Scientific Research Publishing

American Journal of Plant Sciences, 2022, 13, 1448-1459
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Stable Carbon Isotope Discrimination ($\delta^{13}C$) of Cotton Burrs and Seeds as a Season-Long Integrator of Crop Water Stress

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How to cite this paper: Gitz III, D.C., Baker, J.T. and Lascano, R.J. (2022) Stable Carbon Isotope Discrimination ($\delta^{13}C$) of Cotton Burrs and Seeds as a Season-Long Integrator of Crop Water Stress. *American Journal of Plant Sciences*, **13**, 1448-1459.
https://doi.org/10.4236/ajps.2022.1312099

Received: September 26, 2022
Accepted: December 26, 2022
Published: December 29, 2022

Abstract

Plant-based irrigation management schemes typically use surrogates such as canopy temperature, alone, or in conjunction with environmental variables, to infer the degree of "crop stress" (biological strain) induced by drought. Few systematic studies of the relationship between "crop stress", as defined by such surrogates, and physiological estimates of water use efficiency (WUE) exist over both daily and seasonal time scales relevant to agronomic irrigation control. The systematic application of stable carbon isotope discrimination ($\delta^{13}C$) might allow *post hoc* evaluation of irrigation scheduling schemes and might also be a useful germplasm screening tool if the source(s) of variability can be uncovered and/or controlled. Results from preliminary efforts comparing leaf and cotton seed $\delta^{13}C$ to season-long water deficits showed that seeds are more useful indicators of season-long water stress and water use efficiency during crop development.

Keywords

Irrigation-Scheduling, Germplasm Screen, Water Management

1. Introduction

Plant "stress" is an inaccurate but generally accepted term that refers to biologi-

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cal strain resulting from specific stressors, or, more often, deviations from optimal conditions [1]. In an agronomic sense, plant stress results from any deviation from optimal conditions that result in yield reduction. The gross morphology of any given crop plant at any point in time is the result of the cumulation of physiological and developmental processes that have occurred since seed germination. Hence, the yield of any given crop is the culmination, or integration, of plant physiological and developmental processes throughout the growing season as affected by environmental conditions and cues. And so, crop plant performance indicators such as above ground biomass, shoot height, or yield at harvest or growing season end are season-long stress indicators when compared those of crop plants grown under ideal conditions.

In agronomic settings, especially in arid and semi-arid regions, the primary limiter of crop yield is water availability. When rainfed or dryland crop yield is limited by lack of precipitation water, irrigation is used to maintain yields and profitability. Irrigation is simply a replacement for timely rainfall. For example, in the Texas High Plains irrigation-water from the Ogallala aquifer is becoming more limited. For this reason, and for others such as increasing energy costs associated with lifting water from increasingly deeper aquifers, irrigation scheduling has been a topic of research for at least fifty years. And, with increasingly limited irrigation-water, irrigation management research has focused on deficit-irrigation scheduling. Deficit-irrigation scheduling [2] attempts to provide less than optimal water for a smaller but predictable crop yield, ideally maximizing yield returns for amount of water delivered, and minimizing yield variability and risk.

There are three general approaches to deficit-irrigation scheduling. First, by calculating the water demand and delivering a fraction of the calculated demand on a daily or weekly interval; second, by monitoring and maintaining soil moisture, and third, by evaluating and controlling plant water stress. Direct measurement of physiological plant stress is very difficult to measure directly and instead surrogates for stress are usually measured [3].

