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Research Article

Impact of Drought Stress on *Sorghum bicolor* Yield, Deconstruction, and Microbial Conversion Determined in a Feedstocks-to-Fuels Pipeline

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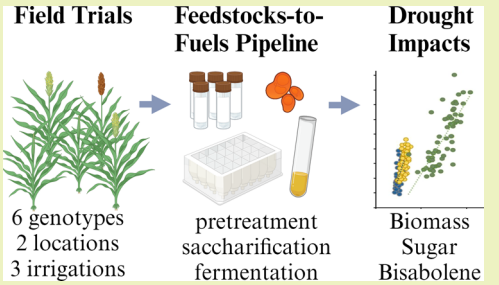
ABSTRACT: Sorghum is an attractive feedstock for biobased fuel and chemical production because it is familiar to farmers, naturally drought tolerant, and versatile as a food, feed, and fuel crop. Although sorghum is a promising feedstock, particularly in regions that experience drought stress, little is known about how drought conditions impact the ease of conversion of sorghum to fuels and products. This study combines agronomic field trials with a high-throughput experimental pipeline to explore the field performance and liquid biofuel (bisabolene) yields resulting from three sorghum types (photosensitive forage sorghum, optimized grain sorghum, and drought-resistant grain sorghum) grown under pre- and postflowering water limitations in two different California locations. Multiple drought treatments are compared to the control, as the timing (preflowering versus postflowering) of drought stress elicits different survival strategies and corresponding impacts on yield and composition. Forage-type sorghum maintained the highest biomass yields across all irrigation conditions and locations. Glucose and xylose yields resulting from ionic liquid pretreatment and enzymatic saccharification were not significantly impacted by irrigation treatments but differed by location and genotype. However, *Rhodosporidium toruloides* grown on the resulting plant hydrolysates unexpectedly produced higher titers of bisabolene for drought-stressed sorghum samples regardless of genotype.



KEYWORDS: *sorghum, pre flowering drought stress, postflowering drought stress, bisabolene conversion, high sample throughput, feedstocks-to-fuels pipeline*

INTRODUCTION

In 2022, the United States (US) produced 15.4 billion gallons of fuel ethanol, primarily from corn (*Zea mays*).[1] Growing corn can be emissions-intensive and resource-intensive. Low-input and high-yielding perennial grasses such as switchgrass (*Panicum virgatum*) and Miscanthus (*Miscanthus* spp.) have been explored as feedstocks for advanced conversion processes capable of converting lignocellulosic material to fuel, but these carry an increased risk for growers: high upfront costs for the establishment, lack of secondary markets, and long-term economic and land use commitments.[2,3] As the frequency and severity of droughts increases due to climate change,[4] successful bioenergy production will require low-risk crops that are also drought tolerant. This raises the question of which biofuel feedstock crops can withstand water stress and maintain high downstream sugar and fuel yields once processed in advanced biorefineries.

Sorghum [*Sorghum bicolor* (L.) Moench] has emerged as an attractive bioenergy feedstock in recent years.[5] Among cereal crops globally, sorghum ranks fifth, behind wheat, corn, rice, and barley in terms of total production, with the US being the largest producer.[6] The familiarity with farmers and the existing commercial supply chain are advantageous in establishing sorghum as an attractive bioenergy crop.[7] Sorghum is grown both for grain and, to a lesser extent, for forage/silage in the US.[8,9] Grain sorghum is mostly used as feed, though both the stover and the grain can be used for fuel production just corn grain and stover are both potential feedstocks for ethanol production.[10] Prior analyses have attempted to identify sorghum ideotypes for biofuel production but had to extrapolate from compositional data rather than using experimental results for each phenotype.[11,12] Sorghum's resistance to drought makes it especially promising in regions where climate change is expected to exacerbate the duration

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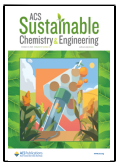

and severity of droughts,[13] but the impact of drought stress on downstream conversion has so far been unexplored.

Testing the viability of sorghum-based bioenergy production under drought stress requires an end-to-end approach that links field-trial data with conversion. In order to test the impact of varied sorghum growth conditions, such as preflowering and postflowering drought stress, on downstream biomass conversion efficiency, a high-throughput analysis approach was developed. The Feedstocks-to-Fuels (F2F) pipeline includes end-to-end conversion of biomass in a small volume, multiwell format, starting with ionic liquid pretreatment, enzymatic hydrolysis, and ending with microbial conversion to a promising biofuel/bioproduct precursor (bisabolene). Glucose and xylose yields can be measured, along with the final bisabolene yield. This conversion pipeline fills a critical research gap: compositional analysis and quality control is done using laborious wet chemistry, while traditional forage quality analysis alone is not an adequate substitute for wet chemistry methods or directly testing conversion yields.[14] Having direct measures for a large number of biomass samples of the relevant downstream conversion metrics allows for the assessment of a variety of feedstocks, growth conditions, and field replicates. This study provides the first-ever demonstration of the F2F pipeline using samples from sorghum field trials including six sorghum genotypes, two California locations, and three irrigation regimes.

