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RESEARCH ARTICLE

High temperatures and low soil moisture synergistically reduce switchgrass yields from marginal field sites and inhibit fermentation

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Abstract

'Marginal lands' are low productivity sites abandoned from agriculture for reasons such as low or high soil water content, challenging topography, or nutrient deficiency. To avoid competition with crop production, cellulosic bioenergy crops have been proposed for cultivation on marginal lands, however on these sites they may be more strongly affected by environmental stresses such as low soil water content. In this study we used rainout shelters to induce low soil moisture on marginal lands and determine the effect of soil water stress on switchgrass growth and the subsequent production of bioethanol. Five marginal land sites that span a latitudinal gradient in Michigan and Wisconsin were planted to switchgrass in 2013 and during the 2018–2021 growing seasons were exposed to reduced precipitation under rainout shelters in comparison to ambient precipitation. The effect of reduced precipitation was related to the environmental conditions at each site and biofuel production metrics (switchgrass biomass yields and composition and ethanol production). During the first year (2018), the rainout shelters were designed with 60% rain exclusion, which did not affect biomass yields compared to ambient conditions at any of the field sites, but decreased switchgrass fermentability at the Wisconsin Central–Hancock site. In subsequent years, the shelters were redesigned to fully exclude rainfall, which led to reduced biomass yields and inhibited fermentation for three sites. When switchgrass was grown in soils with large reductions in moisture and increases in temperature, the potential for biofuel production was significantly reduced, exposing some of the challenges associated with producing biofuels from lignocellulosic biomass grown under drought conditions.

KEYWORDS

bioethanol, fermentation, marginal lands, rainout shelter, soil water stress, yeast inhibition

1 | INTRODUCTION

The impending depletion of conventional fossil fuels, as well as the dominant role of fossil fuel use in causing climate change, have led to a search for alternative sources of energy. The replacement of petroleum-based liquid transportation fuels with renewable and carbon-neutral cellulosic biofuels presents a viable opportunity to reduce reliance on fossil fuels for transportation (Robertson et al., 2017). As bioenergy crops should not compete with arable land use for food production, these species are expected to be tolerant to conditions that render lands unsuitable or unprofitable for food crops, such as limited soil water availability during the growing season. Many grasses can flourish in spite of low soil water (Cui et al., 2020; Drebenstedt et al., 2020; Eziz et al., 2017; Kørup et al., 2018; Oliver et al., 2009; Wang et al., 2007) and are thus potentially suitable as bioenergy crops (Gelfand et al., 2013; Kang et al., 2013).

Switchgrass (*Panicum virgatum* L.) is a productive C_4 prairie grass native to North America that is often used as a forage crop. Switchgrass can be cultivated across diverse climatic and edaphic settings and is relatively drought-tolerant (Emery et al., 2020; Hui et al., 2018) compared to other C_4 bioenergy feedstocks, and likely more resilient to changing climatic conditions. However, when cultivated during extreme drought, switchgrass experienced a severe reduction in biomass yield and caused total inhibition of yeast growth during fermentation (Ong et al., 2016). This would be deleterious if experienced by a biorefinery, making it important to understand the agronomic and physiological factors that impact switchgrass yield and its quality for conversion into biofuels and bioproducts.

Drought is often quantified using hydroclimatic parameters and biomass yields. However, dry periods that are not technically considered drought conditions can still exert water stress on growing plants (Smith et al., 2022; Zhang et al., 2015). Soil water content and air and soil temperatures during the biomass growth phase have been frequently related to crop yields (Drebenstedt et al., 2020; Hui et al., 2018; Wang et al., 2007). Many studies have reported how reduced precipitation and soil warming can affect physiological and morphological changes in crops including switchgrass, barley, and soybean, by directly reducing the aboveground biomass and interfering with photosynthesis, causing notable changes in leaf physiology (Cui et al., 2020; Drebenstedt et al., 2020; Hui et al., 2018). Depending on the timing of water stress and its severity with respect to the growing season, water stress may lead to a severe reduction in biomass yields or have little to no effect (Hamilton et al., 2015; Kørup et al., 2018). It is less well understood how water stress may affect the biochemical quality and potential for biofuel production from feedstocks such as switchgrass.

The main goal of this study was to determine if water limitation affects switchgrass biomass yields and fermentability when grown on low productivity lands. In this experiment, switchgrass was grown over four growing seasons (2018–2021) at five marginal land field sites with diverse soils, spanning a latitudinal gradient within Michigan and Wisconsin, USA. These sites were previously abandoned from food crop production due to low productivity resulting from low soil fertility. During the 2018 growing season, mature stands of switchgrass were either exposed to ambient conditions or ~60% reduced rainfall using rainout shelters. In later seasons (2019–2021), the rainout shelters were modified to exclude 100% of rainfall and increase the severity of the water stress. Switchgrass from each field site was harvested, ground, dried and pretreated using Ammonia Fiber Expansion (AFEX) followed by high solids enzymatic hydrolysis (7% glucan loading; Chandrasekar et al., 2021). Hydrolysates were fermented using engineered *Saccharomyces cerevisiae* yeast, and real-time carbon dioxide and final ethanol production measured. The ethanol yields for switchgrass samples under the rainout shelters were evaluated and compared with ambient conditions (normal precipitation) to understand the influence of the soil temperature and moisture content on biomass yields and quality.

2 | MATERIALS AND METHODS

2.1 | Site details

In 2013, the Great Lakes Bioenergy Research Center (GLBRC) planted six bioenergy crops—switchgrass (var. Cave-in-Rock), miscanthus, native grass mixture, poplar, early successional vegetation, and restored prairie—in five marginal land sites in Michigan (MI) and Wisconsin (WI), USA. Sites were located along a north–south gradient in each state (Figure 1, Table 1): MI North-Escanaba, MI Central-Lake City, and MI South-Lux Arbor, WI North-Rhinelander, and WI Central-Hancock. Going forward, to increase clarity in the text, sites will be abbreviated using only the city name, while the full name will be used in figures.

Each switchgrass field site had four replicate plots, except for Hancock, which had 3 replicate plots. Plot dimensions for Lake City and Escanaba were 19.5 × 19.5 m and for Lux Arbor, Hancock, and Rhinelander were 19.5 × 12.2 m. Mown alleyways that were 3–15 m wide separated adjacent plots. Replicate plots were arranged using a randomized block design as reported previously (Jayawardena et al., 2023). Maximum daily temperatures and total monthly precipitation for each year and location were obtained from nearby National Weather Service (NWS) station data (NOAA, 2018–2021): Lux Arbor (Battle Creek 5NW, MI), Lake City (Lake City Exp Fa, MI), Escanaba (Gladstone, MI—precipitation and Rapid River SSE, MI—temperature and May 2019 precipitation), Hancock (Hancock Exp Farm, WI), and Rhinelander (Rhinelander, WI).

2.2 | Switchgrass establishment, management, and harvesting

Soils were prepared for planting by mowing existing vegetation in fall 2012 and applying pesticides according to recommendations from Michigan State University and University of Wisconsin extension agronomists. Glyphosate and 2,4-D ester were applied to each plot to kill existing vegetation before switchgrass establishment in summer 2013 from seeds. All sites were planted without tillage except for Lux Arbor and Rhinelander, which required conservation tillage before planting to remove legacy furrows. Cave-in Rock switchgrass was planted using a Truax No-Till Seed Drill (Truax Company Inc, New Hope, 168 Minnesota) at a seeding rate of 7.85 kg/ha. Once the crop was established, herbicides were applied as needed. A randomly placed 75 cm × 75 cm quadrat ($n = 4$) was used to count the total number of plants to obtain switchgrass stand counts. Soil pH, potassium (K), and phosphorus (P) content were analyzed on a 3-year cycle between 2013–2021. Soil pH at Lux Arbor in 2016 and 2019 and Hancock in 2019 dropped to below the agronomic optimum, and thus the pH was increased by adding pelletized limestone. Potash addition of 34–56 kg K/ha was