Most stress measurement surrogates such as canopy temperature and biomass accretion are directly related to stomatal aperture. As drought stress increases, stomatal aperture decreases. Stomatal closure results in decreased transpiration and increased canopy temperatures. This also results in increased resistance to CO_2 diffusion into the photosynthetic leaf chlorenchyma resulting in reduced growth as biomass accretion. Of relevance to the current work, stomatal closure also leads to decreased substomatal CO_2 concentration. It should be borne in mind that anything that affects the ratio of atmospheric or ambient CO_2 concentration "C_a" to that of the substomatal internal CO_2 concentration "C_i" will affect discrimination against the heavier $^{13}CO_2$. This, the C_i/C_a ratio, is the fundamental statistical mechanical cause of variation in discrimination against heavier $^{13}CO_2$ in favor of the lighter more rapidly diffusing $^{12}CO_2$ in the assimilate. This can be affected by the net photosynthetic rate between cultivars. Taken together,

photosynthetic rates and stomatal apertures form the basis for relating water use efficiency (WUE) to $\delta^{13}C$ between cultivars. This is the rationale for using stable carbon isotope discrimination, $\delta^{13}C$ to indirectly measure variations in stomatal aperture during growth and development and so, plant water stress [4].

Stable carbon isotope discrimination technique has been used in plant breeding programs. For example, it was used to select for and to develop new wheat cultivars with increased intrinsic WUE [5]. Sampling crop material for $\delta^{13}C$ analysis is usually done by collecting fully expanded canopy leaves in an attempt to obtain a "snapshot" of the Ci/Ca ratio during development. Another approach is to collect leaves from plants that have developed at specific intervals in an attempt to eliminate seasonal variation. However, even though cotton $\delta^{13}C$ shows considerable variation across cultivars, using this technique to select for cotton in breeding programs [5] has not been fruitful.

For stable carbon isotope discrimination to be of use for germplasm selection we must be able to relate $\delta^{13}C$ to season-long physiological performance of the crop as affected by the environment. An integrated seasonal measure of both the intrinsic or potential WUE and the actual efficiency as affected by the environment is needed. Analyzing stable carbon isotope discrimination of stored photosynthate in seeds or structural carbohydrates in ovary walls forming the boll walls (carpels) had not been investigated and could yield an integrated measure of WUE over the period of fruit maturation.

Herein we report results of using seeds and carpels, or "burrs" of the cotton fruits for stable carbon isotope determination as an indicator or the integrated season-long drought stress imposed upon the plants by different irrigation control schemes.

2. Methods

2.1. General Culture Methods

Cotton (*Gossypium hirsutum* L.) cvar. FM-9058 seeds (FiberMax[2], Bayer Crop Science, Research Triangle Park, NC) were obtained from a local supplier and planted in a field at the USDA nursery in Lubbock, TX (33˚35'42.0"N 101˚53'51.9"W, 960 m above sea level) in 2011 on Day of Year (DOY) 151 at a rate of 16.8 kg·ha^{-1} (15 lbs ac^{-1}). Seeds were planted at 0.1-m intervals into north-south oriented raised beds spaced at 1 m. The FM-9058 cultivar is a Roundup® (glyphosate) resistant variety widely adapted to Texas in general and to the Southern High Plains in particular. The soil at this site is an Amarillo fine sandy loam (fine-loamy, mixed, superactive, thermic Aridic Paleustalfs) with a bulk particle size distribution of 75% - 80% sand, 5% - 10% silt, and 10% - 15% clay and an average soil bulk density of ~1.3 g·cm^{-3}. Prior to planting (DOY 144) ~50 mm (2") of water was delivered to the plots by furrow irrigation. Immediately after planting (DOY 152 & DOY 153) an additional 64 mm (2.5") was de-

[2]Mention of this or other proprietary products is for the convenience of the readers only, and does not constitute endorsement or preferential treatment of these products by USDA-ARS.

livered to the plots by furrow irrigation to ensure that the soil profile was filled to capacity and to insure even germination, emergence, and even stand of crop plants. Emergence (50%) was observed on DOY 156. An additional 50 mm (2") of water was applied to all plots via subsurface drip irrigation over 4 days (DOY 174-DOY 179) to insure good crop establishment. Pinhead squares (50%) were observed on DOY 182 across all plots. Irrigation treatments began on DOY 189. Local environmental conditions were recorded by a weather station located 300 m west of the plots [6].

