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ORIGINAL ARTICLE

Production of deuterated switchgrass by hydroponic cultivation

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Abstract

Main conclusion **The bioenergy crop switchgrass was grown hydroponically from tiller cuttings in 50 % D_2O to obtain biomass with 34 % deuterium substitution and physicochemical properties similar to those of H_2O-grown switchgrass controls.**

Deuterium enrichment of biological materials can potentially enable expanded experimental use of small angle neutron scattering (SANS) to investigate molecular structural transitions of complex systems such as plant cell walls. Two key advances have been made that facilitate cultivation of switchgrass, an important forage and biofuel crop, for controlled isotopic enrichment: (1) perfusion system with individual chambers and (2) hydroponic growth from tiller cuttings. Plants were grown and maintained for several months with periodic harvest. Photosynthetic activity was monitored by measurement of CO_2 in outflow from the growth chambers. Plant morphology and composition appeared normal compared to matched controls grown with H_2O. Using this improved method, gram quantities of switchgrass leaves and stems were produced by continuous hydroponic cultivation using growth medium consisting of basal mineral salts in 50 % D_2O. Deuterium incorporation was confirmed by detection of the O-D and C-D stretching peaks with FTIR and quantified by ^{1}H- and ^{2}H-NMR. This capability to produce deuterated lignocellulosic biomass under controlled conditions will enhance investigation of cell wall structure and its deconstruction by neutron scattering and NMR techniques.

Keywords Biomass · Switchgrass · D_2O · Deuteration · NMR · FTIR

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Introduction

Switchgrass (*Panicum virgatum*) is an important forage crop which is currently being developed for production of cellulosic biofuels (Bouton 2007, 2008). It is native to the North American prairie and is a perennial C4 warm-weather grass (Douglas et al. 2009; Parrish et al. 2012). In our previous studies, we have demonstrated the utility of small angle neutron scattering (SANS) to investigate the structural changes of switchgrass during dilute acid pretreatment, revealing the onset of lignin aggregation (Pingali et al. 2010a, b). When complex biological samples are examined, deuterium (^{2}H) substitution enables separation of scattering signatures of components that are otherwise too close to distinguish. This can be accomplished by growth of target organisms in deuterated media containing D_2O. However, when the D_2O concentration is increased from the naturally occurring 0.015–50 % and higher to achieve the deuterium incorporation levels desirable for neutron scattering experiments, the physical and chemical differences between D_2O and H_2O begin to affect metabolism and growth of living organisms. These physical and chemical differences include higher melting point, lower ionization constants, and slower reaction rates resulting from the higher bond strength of deuterium compared to hydrogen. For many unicellular species, these differences can be overcome by adjustment of growth conditions and adaptation to deuterated cultures. This approach has been quite successful for algae, yeast, and bacteria (Katz 1960; Katz and Crespi 1966; Perkins 1981: Langan et al. 2012). However, D_2O concentrations higher than 30 % adversely affect germination, root elongation, growth, and flowering of higher plants (Pratt and Curry 1937; Katz 1960; Katz and Crespi 1966). The highest tolerance of D_2O has been reported for the cold-tolerant species winter rye (Siegel et al. 1964) and Arabidopsis (Bhatia and Smith 1968). Deuterium-labeled spinach, carrots, and kale have been produced for nutritional studies by germination and growth in vermiculite watered with H_2O for 4 days followed by transfer to solutions of 15–30 % D_2O in hydroponic growth chambers (Putzbach et al. 2005; Grusak 2000). Using a similar strategy for cultivation of annual ryegrass in 50 % D_2O, we have obtained 37 % deuterated leaf biomass with morphology and composition similar to H_2O-grown controls (Evans et al. 2014). Our previous investigations have found differential incorporation of deuterium in carbohydrates and lignin from the species kale (Foston et al. 2012) and annual ryegrass grown in 50 % D_2O (Evans et al. 2014). We now report a method for continuous production of highly deuterium-enriched switchgrass under controlled conditions.