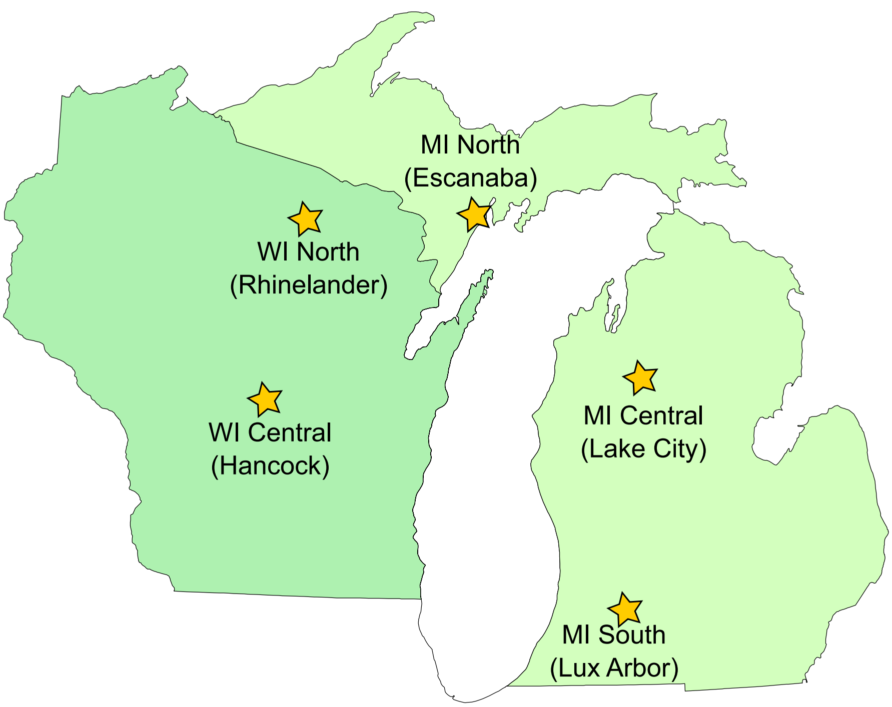

FIGURE 1 Bioenergy Lands Experiment (formerly called the Marginal Lands Experiment) sites in Michigan and Wisconsin. All sites including switchgrass and other perennial biofuel crops were grown in replicated experimental plots (Jayawardena et al., 2023).

TABLE 1 Soil taxonomy and coordinates for each site.

Location	Coordinates	Soil series	Taxonomic class	Supplemental soil information
MI North-Escanaba	*[45.7627, −87.1877]*	Onaway	Inceptic Hapludalf (Alfisol)	Table S1
MI Central-Lake City	*[44.2961, −85.1996]*	Croswell	Oxyaquic Haplorthod (Spodosol)	Table S2
MI South-Lux Arbor	*[42.4764, −85.4519]*	Kalamazoo/Oshtemo	Typic Hapludalf (Alfisol)	Table S3
WI North-Rhinelander	*[45.6656, −89.2180]*	Vilas	Entic Haplorthod (Spodosol)	Table S4
WI Central-Hancock	*[44.1194, −89.5338] and [44.1129, −89.5334]*	Plainfield	Typic Udipsamment (Entisol)	Table S5

Note: From Kasmerchak and Schaetzl (2018).

undertaken in May 2020 for all sites except for Rhinelander (Jayawardena et al., 2023) based on soil test results.

The switchgrass growing season spanned May through mid-October. Michigan sites were harvested in mid-late October while Wisconsin sites were harvested in early November. The switchgrass in Hancock Plot 1 died prior to the beginning of the study, and the switchgrass in Hancock Plot 2 died prior to the 2021 growing season, at which point it was removed from the study. In 2019, the biomass yield was not recorded at Hancock Plot 4 and Rhinelander Plot 2 ambient sites due to sampling errors in the field. Switchgrass under the shelters was hand-harvested while the ambient plots were machine-harvested using a method previously reported (Jayawardena et al., 2023). Following harvest, the switchgrass was oven-dried (Grieve Corporation, Round Lake, IL) at 66°C for 3–4 days and then milled through a 5 mm screen using a hammer mill (Schulte Hammermill WA-8-H, Buffalo, NY). The switchgrass dry matter yield was reported as the weight of switchgrass harvested per hectare of land (Mg/ha).

2.3 | Rainout shelters

Rainout shelters (3.7 × 3.7 m) were installed near the corner of each plot at each field site, with a sampling area of 2.4 × 2.4 m. The shelters had side post vertical heights of 1.8 m to account for switchgrass maximum crop height. Caster wheels were eight inches in diameter, for a total vertical height of 2.0 m. The shelters were constructed with 5 × 5 cm galvanized steel rectangular tubing with vertical supports every four feet for rigidity. Framing and roofing were built to support 145 km/h wind loads. The roof truss network had a slope of 8 cm × 30 cm, and the designed center peak was 0.5 m taller than the side post height. The peak run was in the same direction as the longer crop plot dimension and the side with the open truss peak had slightly more ambient exposure. The corrugated roofing panels were 1.3 m wide by 1.8 m long, with panels overlapped to prevent any rain intrusion. In 2018, the rainfall exclusion was 60% with a 0.23 m panel and 0.15 m openings on the roof. In subsequent years, a full rainfall exclusion was undertaken by eliminating the 0.15 m openings, thus providing 100% roof occlusion to fully exclude rainfall. The corrugated roofing panels (Amerilux Greca Lexan) allowed approximately 90% light transmittance above 385 nm. Panels were weather resistant and had a UV protective coating on one side to prevent gradual color change (yellowing) of the panels over time. A clear Lexan ridge cap was installed to prevent rain intrusion at the length of the shelter peak. Each shelter had a base made of the same galvanized steel shelter material running the length of the shelters in the same direction as the center peak for added strength. A gutter and hose network spanning the length of each shelter was installed on each side and pitched toward the plot edge to allow any rain collected from the roof to be diverted to well outside the plot.

2.4 | Field measurements

Reflectometer (CS655-L) probes (10 cm rods) from Campbell Scientific (UT, USA) were installed horizontally at two soil depths (10 and 25 cm) beneath the rainout shelters and just outside the shelters (for ambient samples). Soil temperatures at both depths were measured using thermistors within each probe. The reflectometer probes detected soil water content continuously at 60-min intervals. The daily average was calculated and reported. Soil water content from the reflectometer probes was not recorded from July to September at Lake City and Rhinelander in 2018 and Lux Arbor in 2021 due to probe installation issues.

2.5 | Near-infrared spectroscopy (NIRS) composition analysis

Switchgrass biomass composition was evaluated using near-infrared spectroscopy (NIRS). Each sample was separately milled through a 2 mm screen using a Cyclotec 1093 Mill (FOSS Analytical, Denmark) and sent to National Renewable Energy Laboratory (Golden, CO) for NIR analysis. Samples were dried for 24 h at ambient temperature under vacuum before analysis. A Metrohm XDS Multivial NIRS Analyzer scanned the biomass, and the spectra were collected using Vision 4.1 (Metrohm). Quartz optical glass ring cups of 36 mm in diameter were used to hold the sample. A diffuse reflectance detector, set to 17.25 mm spot size, scanned the samples at a wavelength of 400–2499.5 nm with 0.5 nm resolution. The dataset was evaluated using a previously developed NIRS composition model based on various biomass types (Templeton et al., 2016; Wolfrum et al., 2020).

2.6 | Ammonia fiber expansion (AFEX) pretreatment

All switchgrass samples were separately processed in triplicate using ammonia fiber expansion pretreatment as reported previously (Chipkar et al., 2022). Ground switchgrass was hand-mixed with distilled water (0.6 g per g dry biomass) and then loaded in custom stainless-steel reactors. Anhydrous liquid ammonia was added (2 g NH_3 per g dry biomass) and then the reactors were heated to 120 ± 5°C and then held for 30 min after which the ammonia was released (Chundawat et al., 2020). The pretreated biomass was then air-dried in a custom acrylic drying box until reaching a moisture content of <12%.