EXPERIMENTAL SECTION

Feedstocks-to-Fuels Pipeline. The F2F pipeline was developed to analyze glucose and xylose yields after pretreatment and saccharification, as well as bisabolene yields from the resulting hydrolysate. The design of the F2F pipeline is based on the one-pot integrated method of ionic liquid pretreatment, described in previously published work.[15] A detailed step-by-step protocol with exact measures and concentrations can be found here.[16] In short, dried whole-plant sorghum biomass samples were finely ground to a 1 mm screen and automatically weighed before ionic liquid was added. The sample volume is less than 2 mL, and 48 samples can be analyzed simultaneously in FlowerPlates. Cholinium phosphate was used as ionic liquid for pretreatment, which was subsequently incubated under heat and pressure (121 °C, 15 psi) for 3 h. Although cholinium phosphate does not result in sugar yields as high as those documented for other ionic liquids such as cholinium lysinate,[17] it was selected to enable clearer differentiation of samples based on their recalcitrance based on the assumption that samples that have higher sugar yields under mild conditions are worthwhile to identify. Additionally, using cholinium phosphate also facilitates easier pH adjustment, as aqueous cholinium lysinate has very high alkalinity (pH ∼ 13).[18] Upon cooling, the pH was adjusted to ∼5.3 with a citrate buffer, and the Ctec/Htec enzyme mix (Novozymes) was added to the slurry. Samples were incubated for 72 h at 50 °C while being agitated for enzymatic hydrolyzation. Once finished, the hydrolysate was then filtered (Pall, AcroPrep) subsequently an aliquot was diluted 1:20 with water, and 5 μL of this diluted hydrolysate was used for HPLC (Bio-Rad aminex 87H column, temp 60 °C, 0.6 mL/min flow rate, mobile phase 4 mM sulfuric acid, detector: refractive index detector (RID)), to determine the glucose and xylose yield. The remainder of the hydrolysate is rebuffered to pH 7.0 using a phosphate buffer and subsequently fermented, using a *Rhodosporidium toruloides* strain previously published under the names GB1 or BIS3.[15,19] Fermentation is conducted at 30 °C for 5 days while continuously shaking, and bisabolene is extracted into Durasyn164 during fermentation. As an internal standard, Durasyn164 is diluted to 0.22% (v/v) with ethyl acetate containing naphthalene. Bisabolene yield is ultimately determined using GC-MS (intuvo 9000 system, Column HP-5 MS, oven temperature cycle, 80 °C hold 1 min, ramp 20 °C per min, 300 °C hold time 2 min).

Plant Genotypes and Growth Conditions. To develop a diverse set of biomass samples for the F2F pipeline, we conducted field trials using commercial sorghum varieties. Two photoperiod sensitive forage sorghum (FT) genotypes SP1615 (Sorghum Partners, Longmont, CO) and SWFS5147 (experimental line from NexSteppe Breeding, acquired by S&W Seeds, Lubbock, TX), two grain sorghum (GS) genotypes (SP7715, SP74C40: Sorghum Partners, Longmont, CO) and two drought-resistant grain sorghum (DR) genotypes (BTx642, RTx7000;[20,21] Texas A&M Agrilife, College station, TX) were selected for this experiment. Agronomic data was collected from two sites in California that contain distinctly different soil types: University of California-Agricultural and Natural Resources (UC-ANR) Kearney Agricultural Research Extension Center (KARE) in Parlier, CA at 36.59507 latitude and −119.50531 longitude and UC-ANR West Side Research Extension Center in Five Points, CA (WREC) at 36.33669 latitude and −120.11636 longitude. The KARE location soil is characterized as a Hanford sandy loam with silty substratum, while WREC is a Panoche clay loam soil. The sandy loam soil at KARE has a lower water-holding capacity than clay loam, meaning the same irrigation treatment typically results in greater drought stress at KARE relative to the WREC location. Across both locations, different irrigation regimes were used to capture the impacts of drought stress prior to flowering (preflowering) versus after flowering (postflowering) because the timing of drought stress relative to flowering elicits different responses from the plants.

Both locations were irrigated prior to planting, and fertilizer was applied at planting time. Plots were planted in six rows: two rows were used to determine grain yield, and two further rows were used to determine the total biomass (stover and grain), while the remaining two rows were discarded for border effects. Harvest lengths for both the biomass and the grain yield were 6.10 m (20 linear ft). Planting was done on June 11 and 12, 2019 and any subsequent irrigations during the growing season were done with a linear move sprinkler system on average once a week at KARE and every other week at WREC. At the KARE location, 61.2 cm of water was applied to the control group, 41.96 cm to the preflowering treatment, and 40.15 cm to the postflowering treatment. At the WREC location, 58.11 cm of water was applied to the control group, 31.69 cm to the preflowering treatment, and 40.38 cm to the postflowering treatment. Neither location experienced rainfall during the growing season, and the maximum daily temperatures were comparable (Figure S1). Plants were in the field for a total of 142 days at KARE and 136 days at WREC. Preflowering drought stress was imposed by halting water after the preplant treatment for 8 weeks at KARE and 10 weeks at WREC. Following these drought periods, treatment groups were watered together with the control group regularly. Postflowering drought stress was imposed by halting irrigation on the same day that preflowering drought stress was alleviated, which corresponded to 50% flowering at KARE and onset of flowering in the drought-resistant types at WREC. For the remainder of the growing season until harvesting occurred (11 weeks at KARE and 9 weeks at WREC), plants under postflowering drought stress did not receive any water. A schematic indicating times and watering regimes is included (Figure S1).