Materials and methods

Switchgrass cultivation

Switchgrass seeds (*Panicum virgatum*, Alamo cultivar) were obtained from the Bioenergy Science Center located at Oak Ridge National Laboratory, Oak Ridge, TN, USA. Deuterium oxide (D_2O), 99.8 %, was purchased from Cambridge Isotope Laboratories (Cambridge, Massachusetts). Distilled water was further purified by filtration through Millipore or Barnstead water purification systems before use. Plant jars, filter-vented lids, basal salt mixtures, agar, and growth hormones were purchased from Phytotechnology Laboratories (Shawnee Mission, Kansas, USA). Perfusion chambers were assembled from 1-L graduated cylinders fitted with rubber closures with inflow and outflow tubes. Chambers were perfused with ambient air supplied by an aquarium pump and dried by passage through in-line tubes containing silica gel desiccant as described previously (Evans et al. 2014). Carbon dioxide fixation rates were measured by attaching a LI-COR CO_2 monitor to the outflow tubing from an individual chamber. Plants were grown in an in-house made plant stand illuminated by a metal halide lamp in a Sun System 2 fixture equipped with a UV-blocking safety screen (Future Garden, North Lindhurst, NY, USA). Incident light intensity was 150 µmol photons m^{-2} s^{-1} and 8930 lux. The diurnal cycle was 12 h light/12 h dark. Growth solution was Schenk and Hildebrandt's basal salts. Potting soil and peat plugs were obtained commercially from garden supply outlets. Switchgrass tillers were harvested at 1–2 month intervals due to height constraints of the growth chambers. Tissue culture was carried out according to published protocols using complete Murashige and Skoog media supplemented with vitamins and maltose (see ESI for details).

Compositional analysis

Deuterium incorporation was estimated by FTIR analysis of leaf and stem samples and quantified by NMR as described previously (Foston et al. 2012; Bali et al. 2013). Infrared (FTIR) spectra were obtained with a Perkin-Elmer 100 Series Fourier Transform Infrared Spectrometer in an attenuated total reflectance (ATR) mode (see ESI Table S-7

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for band assignments). Samples for carbohydrate and acid-insoluble lignin analysis were prepared using a two-stage acid hydrolysis protocol based on Tappi method T-222 om-88. The sugar solution was analyzed for carbohydrate components by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., Sunnyvalle, CA, USA).

Gel permeation chromatography (GPC) analysis of cellulose and hemicellulose

The cellulose and hemicellulose fractions were isolated from extractive-free hydroponic switchgrass samples as described previously (Evans et al. 2014). The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of cellulose were determined by Gel Permeation Chromatography as described in previous publications (Foston and Ragauskas 2010; Bali et al. 2013; Evans et al. 2014). In brief, the isolated cellulose samples were derivatized using phenyl iso-cyanate in anhydrous pyridine (4.0 ml), after which the derivatized cellulose samples were dissolved in tetrahydrofuran (1 mg/mL), filtered through a 0.45-mm filter and placed in a 2-mL autosampler vial. The molecular weight distributions of the cellulose tricarbanilate samples were analyzed on an Agilent GPC Security 1200 system equipped with four Waters Styragel columns (HR1, HR2, HR4, HR5), Agilent refractive index (RI) detector and Agilent UV detector (270 nm) using THF as the mobile phase (1 mL/min) with injection volumes of 0.02 ml. A calibration curve was constructed based on eight narrow polystyrene standards ranging in molecular weight from 1.5×10^3 to 3.6×10^6 g/mol. Data collection and processing were performed using Polymer Standards Service WinGPC Unity software (Build 6807). Molecular weights (M_n and M_w) were calculated by the software relative to the universal polystyrene calibration curve and weight-average degree of polymerization (DP_w) were obtained by dividing M_w by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeat unit. Polydispersity index (PDI) was calculated by dividing M_w by M_n.