2.7 | Production of switchgrass hydrolysate

The method for producing high solids loading (7% glucan) switchgrass hydrolysates has previously been optimized (Chandrasekar et al., 2021). Nalgene Oak Ridge roller bottles (85 mL) were loaded with AFEX-treated biomass and water to reach 7% glucan loading and a final working volume of 35 mL. Pretreatment and hydrolysis were conducted as a unit in triplicate. The protein content of the cellulase (Novozyme 22257) and hemicellulase (Novozyme 22244; Novozymes, Franklinton, NC, USA) enzymes was determined by desalting using a disposable column (PD-10, Cytiva, VWR 95017-001) and followed by Pierce BCA analysis using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology). Enzymes were loaded at 28 mg protein/g glucan consisting of 70% cellulase and 30% hemicellulase on a protein basis. The hydrolysates were maintained at a pH of 5.80 using 0.1 M, pH 3 phosphate buffer containing monobasic and dibasic potassium phosphates.

2.8 | Fermentation

Switchgrass hydrolysates were fermented with engineered *Saccharomyces cerevisiae* strain Y945 (Sato et al., 2016) using a respirometer to quantify volumetric CO_2 production over time (Chandrasekar et al., 2021). Each plot at a specific field site was processed in triplicate (with replication over the combined pretreatment, hydrolysis, and fermentation process) and every rainout sample was paired with an ambient sample to account for biological variability between fermentation runs. Quality controls (synthetic hydrolysate medium [SynH]; Keating et al., 2014), 2008 AFEX-treated corn stover hydrolysate, and/or 2019 AFEX-treated switchgrass hydrolysate) were run with each fermentation trial to ensure consistency. The validity of the method was confirmed using previously characterized inhibitory (2012 AFEX-treated switchgrass) and non-inhibitory (2010 AFEX-treated switchgrass) controls.

2.9 | Statistical analysis

Tukey's pairwise comparisons and ANOVA (general linear model with 95% confidence interval) were performed on dry matter harvest yields using Minitab 20, with field plots as the replicate. ANOVA of biomass glucan and xylan NIR composition, hydrolysate glucose concentration, and ethanol production were evaluated using the lm() and anova() functions in R, with plot nested within location. The carbon dioxide production from triplicate respirometer experiments was averaged using the ggplot2 package in RStudio by applying a generalized additive model (gam) with the formula = 'y ~ s(x, bs = "cs")' (Wickham, 2016). Pairwise comparisons (*t*-test) between ambient and rainout samples for ethanol production, carbon dioxide release, and process ethanol yields were evaluated using the compare_means() function in the ggpubr package (Kassambara, 2023) in R.

To statistically compare the effect of rainout treatment on fermentation profiles, survival analysis (Canales et al., 2018; Marquenie et al., 2002) of the fermentation lag phase was carried out using the survival (Therneau & Grambsch, 2000) and survminer packages (Kassambara et al., 2021) in R. The fermentation lag phase was calculated as the x-intercept of a tangent line to maximum slope of the CO_2 production curve (calculated based on 10 datapoints). Samples that did not have sufficient data to plot a tangent line were coded as Low CO_2 and censored as "0". The other observations, where a lag phase could be calculated, were coded as "1". Log-rank tests (Hosmer & Lemeshow, 1999) were conducted to evaluate the effect of treatment (rainout vs. ambient) for the same location and harvest year, and to compare two pairs of controls (synthetic hydrolysate [SynH] vs. 2008 corn stover and 2010 vs. 2012 switchgrass) on the survival plot distribution. Log-rank *p*-values were reported for all pairwise comparisons.

3 | RESULTS

3.1 | Switchgrass dry matter yields were reduced under full water exclusion treatment at three out of five field sites

Switchgrass dry matter yield (Figure 2) was highly dependent on field site ($p = 0.000$), water exclusion treatment ($p = 0.001$), and year ($p = 0.000$), and there were significant two-way interactions between field site*year ($p = 0.000$) and treatment*year ($p = 0.002$; Table 2). All Michigan and Wisconsin field sites had similar average dry matter yields for the paired ambient and rainout samples in 2018 (Table S6), which led to the decision to modify the shelters in 2019–2021 to fully exclude rainfall with 100% roof occlusion (2019–2021). Full rainfall exclusion led to declines in biomass yields for three of the field sites (Lux Arbor, Escanaba, and Hancock) compared to the ambient control. Between 2019–2021, the Hancock site had the greatest reduction in biomass yields under the rainout shelters compared to the paired ambient treatment, and the difference remained consistent across years (Figure 2; Tables S7 and S8). The Lux Arbor and Escanaba sites also showed a reduction in the biomass yield for rainout treatment compared to ambient but only in 2020 and 2021 (Figure 2; Tables S7 and S8). In contrast to the other three field sites, the Lake City and Rhinelander sites showed a decline in biomass yield over time for both treatments, which had comparable yields within a given year (Figure 2; Tables S7 and S8). Overall, the Escanaba site had the highest yields, and the Hancock site had the lowest biomass yields compared to other field sites (until 2021 when the Lake City and Rhinelander biomass yields declined to similar levels; Figure 2; Table S8).

3.2 | Rainout shelters reduced soil moisture at all locations with the largest reductions at Lux Arbor and Escanaba in 2020 and 2021

Under ambient conditions, soil moisture levels were consistent for the same site across multiple years (Figure 3). Of the locations, Lux Arbor, Escanaba, and Rhinelander had comparatively higher soil water contents compared to Lake City and Hancock (Figure 3). The rainout shelters reduced the soil water content and variability at all locations except for Hancock, with the largest reductions at Lux Arbor and Escanaba in 2020 and 2021, where the ambient plots did not experience significant water depletion between June and September (Figure 3). Although the Hancock location showed an effect of rainout treatment on biomass yields (Figure 2), there was no obvious relationship to soil water content, which was already very low for the ambient plots, and not drastically lower for the

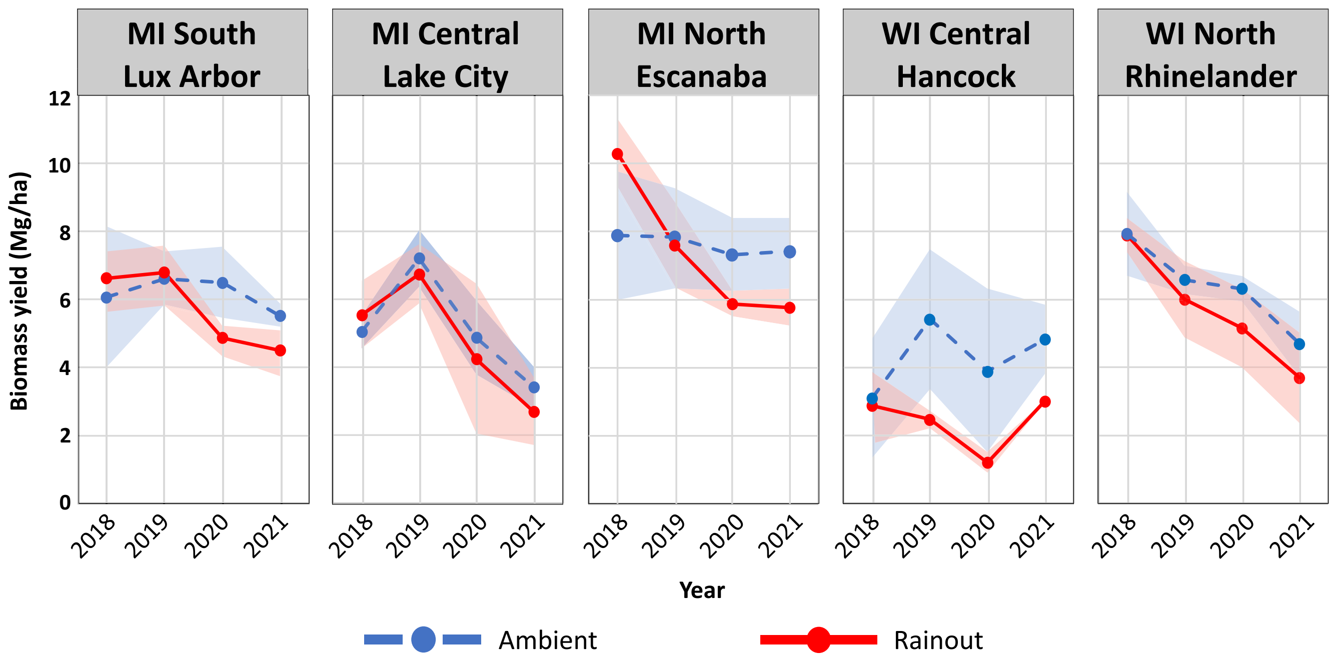

FIGURE 2 Biomass yield reduction was observed for ambient as well as rainout switchgrass samples for the five field plots from 2019 to 2021 except for Wisconsin Central – Hancock in 2021. Shaded region in respective color represents yearly biomass yield (averaged across field plots) ± standard deviation (Wisconsin Central-Hancock [$n = 3$]; all other sites [$n = 4$] in all years except for 2021 Wisconsin Central-Hancock [$n = 2$]).