Biomass Yield, Compositional Analysis. Grain for the grain sorghum types was harvested with a plot combine harvester (Almaco Model SPC40, Nevada, Iowa), while both grain- and forage-type (FT) sorghum were cut with a forage chopper (Wintersteiger Model Cibus S, Salt Lake City, Utah) for total biomass estimates. The fresh weight of bulk harvest from each plot was determined using onboard load-cell-based weighing systems for both the combine and forage chopper. One subsample was collected from each plot bulk harvest, and the sample fresh weight was determined in-field using a calibrated platform scale. Subsamples were dried for 5−7 days at 50−55 °C to determine fresh/dry weight ratios and calculate dry weight. Dry samples were subsequently analyzed for select forage quality parameters (CP, ADF, aNDF, aNDFom, ADL, ESC, WSC, EE, Ash, NFC, starch) by Dairyland Laboratories (Arcadia, Wisconsin) according to standard protocols for forage analysis (https://

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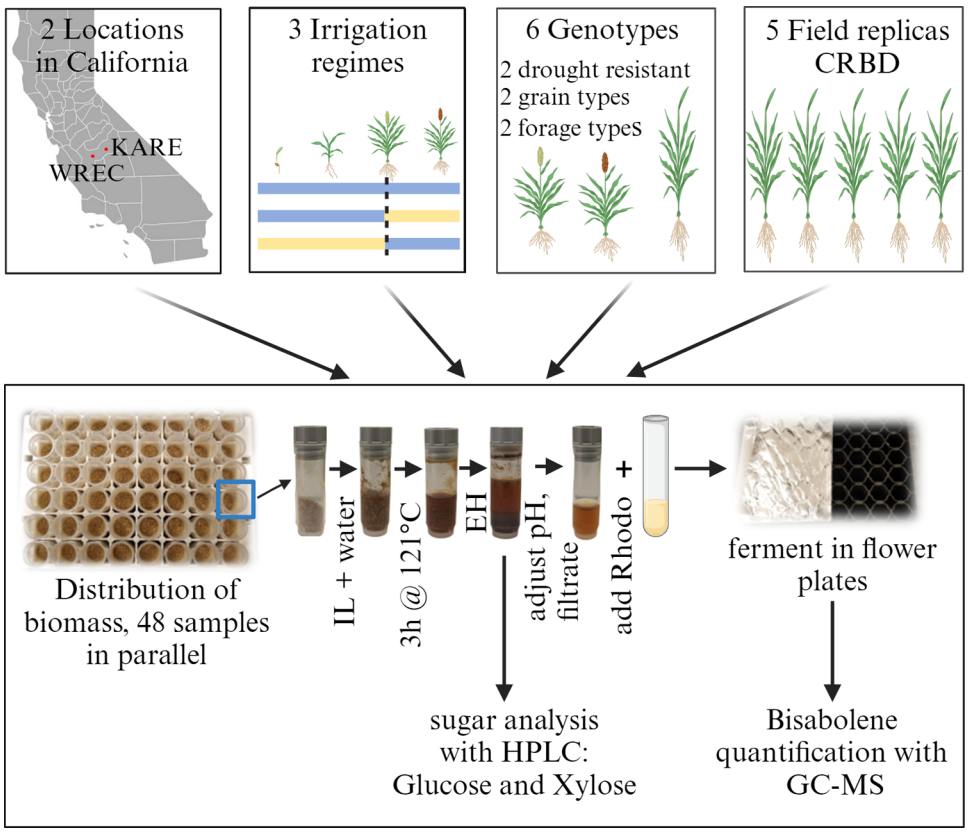

Figure 1. Schematic representation of the F2F pipeline. Sorghum plants were grown in two California locations using 3 irrigation regimes and 6 genotypes in a randomized complete block design with 5 replicas. The resulting fine ground, dry biomass is dispensed, weighed, and pretreated, running 48 samples in parallel. CRBD = Complete Randomized Block Design, IL= Ionic Liquid, EH = Enzymatic Hydrolysis, and Rhodo = Rhodosporidium culture.

dairylandlabs.com/methodology). All samples were prepared in three technical replicates, which were averaged for statistical analyses.

Statistical Models and Measures. The plots were planted in a randomized complete block design (RCBD) at each location with four replicates and a split-plot restriction. Irrigation treatments were the main plots, and cultivars were the subplots, and each trial was considered nested within location (KARE or WREC). The split-plot design was necessary due to practical restrictions on the application of irrigation water at each site. Data collected was analyzed with a split-plot analysis of variance (ANOVA) model nested within the location using the "agricolae" package in R.[22] This ANOVA model is well suited for RCBD experiments with nested levels of experimental factors.[23] We compared the location means using Fisher's least significant difference test (LSD), while pairwise comparisons of group means at the main plot and subplot levels were performed with Tukey's honest significant distance test (HSD). Although the Fisher's LSD test is less conservative than Tukeys' HSD, it is better suited for comparing means between only two groups, while Tukey's HSD test is more appropriate for comparing three or more group means.[24] Linear regressions to analyze pairwise correlations between dependent variables were performed in Microsoft Excel.