2.2. Irrigation Treatments

Irrigation treatments were as detailed by Baker *et al*. [7]. Treatments are simplified and summarized in **Table 1** and briefly described below. There were six individual irrigation treatments in separate irrigation zones running the length of the field. Each irrigation treatment zone was comprised of eight rows. Subsurface irrigation drip emitters were directly under each raised bed 25 cm below the surface. Irrigation zones were further divided into four 25 m plots by cutting 1 m alleys perpendicular to the rows. Treatments ranged from Dryland, or Rainfed (DL), with no irrigation, to irrigation delivered at 110% of the calculated evapotranspiration rate (110ET) based upon daily short grass reference ET (ET_{os}) calculations from a Texas Tech University Mesonet site located about 1 km north of the plots (33.604091N, 101.899195W). The ET_{os} was then multiplied by a crop coefficient K_c obtained from a degree-day driven crop development model and adjusted by a factor of 1.1 (110%) so that the amount of water that would be delivered to the plot was $1.1 \times K_c \times ET_{os}$.

There were four intermediate levels of irrigation controlled by either the Time Temperature Threshold (TTT) or the Stress Degree Hour (SDH) methods. The TTT irrigation scheduling method recorded canopy temperature (T_c) with an infrared thermometer interfaced with a datalogger, and the amount of time of

Table 1. Irrigation treatments arranged by irrigation approach and by increasing water-deficit (Increasing season-long water stress).

Treatment	Irrigation applied when:	Amount of water per irrigation event
110ET	Technical support personnel arrived on business days	110% of calculated ET since last irrigation
TTT5.0	$T_c > 28^\circ$ for >5.5 hours in a day	5.0 mm
TTT2.5	$T_c > 28^\circ$ for >5.5 hours in a day	2.5 mm
SDH15	$\Sigma (T_c - T_a) \times$ Hours >15 degree-hours	5.0 mm
SDH30	$\Sigma (T_c - T_a) \times$ Hours >30 degree-hours	5.0 mm
DL	No irrigations	n.a.

T_c is canopy temperature and T_a is ambient temperature.

time T_c exceeded a critical temperature of 28˚C as detailed elsewhere [7]. An irrigation event delivering either 2.5 mm (TTT2.5) or 5 mm (TTT5.0) was triggered when T_c exceeded 28˚C for more than 5.5 h over the course of a single day (accumulated time was not carried into the next day but was reset to zero). The SDH irrigation scheduling approach [7] recorded the air-canopy differential ($T_c - T_a$), when it was positive (stress degrees), and multiplied the differential by the time elapsed (hours). The resulting product, [$(T_c - T_a) \times$ hours], was summed and an irrigation event of 5.0 mm triggered when the integrated SDH exceeded either 15 degree-hours (SDH15) or 30 degree-hours (SDH30).

2.3. Morphometric and Chemical Analyses

Each week the above ground portion of seven plants from each plot were taken to the laboratory, arranged on the bench according to height and the two outliers discarded, leaving three medial plants for growth analysis. The leaves and fruiting structures were removed from each plant. Leaf area was determined by passing excised leaves through a leaf area meter (LI-COR Biosciences, model LI-3100C, Lincoln NE). Stem height, and node numbers were determined. Plant materials were collected in labelled paper bags, placed in a forced air oven, and allowed to dry at 60˚C until mass did not changed, usually for three days at which time dry biomass was determined.

Leaves were collected at regular intervals for stable carbon isotope analysis. Once each week the most recently fully expanded canopy leaves were taken from four plants in each plot, placed in small, labelled envelopes, taken to a forced air oven, allowed to dry for six days, vacuum sealed with silica gel desiccant, held until the samples could be processed. Samples were then passed through a 60 mesh screen and the dry fine powder, sealed in airtight polyethylene vials and held in the dark at room temperature until analysis could be done. The subsamples from leaves were shaken to insure they were well mixed, and 2 - 3 mg portions placed into tin capsules and submitted to the U.C. Davis Stable Isotope facility for measurement of $\delta^{13}C$ abundance ratios on an elemental analyzer interfaced to an isotope ratio mass spectrometer after combustion. Relative sample abundance, of ^{13}C and ^{12}C were expressed relative to international standards Vienna Pee Dee Belemnite [8] using ‰ notation as $\delta^{13}C = [R_s/R_b] - 1] \times 1000$, where R_s and R_b are the ratio of $^{13}C/^{12}C$ in the sample and standard belemnite, respectively [9].