Determination of cellulose crystallinity with ^{13}C-NMR

Cellulose crystallinity was determined by ^{13}C-NMR. Cellulose samples were prepared by the mild acid (2.5 M HCl) hydrolysis of extractive-free and delignified hydroponic switchgrass. The isolated cellulose I samples were then collected by filtration rinsed with an excess of deionized water and dried in a fume hood. For NMR analysis, 4-mm cylindrical ceramic MAS rotors were filled with the isolated α-cellulose. Solid-state direct- and cross-polarization ^{13}C-NMR measurements were carried out with a Bruker Avance-400 spectrometer operating at a frequency of 100.55 MHz for ^{13}C in a Bruker double-resonance MAS probe at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 ms (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay and 4–8 K scans. All spectra were recorded on equilibrated moisture samples (~35 % water content).

Statistical analysis

Averages, standard deviations, and Student's t test were carried out using the spreadsheet functions of Microsoft Excel. Details and data used for the calculations are given in the Electronic Supplementary Information file (ESI).

Results

Inhibition of seed germination and root elongation by D_2O at concentrations >50 % had been observed since the earliest studies of its biological effects (Lewis 1933; Pratt and Curry 1937; Katz and Crespi 1966; Katz 1960). Initial efforts to cultivate switchgrass (*Panicum virgatum* variety Alamo) for deuteration by direct germination and growth in solutions of 50 % D_2O as had been reported for winter rye (Siegel et al. 1964) and annual ryegrass (Evans et al. 2014) were not successful (see ESI for details). Switchgrass is a warm-weather perennial prairie grass that overwinters by dormancy and its small seeds exhibit low germination rates, even when various stratification and pretreatment protocols are employed (Douglas et al. 2009; Zegada-Lizarazu et al. 2012). In contrast to annual ryegrass, which can be germinated on glass fiber filters and established in hydroponic culture without soil (Evans et al. 2014), switchgrass seedlings required addition of soil for establishment of adventitious roots, exhibiting significantly higher ($P < 0.05$) growth rates (0.133 ± 0.041 cm/day) than controls without soil (0.074 ± 0.019 cm/day).

We attempted propagation of switchgrass from stem sections in nutrient agar using a protocol developed for genetic transformation and clonal production (Conger 2002; Alexandrova et al. 1996), but shoots rapidly died in agar prepared with D_2O (see ESI for details). Transfer of mature plants established in soil to deuterating conditions as described for production of vegetables for nutritional labeling (Grusak 2000; Putzbach et al. 2005; Tang et al. 2009) proved to be more successful than growth from seed or regeneration in tissue culture. Enclosed 1-L chambers equipped with sterile-filtered inflow and outflow tubing and continuously perfused with dry air were used for cultivation (see ESI for detailed description), as described previously for deuteration of annual ryegrass (Evans et al.

2014). Switchgrass plants were germinated and established in soil for 2–4 months before enclosure and watering with 40, 50, or 99.8 % D_2O. As residual H_2O was not removed from the roots and soil, final D_2O concentrations were lower than that of the watering solution. Young switchgrass plants grown for 14 days in soil with H_2O before change to 50 % D_2O initially grew slowly (0.12 cm d^{-1}) for 8 days, then stopped growing, and died at 54 days. Since these plants were at the two-leaf stage at transfer, it is likely that their roots were not sufficiently developed to enable them to survive effects of transfer to D_2O, as 4 weeks of growth are required for growth of adventitious roots, coinciding with the three-leaf stage of the shoots (Zegada-Lizarazu et al. 2012). Plants grown in soil for 2–4 months before the start of D_2O grew slowly with high mortality and fungal contamination rates.