RESULTS AND DISCUSSION

Strengths and Limitations of the Feedstocks-to-Fuels Pipeline. This study serves as both an exploration of drought's effects on biomass convertibility to sugars and bioproducts as well as a demonstration of the strengths and drawbacks of an end-to-end experimental pipeline. An important consideration for research teams seeking to implement end-to-end pipelines like F2F is in defining the types and numbers of replicates (e.g., field, technical, and biological). Testing multiple feedstock genotypes or varying environmental conditions results in large sample numbers, which are subject to further replication once the samples are processed in the pipeline. The analysis presented in this study includes 6 genotypes, 3 irrigation regimes, 2 locations, 5 biological replicates, and 3 technical replicates, resulting in a total of 540 samples (Figure 1). However, the F2F pipeline is designed to run samples simultaneously, thus reducing the time for saccharification and fermentation on all samples to 30 days, compared to a typical timeline of 12 days per sample for bench scale analysis.[15,25] This large number of samples does limit the data that can be practically collected. For example, it is possible to track monosaccharide yields and product yields, but it is not practical to measure the cellulose, hemicellulose, and lignin contents via wet chemistry methods. Instead, samples were sent for forage quality analysis, which provides more limited compositional information but is more practical for implementation with a large number of samples.

In addition to monosaccharides from hydrolysates, the F2F pipeline also determines bisabolene production by *R. toruloides*.[26] Throughout the process, the F2F must, by necessity, be optimized for compressed timelines for sample runs and the ease of differentiation between biomass samples rather than optimized yields (Figure 1). The ionic liquid pretreatment process (cholinium phosphate) and the *Rhodosporidium* strain (GB1, also published as BIS3[15,19]) used in the F2F pipeline are not optimized for maximum sugar or bisabolene output. Instead, the F2F platform aims to be in the midrange of production capability to ensure a large enough

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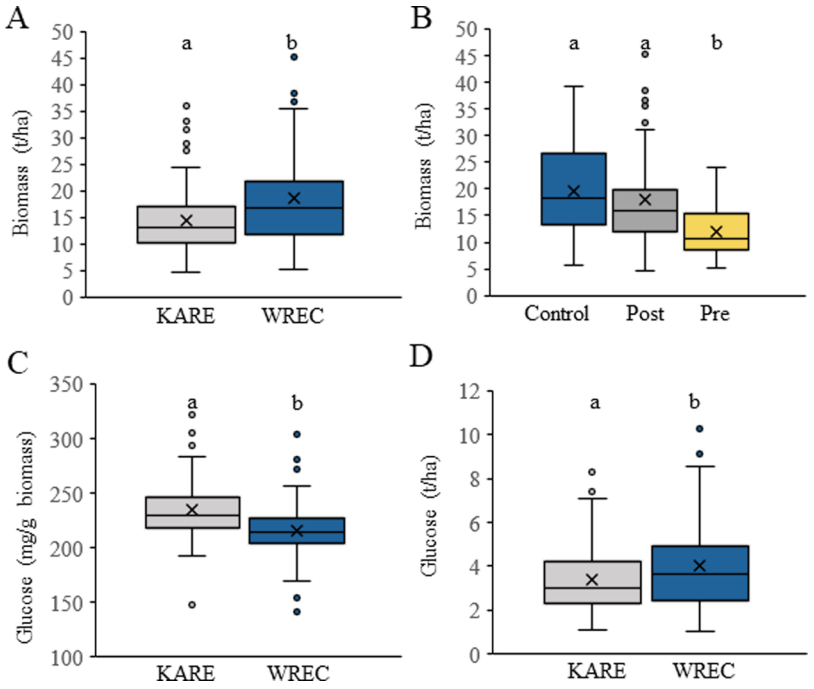

Figure 2. Biomass yield and glucose yield by location. (A) Boxplot of dry biomass in t/ha by location, (B) boxplot of dry biomass in t/ha by irrigation, (C) boxplot of glucose yield in mg/g biomass by irrigation, and (D) boxplot of absolute amounts of glucose calculated on a t/ha basis by location. Each box outlines the first and third quartiles, while horizontal lines within boxes represent medians, and *X* denotes means. Maximum and minimum values are indicated by error bars extending above and below each box. Outlying points are marked with dots. Letters above boxplots indicate significant differences ($p < 0.05$) between groups (i.e., groups that do not share a letter in common significantly differ from one another). "Post" and "Pre" refer to the timing of drought application relative to flowering.