TABLE 2 ANOVA based on the general linear regression for dry matter yield (Mg/ha) of switchgrass harvested from field sites in 2018–2021.

Source	*df*	Adj SS	Adj MS	*F*-value	*p*-value
Field site	4	240.20	60.0505	36.32	0.000***
Treatment	1	19.11	19.1119	11.56	0.001**
Year	3	84.64	28.2137	17.06	0.000***
Field site*Treatment	4	10.84	2.7088	1.64	0.169
Field site*Year	12	64.55	5.3788	3.25	0.000***
Treatment*Year	3	26.14	8.7136	5.27	0.002**
Error	119	196.77	1.6535		
Lack-of-Fit	12	10.83	0.9028	0.52	0.898
Pure error	107	185.94	1.7377		
Total	146	672.93			

Note: Plot was treated as the replicate for statistical analysis.
Significance is represented using asterisks as $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

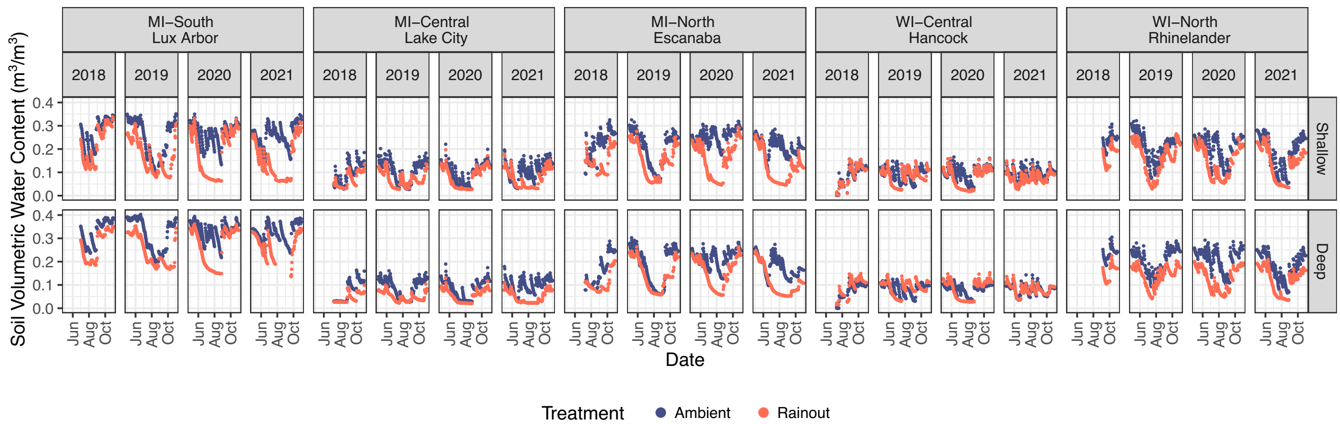

FIGURE 3 Soil moisture was reduced under the rainout shelters for all field sites during the study period, with the largest reduction at Lux Arbor and Escanaba in 2020 and 2021. The probes were installed at two different depths 10 cm (shallow) and 25 cm (deep). The data were recorded during the switchgrass growing season from July to October in 2018 and May to October in 2019–2021. Data points represent the daily average of hourly measurements. Markers on *x*-axis indicate the start of the respective month.

rainout treatment (Figure 3). Although the data were not available for the entire growing season in 2018, the soil moisture patterns seemed similar to those observed following the transition to 100% rainfall exclusion in 2019. There was significant interannual variability in precipitation accumulated during the growing season (Figure 4) that was not reflected in the soil moisture data, which was remarkably consistent between years (Figure 3). Although the Hancock site had the greatest precipitation in 2018 and 2019 (Figure 3), the soil moisture remained low in those years (Figure 4).

3.3 | Soil temperatures at each site were consistent across years, with temperatures comparatively higher at the Hancock site

Soil temperatures were compared at two depths (shallow = 10 cm and deep = 25 cm) for all sites between 2018–2021. For the same location, soil temperatures were highly consistent across the 4 years of the study (Figure 5). All sites, with the exception of Hancock, had very similar maximum soil temperatures of ~22–25°C at 10 cm and ~20–23°C at 25 cm, with minimal differences between the ambient and rainout treatments. In contrast, the maximum soil temperatures at Hancock were significantly higher, between 26–32°C at 10 cm and 24–28°C at 25 cm (Figure 5). Additionally, the Hancock location was the only site that showed a difference in maximum soil temperatures between the ambient and rainout treatments, with maximum soil temperatures ~2–3°C higher under the rainout shelters in 2019 and 2020 (Figure S1). Soil temperatures were largely independent of ambient air temperatures, which were strongly dependent on latitude (Figure 6). There were also significant interannual variations in ambient air temperatures during the study period, with 2019 notably cooler than the other years (Figure 6b).

3.4 | Switchgrass structural carbohydrates and hydrolysate composition were not significantly affected by the rainout shelter treatment

Downstream processing was carried out on switchgrass harvested in 2018 and 2020. Only two of the 4 years were able to be evaluated due to the time-consuming nature of the experiments. The 2019 switchgrass was not included in the analysis due to the lower air temperatures (Figure 6), greater precipitation (Figure 4), and less conclusive effects of rainout shelters on biomass yields (Figure 2) and soil water content (Figure 3). Biomass glucan and xylan contents and hydrolysate sugar concentrations were evaluated to understand their effect on the fermentability of switchgrass. The compositions of the untreated switchgrass samples were determined by near-infrared spectroscopy (NIRS; Figure 7a; Tables S9 and S10) and wet chemistry (2018 only; Table S11). Glucan was not significantly affected by rainout treatment, and xylan was only slightly affected, in contrast to location and year, which had more significant effects (Tables S12 and S13). Glucan and xylan contents were comparable in harvested biomass across all the field sites, with the exception of Lake City 2020, where the glucan was higher in the ambient sample, and Hancock 2020, where the xylan was higher in the ambient sample (Figure 7a; Table S14). There was a slight increase in xylan content between 2018 and 2020 for all locations.

Although hydrolysate glucose concentrations showed some variation between samples (Figure 7b; Table S15), there was no statistically significant effect of rainout shelter treatment on hydrolysate glucose concentrations, only location, plot, and year effects (Table S16). Some of the 2020 Lux Arbor hydrolysates had significantly lower glucose concentrations (<40 g/L) compared to the other samples.

3.5 | Hydrolysate fermentability decreased with rainout treatment and harvest year

Based on our prior work, imposition of drought-like conditions was expected to lead to a reduction in the fermentability of switchgrass hydrolysates compared to ambient conditions (Chipkar et al., 2022; Ong et al., 2016). To investigate this, the potential for biofuel production from the 2018 and 2020 switchgrass was determined by deconstructing switchgrass using AFEX pretreatment followed by high solids enzymatic hydrolysis (7% glucan loading) and yeast fermentation, with carbon dioxide production evaluated in real-time and used to generate growth curves (Cahill et al., 1999; Chandrasekar et al., 2021; Michel et al., 2020). For the 2018 hydrolysates, the Hancock samples showed the largest difference in CO_2 production between ambient and rainout samples, with long lag phases for the rainout samples (Figure 8). The 2020 hydrolysates were less fermentable than the 2018 hydrolysates, with greater lag phases for most of the Lux Arbor, Lake City, and Escanaba rainout plots compared to the ambient plots. Additionally, yeast had long lag phases in both the ambient and rainout 2020 Rhinelander and Hancock hydrolysates.