Cultivation of hydroponic plants from tiller cuttings in the perfusion chambers proved the most successful of the cultivation techniques investigated (detailed description is given in ESI). The earlier results indicated that prior establishment of roots is essential for successful cultivation in high D_2O concentrations. Young tillers (auxiliary shoots) growing from the crowns of established switchgrass cultivated in soil with H_2O for periods ranging from 4 to 60 months were cut and incubated in plant jars containing sterile Schenk and Hildebrandt's basal salts in H_2O until appearance of roots, usually 5–12 days. No sugars, vitamins, or rooting hormones were added. Light period was 12 h d^{-1} at 8930 lx. Rooted shoots were transferred to growth chambers and grown under continuous air perfusion, with bimonthly trimming of leaf shoots (Fig. 1a, b). Establishment of switchgrass tiller cuttings in hydroponic culture showed higher success rates (45.8 ± 16.7 %, $N = 6$) than germination and growth in soil from seed (6.5 ± 3.5 %, $N = 6$) under these laboratory conditions (Student's t test, $P < 0.05$). During the first month, growth rates of the hydroponic plants in perfusion chambers (1.1 ± 0.03 cm d^{-1}, $N = 8$) were significantly higher (Student's t test, $P < 0.01$) than those of seedlings germinated in potting soil (0.57 ± 0.16 cm d^{-1}, $N = 6$), and compare favorably to the elongation rates of 1.4–2.8 cm d^{-1} reported for field-grown switchgrass (Zegada-Lizarazu et al. 2012) (see ESI Tables S-1, S-2, S-3, S-4 for detailed data and analysis).

Deuteration was carried out by transfer of 4-month-old hydroponic switchgrass trimmed to 12 cm height to growth medium containing 50 % D_2O. The plant was maintained in 50 % D_2O under continuous air perfusion for 17 months, with monthly harvests of leaf shoots (total 31 g fresh weight). New tillers grew from the crown (Fig. 1c, d) at rates (0.89 ± 0.18 cm d^{-1}, $N = 14$) matching controls in H_2O (Student's t test, $P > 0.10$). Gross morphology of tiller leaves from deuterated switchgrass resembled that of H_2O-grown controls (Fig. 2).

Tiller numbers and average tiller heights of the 50 % D_2O-grown hydroponic switchgrass were within standard deviation of those of both H_2O-grown hydroponic switchgrass and a switchgrass control grown from seed in a peat plug in a perfusion chamber (details are given in ESI Table S-5). Photosynthetic CO_2 fixation rates measured at chamber outflow tubes and normalized for tiller height were within 20 % for the three plants (averaged value 0.0100 µmol min^{-1} cm^{-1}; see ESI Table S-6 for details).

Extent of deuterium substitution was determined by FTIR and $^1H/^2H$-NMR as used previously for analysis of deuterated bacterial cellulose (Bali et al. 2013) and annual

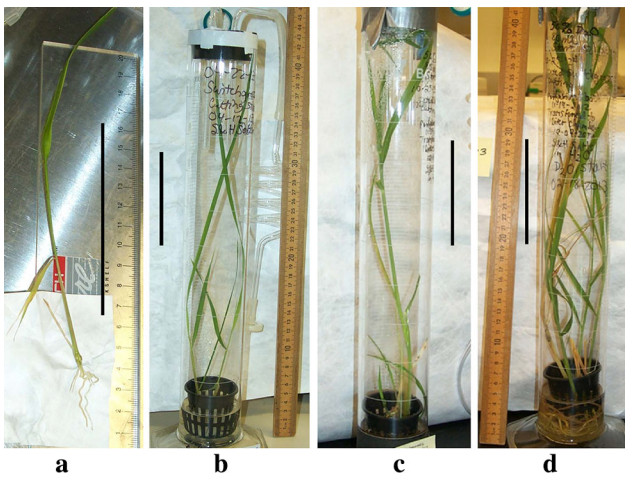

Fig. 1 A rooted switchgrass tiller cutting is shown at 10 days (**a**) and the same plant after 1 month growth in H_2O medium (**b**). Growth of new tillers from the root crown of a switchgrass plant maintained in 50 % D_2O medium: growth of new tillers at 9 months after deuteration start (**c**) and at 10 months (**d**). *Bars* 10 cm