At season end, cotton was hand harvested in each of the two 5 m lengths of row from the middle two rows of each eight-row plot. All open mature bolls were harvested. The seed cotton was separated from the burrs, cotton lint separated from seeds and the seeds acid de-linted. The burrs were further dried as for the leaf samples, passed thru a 60 mesh screen and then held in plastic bags at −10˚C. Whole de-linted seeds were held at −10˚C until they could be further processed for analysis. Seeds were flaked by passing thru a 10 mesh screen, the flakes well mixed, and 10 g of flakes comminuted to a fine powder under liquid

nitrogen in a mortar and pestle. Seed and burr powder were submitted for $\delta^{13}C$ as for the leaf material.

3. Results

3.1. Irrigation and Precipitation

The total water delivered to the experiment, including both from rainfall events and irrigations are shown in **Figure 1**. Total water delivered to the systems over the growing season decreased in the order:

$$110ET > TTT5.0 > SDH15 \approx SDH30 > TTT2.5 > DL.$$

Since the experiment was conducted during the greatest recorded regional drought in over a half century [10] and because the DL treatment received no irrigation, total precipitation and the timing of precipitation events can be visualized graphically in **Figure 1**, Dryland. The amount of precipitation-water received by the plots was well below average. Excluding the pre-emergence and post-emergence seedling establishment furrow irrigations, only 78 mm of precipitation was received by the plots.

In (extreme) contrast to the DL treatment, the 110ET treatment received 715 mm of water over the year. Except for the exponential growth and development phase, the increase in the amount of water delivered to the 110ET plot was rather constant throughout the season rather than decreasing later in the season as with most thermally driven irrigation approaches [11].

Throughout the growing season, both SDH15 and SDH30 scheduling methods applied about the same amount of water as the TTT2.5 method. However, the

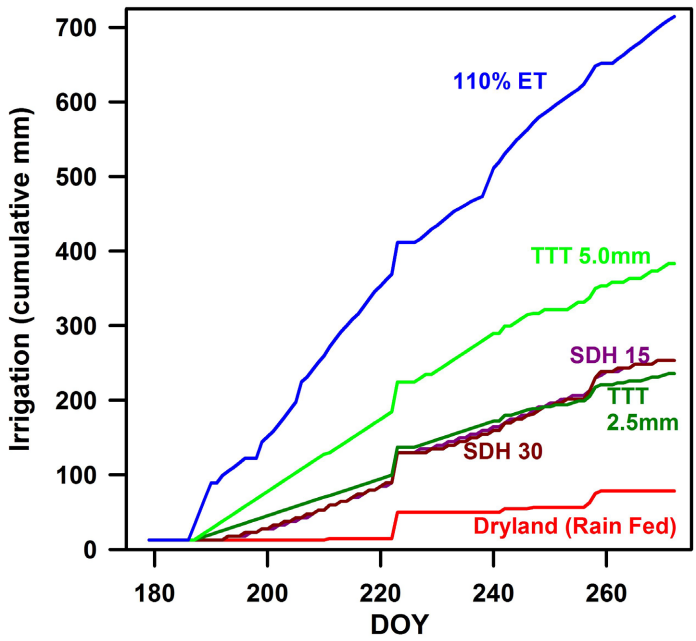

Figure 1. Total water (Irrigation and precipitation) delivered to field grown cotton plots. Treatments are as described in **Table 1**, text, and referenced methods [7].

SDH method applied water less frequently than the TTT2.5. This resulted in the "saw-tooth" pattern Figure 1. Season-long, these three methods used about 60% of the amount of irrigation-water as the TTT5.0 approach. Interestingly, both SDH approaches used less water early in the season and a bit more later in the season as compared to the TTT2.5 treatment.