Survival analysis was used to evaluate the statistical significance of treatment (ambient vs. rainout) on the length of the fermentation lag phase, with longer lag phases indicating stronger fermentation inhibition. Survival analysis is commonly conducted in studies on equipment failure or in medical research on the survival of subjects in response to a treatment. In these types of analyses, some individuals have survived past the end of the experiment and so the exact time to failure/death is not known. The fermentation experiments were the inverse of this system as some of the microorganisms had not started actively fermenting by the end of the experiment and so the lag phase for those

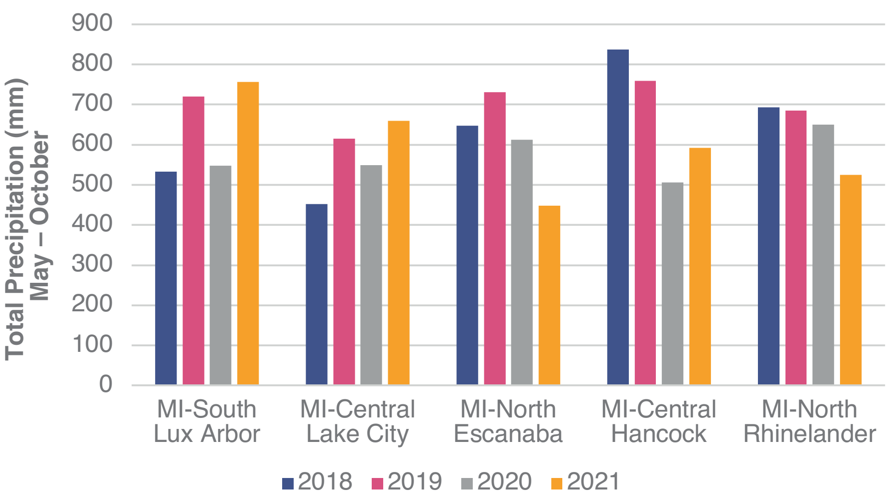

FIGURE 4 Cumulative precipitation varied by location and year, with greatest precipitation in Hancock 2018–2019. Data is reported as the total precipitation from May–October in each year.

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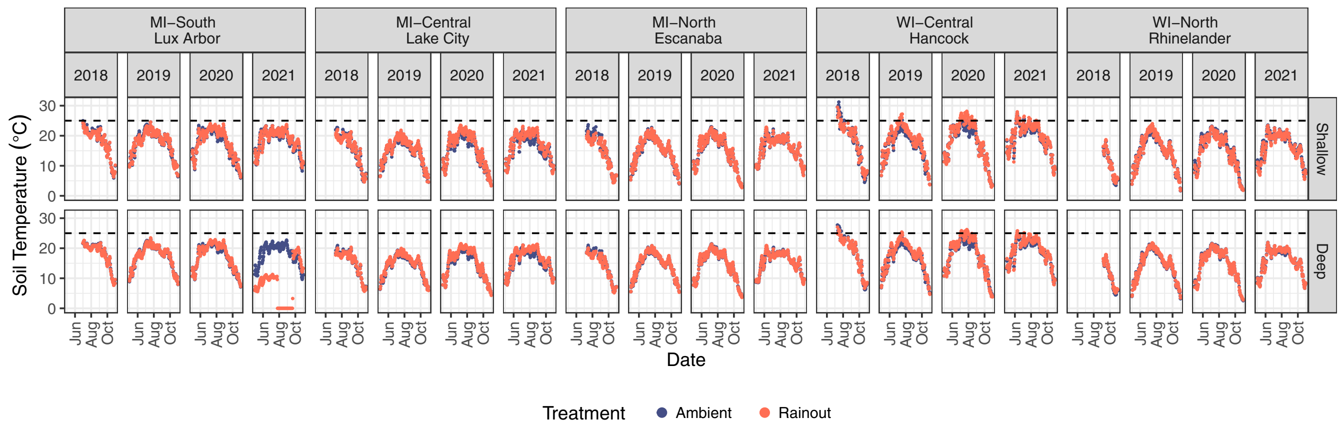

FIGURE 5 Rainout shelter soil temperatures (shallow – 10 cm and deep – 25 cm) showed similar trends across years for the same location, with consistently high soil temperatures at the Hancock location. Temperatures are reported as daily averages of hourly measurements from July–October (2018) and May–October (2019–2021). Markers on the *x*-axis indicate the start of the respective month. The dashed black line at 25°C is included as a visual reference point.

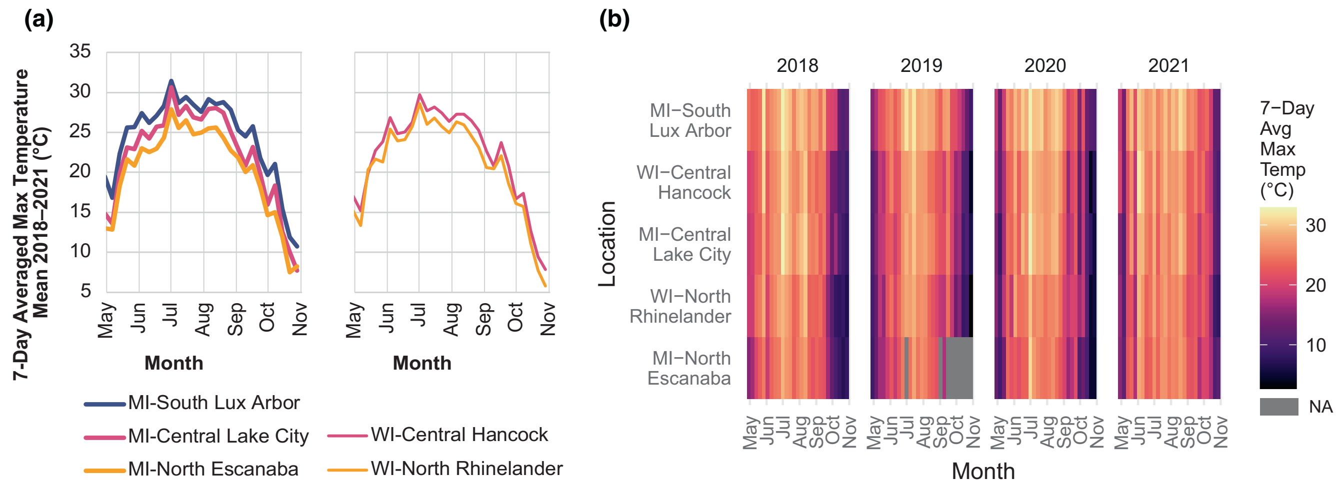

FIGURE 6 Air temperatures were largely dependent upon latitudinal location. (a) Maximum air temperatures (mean 2018–2021) decrease with increasing latitude, with similar temperatures for the comparable latitudes in Michigan and Wisconsin. (b) Escanaba is the coolest location and 2019 is the coolest year, based on a heat map of 7-Day averaged maximum air temperatures. Data are reported as the maximum temperature averaged across 7 days for the switchgrass growing season (May–October) and sites are organized by increasing latitude.

experiments was unknown. Log-rank tests were used to assess the effect of ambient versus rainout treatment on fermentation lag phase, as this test is sensitive to variability at the end of the observation period, where the CO_2 production varied more between samples. Hancock was the only site with a significant difference ($p < 0.05$) in the length of the lag phase for the 2018 rainout and ambient samples (Figure 9). Most of the 2018 Hancock ambient fermentations had lag phases <30 h, while the paired rainout fermentations had lag phases >30 h. A similar trend was observed for control samples where fermentation of the inhibitory switchgrass hydrolysate from a drought year (2012) had a longer lag phase (>20 h) compared to the paired non-inhibitory sample from 2010 (lag phase <20 h; Figure 9). For the 2020 samples, the Hancock and Rhinelander hydrolysates showed no significant differences between rainout and ambient samples, as both were strongly inhibitory (Figure 9). In contrast, the Lux Arbor, Lake City, and Escanaba samples all showed a significantly greater lag phase for the rainout compared to ambient hydrolysates (Figure 9), aligning with the CO_2 production curves (Figure 8).