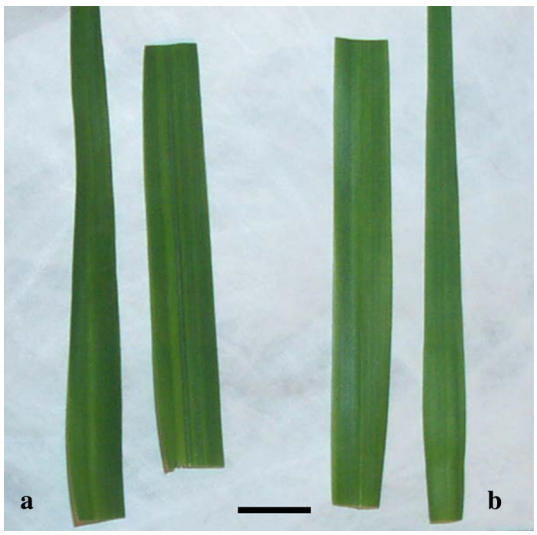

Fig. 2 Leaf morphology of hydroponic switchgrass grown in **a** H_2O and in **b** 50 % D_2O appears similar. *Bar* 1 cm

ryegrass (Evans et al. 2014) (see ESI for details). Incorporation of non-exchangeable deuterium in the switchgrass leaf and stem samples was confirmed by appearance of the C-D stretching band in FTIR (Fig. 3). The deuterium incorporation in hydroponic switchgrass grown in 50 % D_2O under air perfusion was determined at harvest times of 36, 172 and 227 days. FTIR analysis detected the appearance of O-D and C-D bands that were not present during initial growth in H_2O already after 1 month of growth in 50 % D_2O. FTIR also detected evidence of D-N substitution in proteins in the deuterated switchgrass biomass before extraction. FTIR spectra of samples harvested over the course of more than 7 months appeared qualitatively identical (Fig. 3).

Quantitative analysis by $^1H/^2H$-NMR of milled leaf and stem samples pooled from harvests at 36, 172, 227, and 322 days growth in 50 % D_2O found deuterium incorporation of 34 % in the extractives-free lignocellulose. The level of deuterium incorporation appeared to remain fairly constant during the cultivation period, with 32, 41, and 32 % measured in the separate samples harvested after growth for 36, 172, and 227 days, respectively. No significant selectivity of the ^2H incorporation was found with respect to structural features such as stems, nodes, and leaves.

The chemical composition of the hydroponic switchgrass grown in H_2O and in 50 % D_2O was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., Sunnyvale, California, USA) (see ESI for detailed description of carbohydrate and lignin analysis techniques). The carbohydrate composition of the hydroponic samples did not differ significantly from those harvested from the control switchgrass plant that was grown in a similar perfusion chamber from seed in a commercial peat plug (Table 1). Detailed compositional analysis is given in Table S8 (see ESI). Glucan and xylan were the major carbohydrates present in both switchgrass samples with minor amounts of arabinan, galactan, and mannan. The content of these component carbohydrates in the switchgrass grown in D_2O was not statistically different ($P \geq 0.05$) from the control sample as analyzed by Student's t test, except for that of arabinan, which was increased from 3.45 to 3.65 % ($P = 0.02$). Cellulose content measured as glucan in hydroponic 50 % D_2O-grown switchgrass was slightly lower than that of switchgrass grown in H_2O, but not statistically different ($P = 0.09$) by Student's t test (Table 1 and ESI Table S-8). The total hemicellulose content (calculated as the sum of arabinan, galactan, mannan, and xylan) content in the switchgrass grown in 50 % D_2O (Table 1) was not statistically different from the control samples as evaluated using Student's t test (ESI Table S-8). In comparison to the reported composition of field-grown Alamo switchgrass (33–38 % cellulose, 26–32 % hemicellulose, 17 % lignin) (David and Ragauskas 2010), the cellulose content is similar, while hemicellulose and lignin content are lower, probably due to the difference in tiller height and age at harvest (50–60 cm vs. 2–3 m, 1–3 months vs. seasonal).