3.2. Growth and Development (Morphometry)

Leaf area and shoot biomass during the growing season are shown in Figure 2. Lint yields were reported elsewhere [7]. In general, end of season growth followed water amounts delivered. Above ground biomass appeared somewhat "noisy" as compared to leaf area. This was attributed to delayed fruit development in the 110ET treatment [7]. Season-long decrease in dryland leaf area and biomass were attributed to boll and leaf drop.

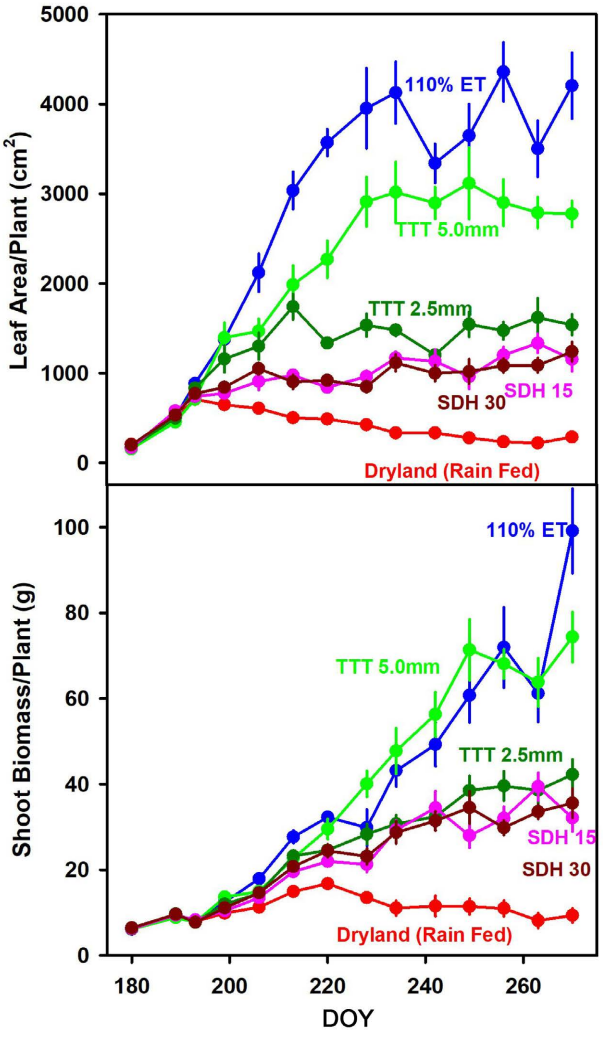

Figure 2. Above ground growth parameters of cotton plant shoots grown under different irrigation scheduling regimes. Leaf area (Top) and whole shoot biomass (Bottom) are shown. Bars are S.E.

3.3. Stable Carbon Isotope Discrimination

Isotope discrimination of leaves gathered during crop development exhibited differences with irrigation-scheduling approach and the amount of water delivered, but no clear pattern across treatments was found. Including all data on a single plot proved confusing and very difficult to interpret. To ease visualization, these data were grouped by scheduling approaches (Figure 3). The DL and 110ET treatments exhibited rather constant levels of discrimination against ^{13}C, though the DL treatment exhibited higher (less negative) values as compared to