In addition to evaluating CO_2 production, the final ethanol titers were compared to three positive control (non-inhibitory) hydrolysates: AFEX-treated corn stover harvested in 2008, AFEX-treated switchgrass harvested in 2010, and synthetic hydrolysate (SynHv2.13; Keating et al., 2014); and one negative

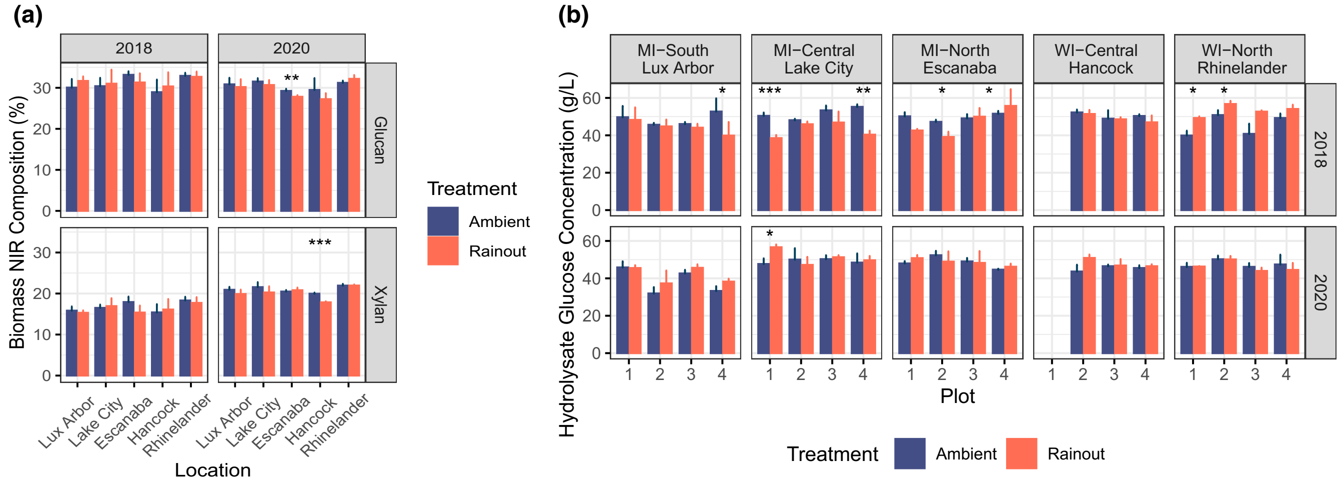

FIGURE 7 (a) Switchgrass showed largely no difference in biomass composition between paired ambient and rainout samples. (b) Hydrolysate glucose concentrations showed some variation between paired samples but did not follow any distinct pattern. Glucan and xylan were determined by NIRS compositional analysis and hydrolysate glucose composition was determined using HPLC. Error bars represent the mean ± standard deviation across field plots ($n = 3$–4). The full NIR dataset is included in the supplemental information. Asterisks indicate statistically different ambient/rainout samples based on paired *t*-test: *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$.

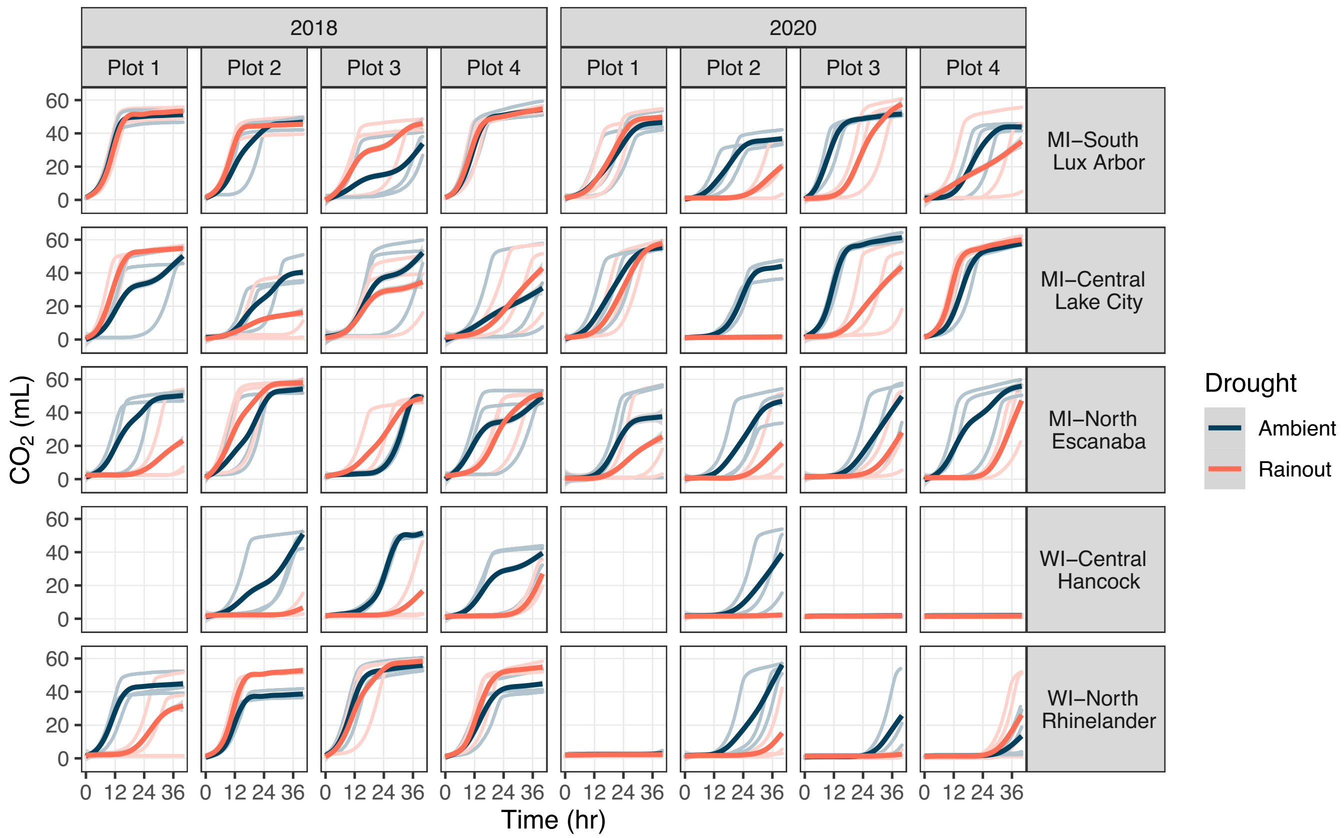

FIGURE 8 Hancock in 2018 and the Michigan sites in 2020 displayed either delayed or no yeast growth for switchgrass grown under rainout shelters versus ambient conditions. Light colored lines represent individual replicates ($n = 3$) of the combined pretreatment, hydrolysis, and fermentation process, while the dark line is the mean for all replicates generated using a generalized additive model.

control (inhibitory) hydrolysate: AFEX-treated switchgrass harvested in 2012 (Ong et al., 2016). Although ethanol titers were highly dependent on all main factors (year, location, plot, and treatment; Table S17), ambient and rainout switchgrass samples showed statistically comparable final ethanol titers for most plots despite

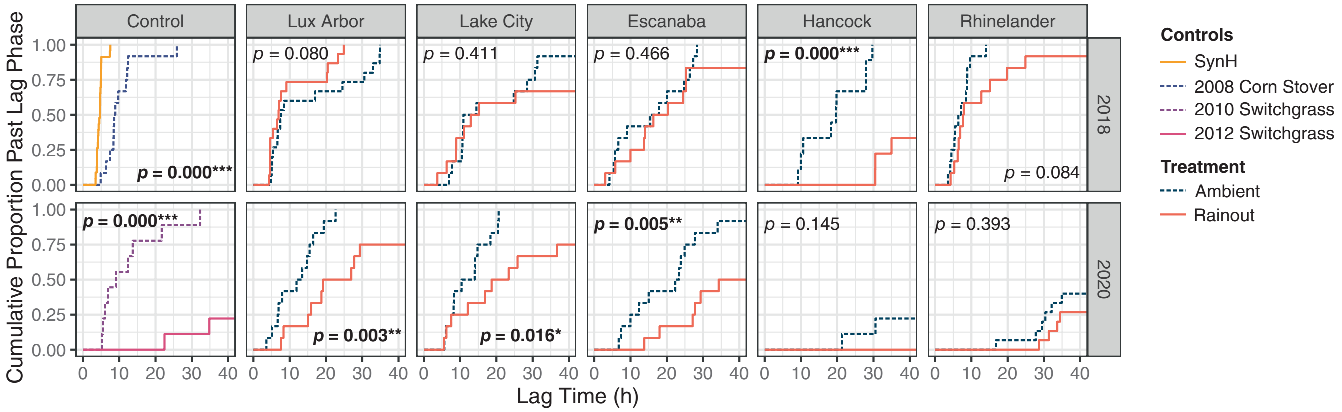

FIGURE 9 More yeast fermentations in 2020 versus 2018 had statistically longer lag phases in the rainout compared to ambient hydrolysates based on survival analysis (Kaplan–Meier curves). The y-axis represents the proportion of replicates from all plots that had completed the lag phase by the time specified on the *x*-axis. Control samples were not associated with a specific harvest year but were plotted in this manner for convenience. Log-rank tests were used to evaluate statistical significance of the difference in paired samples within the same box. Asterisks indicate statistically different ambient/rainout samples: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