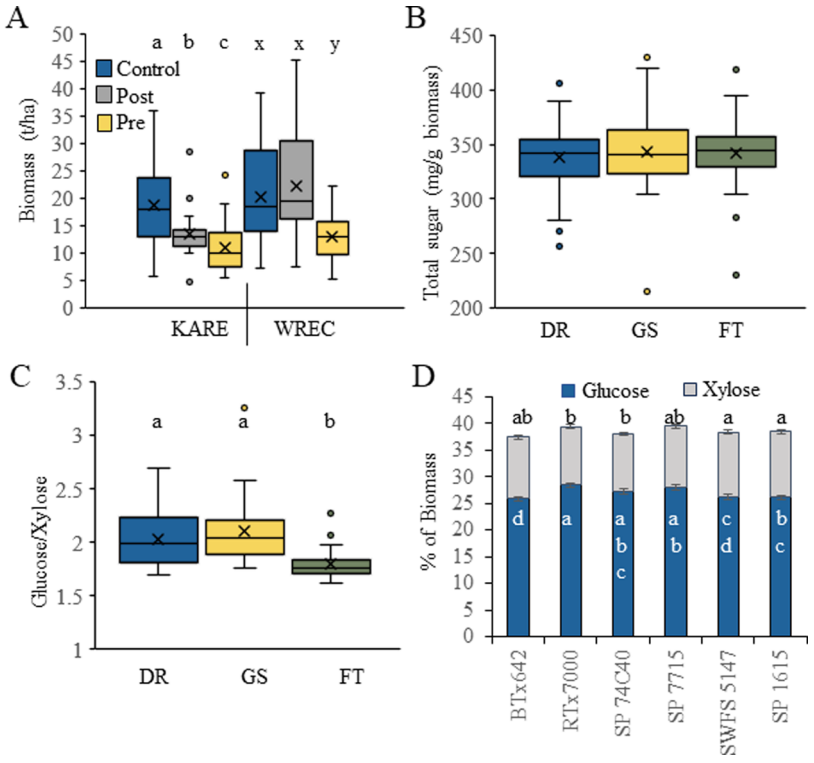

Figure 3. Sugar yield is comparable among types, while glucose to xylose ratio is type specific. (A) Boxplot of biomass yield in mg/g biomass by location and irrigation. (B) Boxplot of bisabolene yield in mg/g biomass by type, (C) boxplot of glucose to xylose ratio by type, (D) boxplot of glucose and xylose yield in % biomass by genotype. Letters above boxplots indicate significant differences ($p < 0.05$) between groups (i.e., groups that do not share a letter in common significantly differ from one another). Boxplot format is described in the legend of Figure 2.

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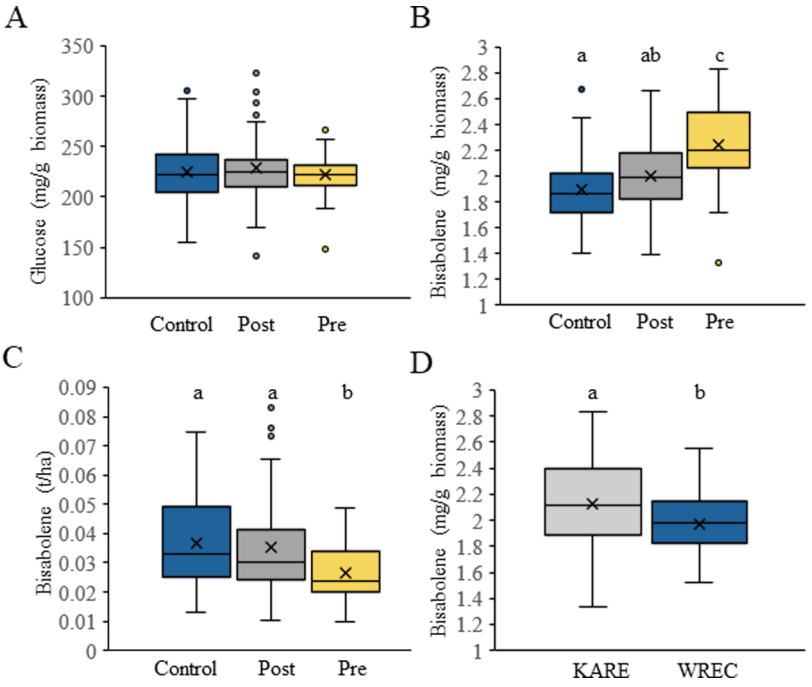

Figure 4. Bisabolene yields are increasing under drought conditions. (A) Boxplot of glucose yield in mg/g biomass by location, (B) boxplot of bisabolene yield in mg/g biomass by irrigation, (C) boxplot of absolute bisabolene amount in t/ha by irrigation, and (D) Boxplot of bisabolene yield in mg/g biomass by location. Letters above boxplots indicate significant differences ($p < 0.05$) between groups (i.e., groups that do not share a letter in common significantly differ from one another). Boxplot format is described in the legend to Figure 2.

dynamic range to detect differences between genotypes and treatments. Because this platform is designed for high throughput, fermentations are performed in volumes smaller than 2 mL. Ultimately, the F2F pipeline is best used to screen feedstocks for changes in recalcitrance across phenotypes and growth conditions and to identify feedstocks that produce compounds that either enhance or inhibit downstream microbial conversion. Further experiments and optimization under more realistic (and likely severe) conditions are required to gauge the potential performance at larger scales.

Sugar Yields and the Fate of Sorghum Grain during Conversion. Although sorghum is a drought-tolerant crop, the deficit irrigation treatments in this study did result in lower overall biomass yield measured as a dry mass in t/ha (Figure 2B). The same irrigation treatments also produced different sorghum yield effects across the two locations (Figure 3A), primarily due to the different soil types. As noted in the Experimental Section, the KARE location soil is characterized as a Hanford sandy loam with a silty substratum, which results in lower water-holding capacity, while WREC is a Panoche clay loam soil (higher water-holding capacity). Therefore, water limitations at KARE show a more drastic effect than those at WREC, where more plant-available water can be stored per unit soil volume (Figure 3A). The median biomass yield across all irrigation treatments was 22.4% higher in the WREC location (Figure 2A), while the glucose yield in the same location was significantly lower (Figure 2C).