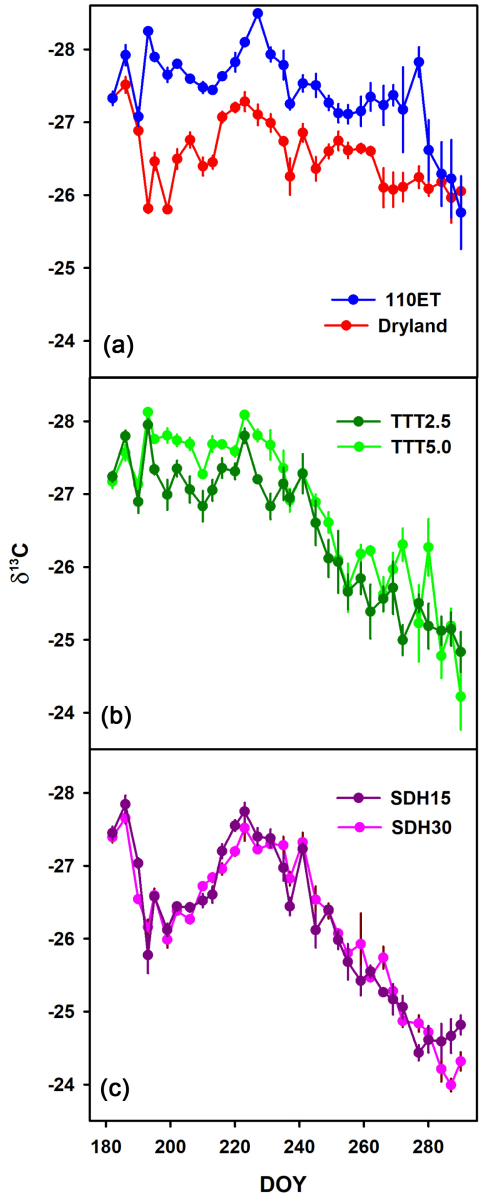

Figure 3. Stable carbon isotope discrimination of cotton leaf samples taken weekly during crop development. (a) Continuous replacement of water (TTT110%) vs. No irrigation (Dryland), (b) Time Temperature Threshold triggered irrigation of either 2.5 mm (TTT2.5) vs 5.0 mm (TTT5.0) in each irrigation event and, (c) Triggered irrigations of 5.0mm when stress exceeded 2.5 (SDH2.5) or 5.0 degree-hours (SDH5.0). Bars are S.E.

that of 110ET. Consistent with this, the TTT2.5 exhibited higher values as compared to those of TTT5.0. Such patterns were less obvious when comparing SDH15 and SDH30 treatments, which were not clearly separated. No clear consistent pattern of season long isotope discrimination was noted other than a clear progression of seed and carpel stable isotope discrimination exhibited very similar, nearly identical patterns (Figure 4). The magnitude of seed and carpel end of season stable carbon isotope ($\delta^{13}C$), as opposed to absolute values, of both seeds and carpels (burrs)relative over the growing season increased in the order:

$$110ET > TTT5.0 > SDH15 \approx SDH30 > TTT2.5 > DL.$$

The apparent response of seeds was clearer and more pronounced than that of carpels primarily due to the clearer separation of the DL $\delta^{13}C$ from those taken from other irrigation treatments.

4. Discussion

The main purpose of this work was to examine whether physiological effects of irrigation control schemes could be assessed with simple and relatively inexpensive end of season stable carbon isotope discrimination analyses. Since isotope discrimination has long been held to be related to water use efficiency it was thought that using this method might be used as a surrogate for water stress induced changes in stomatal aperture leading to carbon fractionation. Stable carbon isotope discrimination is also a heritable trait that has been suggested as a selection factor in breeding programs [5].