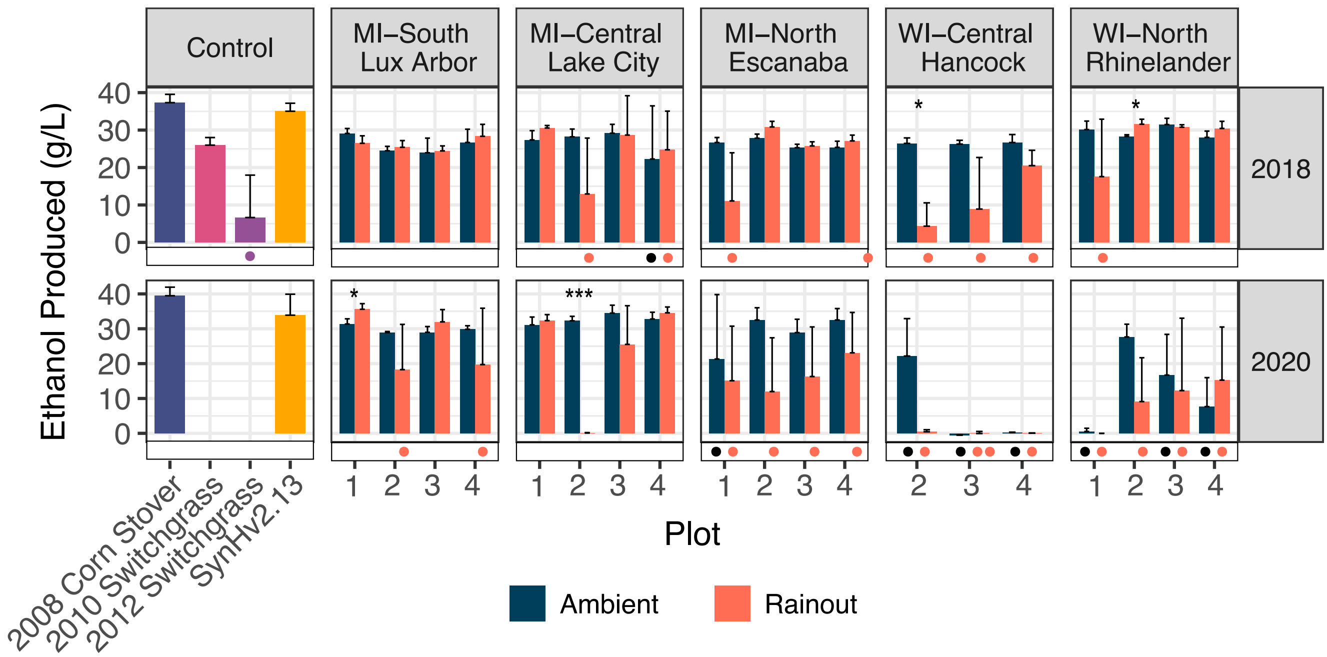

FIGURE 10 Switchgrass harvested in 2020 showed reduced ethanol production under ambient and rainout conditions compared to 2018. The control samples were used as examples of fermentable (2008 corn stover [$n = 27$], 2010 switchgrass [$n = 9$], synthetic hydrolysate [SynHv2.13; $n = 64$]) and inhibitory (2012 switchgrass [$n = 9$]) hydrolysates. Experimental samples were processed in triplicate and the error bars represent the average + standard deviation. A circle '•'at the bottom of the bar indicates samples that had one or more replicates with low ethanol titers in which yeast failed to reach stationary phase by the end of the fermentation. Asterisks indicate statistically different ambient/rainout samples based on paired *t*-test: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

variable CO_2 production between replicates (Figure 8). Based on paired *t*-tests, only two plots (Hancock plot 2 in 2018 and Lake City plot 2 in 2020) had statistically lower final ethanol titers (Figure 10; Table S18) and displayed delayed or no fermentation of hydrolysates from switchgrass cultivated under rainout shelters compared to the ambient samples (Figure 8). High variability in the ethanol production for a given plot was generally due to a subset of replicates failing to reach the stationary growth phase by the end of the experiment. Glucose consumption showed a similar pattern as ethanol production (Figure S2), indicating that redirection of glucose to products other than ethanol was not responsible for the low ethanol titers.

4 | DISCUSSION

To investigate the effect of moisture stress on biofuel production from switchgrass grown on low productivity fields, rainout shelters were installed in replicate plots at five locations in the Great Lakes Region. After the shelter roofs that excluded ~60% rainfall appeared insufficient to induce water stress and significantly reduce switchgrass yields in 2018, the shelters were structurally altered in 2019 to exclude 100% rainfall. This led to reductions in biomass yields for three of the five field sites in 2020 and 2021 (Figure 2; Tables S6–S8). Most of the results observed were highly related to the soil types at each location. The soils at the five locations were classified as either Alfisols, Spodosols, or Entisols (Tables S1–S5; Kasmerchak & Schaetzl, 2018). In spite of all soil types being considered acidic and sandy (Adams, 1984; McLean & Brown, 1984; Yost & Hartemink, 2019), they varied in their fertility and proportions of sand, clay, and loam (Tables S1–S5; Kasmerchak & Schaetzl, 2018). The Lux Arbor and Escanaba sites had Alfisol soils and were largely characterized as loams or sandy loams (Tables S1 and S3). Alfisols are common throughout the U.S. Midwest and have high native fertility and high base levels of saturation (Omonode & Vyn, 2006; Roley et al., 2018; Toliver et al., 2018; Woli et al., 2010), which corresponded to the high background soil moisture observed at the two locations (Figure 3). These soils would be the highest fertility of the five locations and correspond to the highest and most stable yields under normal precipitation (Figure 2). However, the imposition of the rainout shelters with 100% roof occlusion imposed significant water stress at Lux Arbor and Escanaba, reducing the soil water content in 2020 and 2021 to levels observed at the sandier Lake City and Hancock sites (Figure 3) and reducing biomass yields by ~1–2 Mg/ha (Figure 2). It is unclear why the shelters did not reduce biomass yields at these locations in 2019. The difference in soil moisture between ambient and rainout in 2019 was not as pronounced as in later years, but this was due to the lower soil water content in the ambient plots, as rainout plot water content was similar in all 3 years.

Compared to the other locations, the Hancock and Lake City field sites had the lowest ambient soil water content throughout the soil profile, with an additional reduction due to the rainout shelter in July–August (Figure 3). However, at Hancock, the soil temperatures were ~2–6°C warmer in July (Figure 5; Figure S1) when the plants were actively growing. The Hancock site was also the only location that had elevated temperatures (~2–3°C) under the rainout shelters compared to the ambient conditions (Craine et al., 2012; Havrilla et al., 2022). These elevated soil temperatures, in combination with low soil moisture, may have contributed to the reduction in biomass yields. Air temperatures at Hancock were lower than Lux Arbor and similar to Lake City and probably not responsible for the elevated temperatures. Instead, the low biomass cover likely led to reduced shading of soils on the plots, resulting in heating. This could also explain why the rainout shelter plots, which had significantly lower biomass yields than the ambient plots (~1–3 Mg/ha vs. ~4–5 Mg/ha), had higher soil temperatures than the ambient plots. It is also likely that the low water content of the Hancock soil limited the capacity for thermal regulation and exacerbated the heating of the soils. The Hancock soil was a Typic Udipsamment (Entisol) in the Plainfield soil series (Table S5) and contained the highest percentage of sand in excessively drained soils compared to all other field sites, which were either moderately or well-drained (Jayawardena et al., 2023; Kasmerchak & Schaetzl, 2018). Psamments are basically unconsolidated sand with no soil horizons, low soil nutrients (particularly phosphorus), high acidity, and very low water-holding capacities (Grossman, 1983). These soil properties led to the Hancock site having among the lowest volumetric water content under ambient and rainout treatments despite receiving the highest rainfall compared to other sites in 2018 and 2019 (Jayawardena et al., 2023). Of the five locations, the Hancock site represents the most challenging growth environment and had consistently low biomass yields throughout the study period.