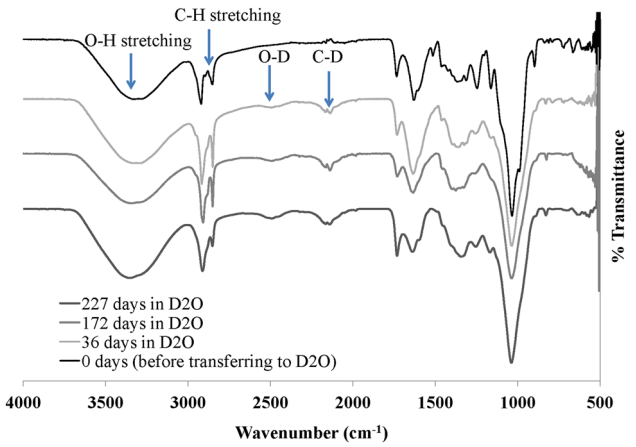

Fig. 3 FTIR spectra of leaf samples of hydroponic A rooted switchgrass show appearance of O–D and C–D bands following growth in 50 % D_2O (band assignments are given in ESI Table S-7)

The molecular weight and crystallinity of the cellulose isolated from the hydroponic switchgrass samples are presented in Table 2, and the molecular weights of the hemicelluloses are presented in Table 3. The crystallinity index (CrI) as determined by solid-state NMR of cellulose isolated from the partially deuterated switchgrass (CrI 42.0 ± 1.6 %) was similar to that of the control hydroponic switchgrass (CrI 44.0 ± 1.6 %). This is consistent with the results previously reported for deuterated bacterial cellulose (Bali et al. 2013) and for partially deuterated annual rye grass (Evans et al. 2014).

The number-average molecular weight (M_n) and weight-average molecular weight (M_w) of isolated switchgrass cellulose and hemicellulose were determined by gel permeation chromatography (GPC), a method frequently used to determine the molecular weight distributions of cellulose and other component polymers isolated from plant biomass (Foston and Ragauskas 2010, 2012). Cellulose samples were solublilized by derivatization with tricabanilate groups as described above in "Materials and Methods". Cellulose isolated from deuterated plants had M_w of $2.8 \pm 0.1 \times 10^5$ g/mol and M_n of $5.7 \pm 0.2 \times 10^4$ g/mol, while its control counterpart had M_w $2.3 \pm 0.3 \times 10^5$ g/mol and M_n $6.1 \pm 0.8 \times 10^4$ g/mol. The weight-average degree of polymerization (DP_w) calculated for cellulose isolated from the partially deuterated switchgrass (DP_w 539.5 ± 18.4), assuming the contribution of non-exchangeable deuterium to be negligible with

Table 1 Composition as percent dry weight of tiller biomass from switchgrass grown in perfusion chambers with H_2O and with 50 % D_2O (calculated from data in ESI Table S-8 assuming glucan content to be equivalent to cellulose)

Switchgrass sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
H_2O peat plug	39.46	23.69	11.24
H_2O hydroponic	36.58	21.05	9.29
50 % D_2O hydroponic	34.79	21.26	16.00

Table 2 Molecular weight and crystallinity of cellulose isolated from hydroponic switchgrass samples

Sample	$M_n \times 10^4$ (g/mole)[a]	$M_w \times 10^5$ (g/mole)[a]	DP_w	PDI	Crystallinity index (%)[a]
Hydroponic switchgrass grown in 100 % H_2O	6.1 ± 0.8	2.3 ± 0.3	443.2 ± 55.8	3.8	44.0 ± 1.6
Hydroponic switchgrass grown in 50 % D_2O	5.7 ± 0.2	2.8 ± 0.1	539.5 ± 18.4	4.9	42.0 ± 1.6

DP_w weight-average degree of polymerization, *PDI* polydispersity index

[a] Standard deviation: calculated from three measurements/sample: 0.2 × 10^4 g/mol to 0.8 × 10^4 for M_n, 3.0 × 10^4 g/mol and 1 × 10^4 for M_w, and 1.6 % for crystallinity index