For the purposes of bioenergy facilities, downstream conversion efficiency is also important; a lower-yielding crop may be preferable if the sugar and fuel yields are substantially higher. Based on the F2F results, the glucose yield per kg of dried biomass across all sorghum varieties and irrigation treatments was significantly lower in WREC as compared with KARE (Figure 2C). However, the glucose yield was only 8% lower at WREC on average, and this difference is comparatively smaller than the difference in the biomass yield. Therefore, the total glucose yield per hectare remains higher in WREC (Figure 2D). It is not clear why there was a lower glucose yield at WREC compared to KARE. This difference was not reflected in a change in neutral detergent fiber (NDF, Table S2), suggesting that the biomass from KARE is slightly less recalcitrant. Noteworthy, there was also no change in lignin (Table S2) between the two locations, which could have explained reduced recalcitrance.[27,28]

One challenge when biomass from forage-type sorghum is compared with grain-type sorghum (GS) is the heterogeneity of the samples. The experiences and results documented here offer useful insights for future studies. Whole-plant samples have the advantage of being most representative of feedstock intake at a commercial biorefinery, which would likely process whole plants from forage-/biomass-type sorghum rather than separating grain heads for separate conversion.[29] The use of these samples also avoids further expanding the number of samples by separating different plant parts (e.g., stalk, leaves, and grain). However, unlike grasses, such as switchgrass, whole-plant sorghum samples include substantial and varying quantities of starch. Detergent fiber analysis on our samples indicated an average fraction of 13.8% starch in our samples (Table S2). Fully converting this starch to fermentable sugars requires amylases; to simplify the comparison and maintain the focus on stover conversion, we omitted any amylases in the F2F processing used here. This also avoided large differences in sugar concentrations across samples (Figure 3D), which could have an impact on fermentation yields. The hydrolysates of all three types of sorghum contained comparable amounts of total sugar (Figure 3B).

Although amylases were not used in the conversion pipeline, there are significant differences in the glucose/xylose ratios

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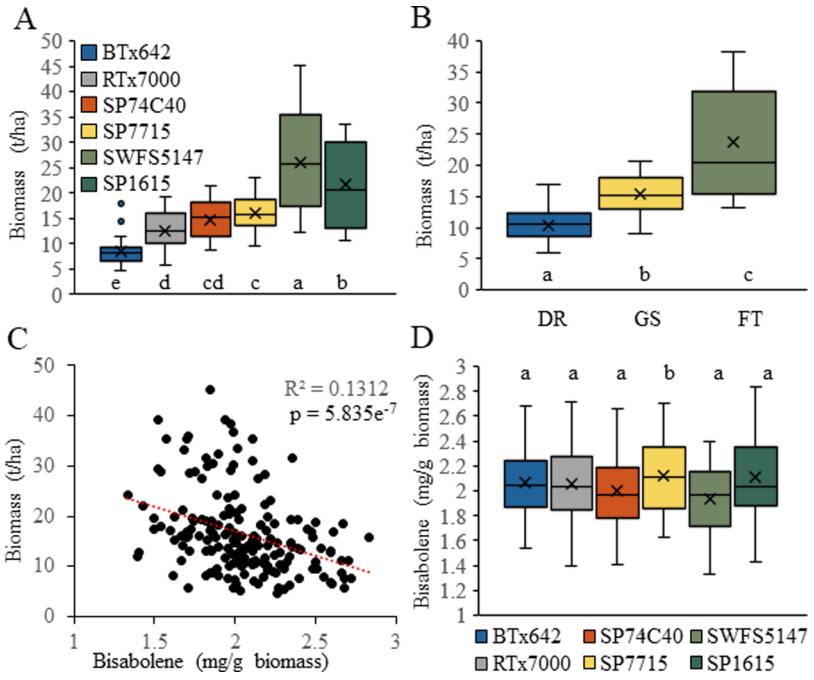

Figure 5. Bisabolene concentration is inversely correlated to biomass. (A) Boxplot of biomass yield by genotype. (B) Boxplot of biomass yield by type. (C) Correlation of biomass yield per hectare and bisabolene yield per unit biomass. Red shows the linear trend line, and correlation coefficient R^2 is shown in the upper right corner. (D) Boxplot of bisabolene yields in mg/g biomass by genotype. Letters above boxplots indicate significant differences ($p < 0.05$) between groups (i.e., groups that do not share a letter in common significantly differ from one another). Boxplot format is described in the legend in Figure 2.

between sorghum types (Figures 3C,D and S2). Both grain sorghum types (DR and GS) contain higher amounts of glucose compared to forage-type sorghum, which could indicate a partial breakdown of starches during the F2F process (Figures 3C and S2). Although not the original goal of the study, our results highlighted the need to develop strategies for effectively pretreating mixtures of starch and lignocellulosic biomass.

Although neither glucose nor xylose yield responds significantly to changes in irrigation (Figure 4A and SI Table 1), there are small but significant differences among genotypes (Figure 3D). It has previously been observed that drought stress did not impact saccharification efficiency in two sorghum cultivars.[30] However, because that analysis was only done on leaves, we can now add that whole biomass including stalks do not undergo a significant change in saccharification during pre- and postflowering drought stress as well as confirm the observation using a larger range of cultivars. Sorghum does use sugar relocation for osmoregulation; however, those are soluble sugars, mostly sucrose.[31,32] With the vast majority of biomass in our study being structural, there is no indication that cell wall deposits are restructured once in place, even under unfavorable conditions.

Effect of Drought Stress on Bisabolene Yields. Once the biomass was pretreated and enzymatically saccharified, *R. toruloides* was grown on the resulting mixed hydrolysate, and bisabolene yields were measured. While biomass production of preflowering and postflowering drought-stressed plants was reduced compared to controls by 38.8 and 8.4%, respectively (Figure 2B), bisabolene yields per gram of biomass were significantly greater (by 15.4% in preflowering and 5.2% postflowering) in drought-stressed plants (Figure 3B). Without a clear indication of the cause of higher bisabolene yields in drought-stressed sorghum, it is only possible to speculate until follow-up studies can be conducted. For example, it is possible that sorghum naturally produces inhibitors of *Rhodosporidium* fermentation but expends less energy to produce these under unfavorable conditions. It is noteworthy that drought induced inhibitors have been investigated for *Saccharomyces* already and are possibly not uncommon.[33] Alternatively, *Rhodosporidium* might use a second carbon source that is more abundant in the drought-stressed samples to boost bisabolene metabolism. Further studies are required to test both hypotheses.