The final two locations, Lake City and Rhinelander, did not show large effects of the rainout treatment on either soil water content or biomass yields. In contrast, the biomass yield for these two field sites followed a similar trend, declining across the 4 years of the study, with nearly consistent biomass yields for the ambient and the rainout treatments within the same year (Figure 2). Lake City and Rhinelander field sites both have Orthod soils, a suborder of Spodosols, however, Lake City had a greater percentage of sand compared to Rhinelander, resulting in significantly lower soil water retention (Figure 3). Spodosols are acidic, naturally infertile (Yost & Hartemink, 2019), and form under coniferous forests (Schaetzl et al., 2018; Schaetzl & Isard, 1991), often requiring lime addition to be agriculturally productive (Adams, 1984; McLean & Brown, 1984). It is possible that low fertility and high acidity may have led to the slow decline in switchgrass yields at Lake City and Rhinelander over the study period.

Many studies that evaluate bioenergy feedstocks in response to environmental conditions rely on composition data to estimate biofuel production potential (Emerson et al., 2014; Sanford et al., 2017). However, such data only represent the potential for biofuel production and may not reveal biochemical variability that potentially limits fermentation performance (Ong et al., 2016). Although there were significant effects of the rainout treatment on fermentation performance (Figures 8, 9 and 10; Table S17), there were no significant effects of treatment on biomass composition (Figure 7a; Tables S12 and S13). In 2020, the rainout shelter treatments at Lux Arbor and Escanaba had reduced biomass yields and increased fermentation inhibition under the rainout shelters. However, there was no statistical difference in biomass composition or hydrolysate composition for the rainout and ambient treatments for these materials (Figure 7). The 2020 Lake City and 2018 Hancock samples also showed a negative impact on fermentation performance for the rainout treatment versus the control (Figures 8, 9 and 10), which was not reflected in the biomass yields. It is possible that for the 2018 Hancock and the 2020 Lake City rainout treatments, the stress induced by the shelters was sufficient to affect biomass quality, while not affecting biomass yields. It is unclear why there was a large increase in fermentation inhibition for the 2020 Rhinelander ambient and rainout switchgrass compared to 2018, although this may be related to the consistent decline in biomass yields (Figure 2). In total, our data indicate that factors that are inducing fermentation stress at these locations are not being captured using standard metrics of biomass and hydrolysate quality.

Plant secondary metabolites, that are produced in response to heat and water stress are of potential concern as fermentation inhibitors, particularly for one-pot processing approaches (Dickinson et al., 2016; Öhgren et al., 2007) where all the compounds in the biomass are carried through to fermentation. Unfortunately, these compounds are largely unidentified and not routinely characterized using standard biomass composition assays. Common triggers for production of these secondary metabolites include stresses such as drought and water salinity (Liu et al., 2015; Wang & Chen, 2019); pathogen or herbivore attack, or nutrient deficiency (Burda & Oleszek, 2001; Cowan, 1999). Previous studies have shown that plants grown in Entisol soil types, such as those at the Hancock site, produce secondary metabolites under combined water and heat stress (Alhaithloul, 2019; Pu et al., 2000; Wu et al., 2016) as a defense mechanism to survive harsh conditions. Depending on the type of external stress, grasses like switchgrass may respond by generating defensive compounds like flavonoids, alkaloids, terpenes, phenols, anthocyanins, tannins, and quinones (Chipkar et al., 2022; Gregorova et al., 2015; Lindsey et al., 2013; Tao et al., 2019). Some of these have been observed to be deleterious to yeast, such as triterpene glycosides (i.e., saponins) isolated from drought-stressed switchgrass in our prior work (Chipkar et al., 2022). Other papers have reported the production of primary and secondary metabolites such as trypsin proteinase inhibitors and phytoanticipins that may induce biocidal action on fermentation organisms during bioethanol production (Tao et al., 2019; War et al., 2012). The presence of these plant-generated secondary metabolites may pose a concern to a commercial bioethanol processing industry as it is desirable to maintain a consistent yield of bioethanol despite varying feedstock sources (Michel et al., 2020; Westman & Franzen, 2015). Although *Saccharomyces cerevisiae* is a robust yeast strain that is widely available, it is sensitive to secondary metabolites generated in second-generation bioenergy crops (de Klerk et al., 2018; Eardley & Timson, 2020; Ong et al., 2016; Sjulander & Kikas, 2020). Hence, researchers are looking into other wild-type yeast strains with greater inhibitor tolerance traits than *S. cerevisiae* (Mukherjee et al., 2017; Radecka et al., 2015; Zheng et al., 2013).

5 | CONCLUSION

While it has been proposed to cultivate bioenergy crops on 'marginal lands' to avoid competition with arable cropland, there are limitations on how productive these sites can be for bioenergy crop and biofuel production, particularly when exposed to stressful growth conditions. Although biomass yields and fermentation performance were adequate under ambient growth conditions for most locations, when exposed to a simulated severe drought causing water and/or temperature stress, even the more productive locations, such as Lux Arbor, showed significant declines in productivity and fermentation performance. Modification of the rainout shelters to fully exclude precipitation resulted in a decrease in soil water content at all locations, higher soil temperatures at Hancock, reduced biomass yields at Lux Arbor, Escanaba, and Hancock, and inhibited fermentation of 2020 switchgrass from Lux Arbor, Escanaba, and Lake City. The susceptibility of a location to water or temperature stress was linked to the soil type for low fertility fields, with sandier and well-drained soils experiencing more severe effects on switchgrass productivity and fermentation performance. The decreased biomass yields between 2018 and 2021 at Lake City and Rhinelander appeared related to their soil classification as Spodosols, suggesting enhanced stress in these soils. Finally, the rainout treatment reduced biomass quality and strongly hindered fermentation by the yeast in many of the treatments. As some of the sites, such as Hancock in 2018, did not show a simultaneous reduction in biomass yields due to the rainout treatment, this might indicate that a low level of water or temperature stress during the active growing season, even though not sufficient to reduce biomass yields, might still degrade biomass quality for fermentation. Conventional metrics for biomass quality (composition and digestibility) showed no differences between inhibitory and non-inhibitory hydrolysates, indicating that the effect of environmental growth conditions on biomass fuel production could be missed unless fermentation studies are carried out or the underlying mechanism of inhibition is determined.

AUTHOR CONTRIBUTIONS

Sarvada Chipkar: Data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Kevin Kahmark:** Data curation; methodology; writing – review and editing. **Sven Bohm:** Data curation; methodology; software; writing – review and editing. **Mir Zaman Hussain:** Data curation; methodology; writing – review and editing. **Leela Joshi:** Data curation; methodology. **Karleigh M. Krieg:** Data curation; methodology. **Jacob Aguado:** Data curation; methodology. **Jasmine Cassidy:** Data curation; methodology. **Pablo Lozano:** Data curation; methodology. **Kevin Garland:** Data curation; methodology. **Andrea Senyk:** Data curation; methodology; writing – review and editing. **Derek J. Debrauske:** Data curation; methodology. **Elizabeth Whelan:** Data curation; methodology. **Morgan Davies:** Data curation; methodology. **Paul Urban:** Data curation; methodology. **G. Philip Robertson:** Conceptualization; funding acquisition; investigation; writing – review and editing. **Trey K. Sato:** Conceptualization; data curation; funding acquisition; investigation; methodology; visualization; writing – review and editing. **Stephen K. Hamilton:** Conceptualization; data curation; funding acquisition; investigation; methodology; supervision; visualization; writing – review and editing. **Kurt D. Thelen:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; writing – review and editing. **Rebecca G. Ong:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; software; visualization; writing – original draft; writing – review and editing.

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