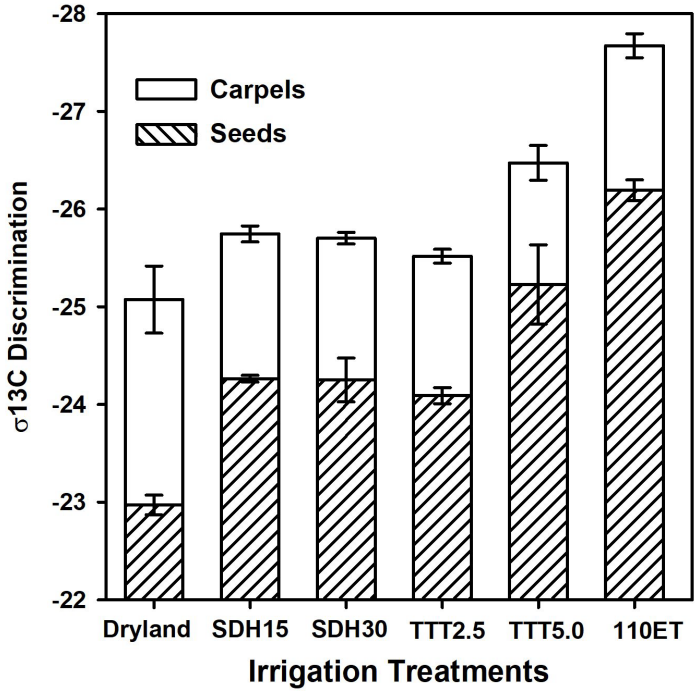

Figure 4. Stable carbon isotope ($\delta^{13}C$) fractionation response of Carpels (Burrs) and Seeds from field grown cotton plants and collected at end of season. Bars are S.E.

Samples used in such work are usually taken from leaf material. Unfortunately, environmental effects such as water availability can vary through the season introducing variability in vegetative samples. This is clearly demonstrated in Figure 3 where all of the first samples taken earliest in the season have identical $\delta^{13}C$ values that begin to diverge later in the season. The effect of water availability means that samples acquired from different years or perhaps even from different fields cannot be compared successfully. For this reason, workers have suggested sampling schemes such as sampling on two different dates corresponding to mid-flowering and mid-squaring [5], leaf sampling during boll ripening [12] [13], analyzing leaves developing under water stress [14], etc. Season-long variation in leaf $\delta^{13}C$ (Figure 3) explains conflicting recommendations.

Sampling methods could also introduce variability into such approaches. Consider that a scant 2 - 3 mg of dried vegetative materials are required for each datum. Simply grinding a small, desiccated leaf disk from a single plant will undoubtedly lead to acquisition of samples with unique developmental histories and results. The goal of such work with agronomically important plants, whether to assess physiological response to water availability or to screen germplasmis to acquire information that is representative of field rather than a single sample. The problem becomes more apparent when sampling cotton seeds, or carpels. Because cotton is a determinate plant, the photosynthetic assimilate that is diverted to these tissues should vary as with the developing leaves. Taking a 3 mg sample from a single seed, or from a single node, or from a single plant does not guarantee that the chemical properties are representative of the entire stand. In an attempt to eliminate, or at least reduce this source of error a concerted effort was made and considerable resources expended to ensure that samples obtained from different treatments and plots were representative of the individual treatments. For canopy leaves, this was done by selecting large numbers of leaves from each plot for each of the three replicates along the field and mixing each sample well after grinding. For seeds and carpels, this was done by processing seeds and fruit walls from large numbers of plants following yield determination. The seed samples flaked were on the order of 250 to 500 g.

While flaking the seeds was not problematic, grinding the oily seeds can be quite difficult. Passing the cotton seed subsamples through a fine screen was challenging due to the oil building up and clogging the screens. It was due to this that were sorted to grinding the flaked seeds under liquid nitrogen, and included the more easily ground carpels in the analysis. Although there was variation between the seeds and the carpels (Figure 4) the relative discrimination between the different treatments was identical. Most importantly, the response pattern of both the carpels and of the seeds mirrored the seasonal water use. The difference between the carpels and the seeds is unknown but must have resulted to biochemical process differences between the two tissues.

5. Conclusion

Sampling leaves to assess the effects of irrigation or to screen germplasm for en-

hanced WUE results in considerable variation throughout the season. To understand season-long processes from leaf samples large numbers of leaves must be collected for each sample and large numbers of samples collected throughout the season. We concluded that as compared to leaf sampling, isotope fractionation analysis of cotton seeds is a more useful indicator of season-long water stress and WUE during crop development.

Acknowledgements

We gratefully acknowledge John Stout of the ARS-USDA in Lubbock, TX, and Wesley Burgett of the Texas Tech University West Texas Mesonet for their assistance in providing access to and allowing use of weather data. This research was supported in part by the Ogallala Aquifer Program, a research consortium between ARS and regional universities.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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