAH et al., 2008). Thus, the adoption of direct screening methods that use integrative variables that are easy to measure, such as  $\Delta$ , may make selecting for cotton plants with high-performing yield components under water deficiency or for cotton germplasms with more robust root systems feasible on a large scale. However, it is very important to consider that more selection studies relating crop performance to  $\Delta$  also need to be performed using the progeny of crosses that are constructed specifically for this purpose. These studies should be performed in different environments with different soil characteristics, considering that there are several critical areas of research that need to be addressed if the potential of  $\Delta$  as an indicator of this variation is to be exploited effectively (CONDON; HALL, 1997).

The fact that there are many studies that have shown positive associations between  $\Delta$  and biomass production (including grain yield), which have been observed with many crop species and environments, including dry environments, warrants discussion concerning possible mechanistic and evolutionary causes (CONDON; HALL, 1997). In many cases, the association is linked to a positive association between  $\Delta$  and *gs*. The use of Pima cotton as a model species (LU et al., 1996) provides additional evidence for this phenomenon. There has been speculation that the evolution of some crop plants, as such cotton, resulted in conservative performance with respect to *gs*; that is a tendency for stomata to be at least partially closed on many occasions. This evolution could have occurred as a results of plants submitted to stress by years and under severe drought stress conditions, when conservative stomatal performance may be adaptive, had disproportionate influences on seed production and long-term evolutionary success over many years due to soil "seed banks" being much less effective after 1

year. This suggestion leads us to comment on the influence on water use of the less conservative stomatal performance of newer cultivars. Therefore, although  $\Delta$  has been extensively used by physiologists, agronomists and plant breeders, further studies aiming to clarify the physiological or genetic mechanisms of their yield component associations are necessary.

## Conclusion

In two locations, the use of  $\Delta$  allowed discrimination between MT 04-1540 genotypes that showed low  $\Delta$ . They also showed higher values for lint yield, at least in the Montividiu site, and they showed a similar performance to genotypes evaluated in Santa Helena de Goiás. Additionally, in this site, the negative correlations found between  $\Delta$  and fiber percentage strongly suggest an integrative effect of gas exchange on  $\Delta$  and its association with yield components. Therefore, cotton breeders could use  $\Delta$  values as a criterion for plant selection to obtain plants with higher efficiency for gas exchange and/or water use when growing in unfavorable conditions without the need for many years of laborious and expensive field testing.

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