Although this bisabolene yield increase is significant on a per-gram biomass basis, it does not compensate for the loss in biomass yield due to drought stress. Drought-stressed sorghum still results in less bisabolene production per hectare of feedstock planted relative to the control (Figure 4C).

The observed increased fermentation yield per unit sugar in drought-stressed biomass is in strong contrast to observations made in switchgrass, where growth and fermentation of *Saccharomyces cerevisiae Y128* were severely inhibited in hydrolysates from drought-stressed plants.[33,34] Therefore, either sorghum is not producing similar fermentation inhibitors during drought stress or *Rhodosporidium* is not responding the same way as *Saccharomyces* to such components. Both possibilities are supported by reports that neither drought-stressed corn stover hydrolysates are inhibiting growth and fermentation of *S. cerevisiae* nor does *Zymomonas mobiliz*, a bacterium, respond negatively to drought-stressed switchgrass hydrolysate.[33] The counterintuitive findings here warrant further study and comparisons among different microbial hosts and feedstocks. Experimental pipelines such as the one documented here are ideally suited for exploring a large number of feedstocks and environmental conditions, with the goal of generating hypotheses that can be further explored through more detailed studies. Biomass from bioenergy grasses contains large amounts of "NFC" labeled as "nonfibrous

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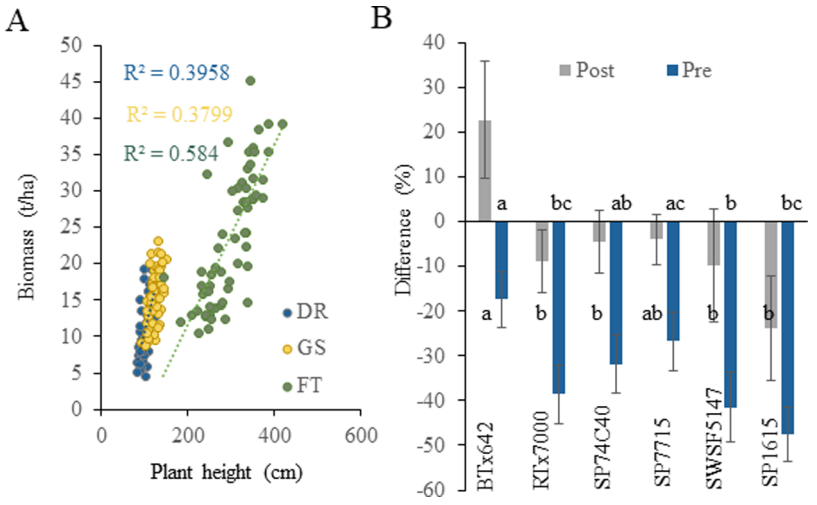

Figure 6. Drought response assessment. (A) Correlation of plant heights with biomass. (B) Biomass yield of each genotype under drought conditions. The bar graph shows the relative difference to the control treatment in % from the average.

carbohydrates" in standard forage analysis even though this fraction represents many different types of compounds, including organic acids and sugar alcohols. This is a residual fraction in the forage analysis that has not been well characterized. In our study, it represents around 40% of the biomass. NFC is a fraction that has been largely ignored in lignocellulosic biofuels research, and it is clear from our study that NFC warrants much more attention in future research to determine to what extent NFC may contain potential inhibitors and potential carbon sources for microbial conversion and how that varies with environmental conditions.

We also compared the bisabolene yield per gram of biomass by location and found yields to be significantly greater at KARE (Figure 4D), again suggesting that *R. toruloides* produces more bisabolene when consuming the hydrolysates of sorghum plants that experienced greater drought stress. We then explored the impact of irrigation conditions (preflowering drought, postflowering drought, and the control) by location (Figure 3A). While there is a clear reduction in biomass under preflowering drought stress at both locations, only samples from the KARE site showed a significant change in yield under postflowering drought conditions compared to controls (Figure 3A).

The bisabolene yield increased significantly under preflowering drought conditions, but not under postflowering drought conditions in the WREC location (Figure S2). Bisabolene yield therefore seems to exhibit an inverse relationship with the biomass yield. This observation prompted us to quantify the correlation between biomass and bisabolene yields. Biomass and bisabolene yields indeed correlate with each other inversely and significantly, but weakly (R^2 = 0.1312, p = 5.835×10^{-7}), indicating a secondary relationship instead of a direct effect (Figure 5C). We did not detect a similar pattern when looking at the bisabolene yield by genotype. The genotypes exhibited very different biomass yields (Figure 5D), but only the late flowering grain-type hybrid SP7715 had a small but significant increase in bisabolene yield when compared to all other sorghum genotypes (Figure 5D). Clearly, we can establish a dependency on both location and irrigation regime, but bisabolene yield remains independent of genotype, indicating a malleable, complex change in the fermentability of biomass.

Since we found a negative correlation between biomass and bisabolene yield, we further analyzed the data to identify other statistically significant correlations with bisabolene yields. We therefore tested glucose yield per g biomass and bisabolene yield per g biomass directly and surprisingly found that they do not significantly correlate (R^2 = 0.017, p = 0.0799) (Figure S2). This is also supported by the significant interactions in Table S1. Other compositional elements from our analysis were then tested for their correlation to bisabolene production. However, no additional significant correlations were identified. It is possible that there are nonlinear relationships, and additional data could shed light on possible methods to predict the bisabolene (or other microbially produced products) yield.

Biomass Yield in Sorghum Varieties and Types. The third factor we examined was the performance of three different types of sorghum: forage-type sorghum (FT), grain-type sorghum (GS) and drought-resistant grain-type sorghum (DR). FT sorghum lines are bred to have high biomass yields, as these plants are typically harvested, and the whole plant is ensiled to produce animal feed. The difference in biomass by all three types was highly significant: Both FT sorghum hybrids (SWFS5147 and SP1615) produced, on average, more than double the amount of biomass of the DR genotypes (BTx642 and RTx7000) and 64% more than the GS sorghums (SP74C40 and SP7715) (Figure 5A,C). The genotype that produced the most biomass in our experiment was SWFS5147, averaging 25.9 t/ha of dry weight. The total amount of sugars released per gram of biomass does not significantly change by type (Figure 3B).

The strongest factor correlating with biomass is plant heights (R^2 = 0.631, p = 2×10^{-40}), unsurprisingly, which also holds true for the grain-type hybrids (Figure 6A). This means that even in the grain hybrid lines tested here, the major source of biomass is coming from the stover. Notably, we did not find any correlation between plant heights and lodging (referring to sorghum plants falling over in the field) either. Lodging negatively impacts yields and makes harvesting more challenging. Lodging only occurred for both FT sorghum hybrids, predominantly though in SWFS5147, and it only occurred in the KARE location in the control and postflowering watering conditions, but not during predrought stress or at the WREC location. This indicates that lodging is

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not dependent on any single variable tested here but rather a complex trait depending on an interplay of all three: location, irrigation, and genotype.[35]

Performance under Drought. Despite the unexpected impacts of drought stress on bisabolene yields, there is a significant overall loss in biomass under both post- and preflowering drought stress (Figure 2B), and this effect outweighs the positive impacts on product yield. Overall biofuel yield per unit of land will remain higher for feedstocks not subjected to drought stress. The drought response by genotype was analyzed in more detail by looking at the relative differences between treatments (Figure 6B). As all genotypes have a significantly different biomass yield, the data was normalized to the average yield under control conditions for each genotype. Although RTx7000 has been described as preflowering drought resistant,[36,37] it did not show a significantly different yield performance compared to the GS or FT sorghum genotypes in this study (Figure 6B). It is noteworthy that tolerance to preflowering drought stress is measured by the recovery response after water availability is restored, not while plants are water deprived, and additionally, most preflowering drought-resistant sorghum lines subsequently undergo a full new cycle of tillering, thus ultimately requiring more water overall in order to reach maturity. In our conditions, plants were all harvested at the same time when the watering control plants reached maturity, contributing to the observed lower yield in RTx7000.

The variety that performed the best under preflowering drought conditions surprisingly has been BTx642 (Figure 6B), which also showed an increase in productivity under postflowering drought. As BTx642 is also the least productive of all of the tested genotypes, it will have less transpiration than the GS or FT hybrids, and therefore, drought will affect this genotype by design less than the other types. Nevertheless, BTx642 has well-characterized adaptation mechanisms,[38] which are contributing to the observed resistance under postflowering drought stress.[39,40]

SUMMARY

We successfully employed a novel high-throughput feedstocks-to-fuels platform for the first time to assess the influence of three different irrigation treatments in two California locations on six genotypes of sorghum. This approach makes it possible to more closely link field trials, which produce large numbers of variants and field replicates and downstream conversion. The sorghum types that produced the most biomass under all conditions were the forage-type hybrids. While more biomass was produced in the WREC location, the location that is better able to retain water in the soil, the biomass had a significantly lower glucose yield. Additionally, fermentability into bisabolene also showed a significant dependence on location but even more so on irrigation. Even though biomass decreased under both tested drought conditions (post- and preflowering drought), bisabolene yield surprisingly increased in the drought-stressed samples significantly. The results demonstrate the value of these pipelines in identifying statistically significant relationships between plant growing conditions and phenotypes with downstream yields but also highlight the challenges in elucidating causal linkages without full compositional analysis for every sample. Future studies on strengthening this linkage between compositional factors and high-throughput experimental pipeline results can further advance the effectiveness of this approach.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.4c05826.

Experimental design and conditions correlation of bisabolene with glucose split−split-plot ANOVA table for glucose, xylose, bisabolene, and biomass split−split-plot ANOVA table for cell wall components analyzed by forage analysis (PDF)

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M.L., V.P., C.H., and J.G. contributed to investigation; V.P., B.A.S., and J.G. contributed to developing the methodology; J.C.D., C.D.S., H.V.S., and D.P. conceptualized the project; D.P., J.G., B.A.S., B.H., C.D.S., and H.V.S. were project administrators; J.C.D. and T.H. did formal analysis; J.G., C.D.S., and H.V.S. did supervision, B.A.S., C.D.S., and H.V.S. were responsible for funding acquisition; J.C.D. did visualization and writing of the original draft; C.D.S. and H.V.S. contributed to editing.

Notes

The authors declare the following competing financial interest(s): B.A.S. has a financial interest in Erg Bio.

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