Table 3 Molecular weight of hemicellulose isolated from hydroponic switchgrass samples

Hemicellulose	$M_n \times 10^4$ (g/mole)[a]	$M_w \times 10^4$ (g/mole)[a]	PDI
Hydroponic switchgrass grown in 100 % H_2O	1.7	2.9	1.7
Hydroponic switchgrass grown in 50 % D_2O	1.8	3.9	2.1

[a] Standard deviation: calculated from three measurements/sample: 0.4 × 10^4 g/mol to 0.02 × 10^4 for M_n, 0.4 × 10^4 g/mol and 0.08 × 10^4 for M_w, and $P = 0.03$

respect to the molecular weight of tricarbanilate-glucose units (519 g/mol), appeared to be comparable to that of the control hydroponic switchgrass (DP_w 443.2 ± 55.8), within the range expected with this method. This is consistent with the results previously reported for deuterated bacterial cellulose (Bali et al. 2013) and for partially deuterated annual rye grass (Evans et al. 2014). Since M_w determined by GPC corresponds to the first statistical moment of the elution, a slightly higher value would be consistent with higher molecular weight resulting from deuterium incorporation eluting in the front of the polymer distribution curve. However, the polydispersity index (PDI) calculated from the GPC results for M_w and M_n increases from 3.8 for the control cellulose to 4.9 ($P = 0.03$) for the partially deuterated cellulose. It appears that growth in 50 % D_2O results in more dispersed molecular weights of these constituent polysaccharides than is observed in corresponding samples isolated from plants grown in H_2O. This change in molecular weight distribution may include different degrees of polymerization, possibly due to kinetic isotope effects, as well as differences in deuterium incorporation throughout the distribution. Hemicellulose isolated from deuterated switchgrass had significantly higher ($P = 0.03$) M_w and M_n values, as well as PDI (3.9 × 10^4, 1.8 × 10^4 g/mol, and 2.1, respectively), than the hemicellulose isolated from the H_2O-grown control (2.9 × 10^4, 1.7 × 10^4 g/mol, and 1.7, respectively) as shown in Table 3. As PDI is a measure of broadness of molecular weight distribution, this suggests that variation in both deuterium content and arabinose side chains, as indicated by compositional analysis, result in a wider and slightly increased molecular weight distribution in GPC analysis.

Discussion

For the first time, a protocol has been devised and demonstrated for continuous long-term production of highly deuterium-enriched switchgrass under controlled conditions. Propagation from cuttings and hydroponic growth enable faster and cleaner deuteration by avoiding germination inhibition, limiting microbial contamination, and eliminating difficult to quantify D-H exchange from soil components. Hydroponic growth did not significantly change chemical composition and leaf morphology compared to control plants grown from seed in soil under the same conditions, and resembled that reported for field-grown switchgrass. Gross morphology and growth rates of the tillers grown in 50 % D_2O appeared similar to H_2O-grown controls. The

chemical and physical properties of the 34 % deuterated stem and leaf biomass from the switchgrass cultivated in 50 % D_2O resembled that of H_2O-grown controls. The differences in response of young seedlings and established switchgrass to cultivation in 50 % D_2O may be correlated to the development of C4 vascularization, characterized by closer vein spacing and more veins per leaf than comparable C3 plants, which may lessen the impact of D_2O on root–stem translocation (Dengler and Nelson 1999; Brown 1999). Inhibition of cation and water translocation from roots to stems correlated with sensitivity to D_2O in rice as compared to the D_2O-tolerant winter rye (Shibabe and Yoda 1984a, b, 1985). The ability to establish and maintain long-term cultivation of higher terrestrial plants under deuterating conditions can provide regular harvests of deuterated materials of uniform properties for experimental studies. This capability will expedite neutron scattering and NMR studies of lignocellulosic biomass deconstruction and conversion to biofuels using a key energy crop, providing fundamental understanding of these processes. It also provides opportunities for experimental investigations of growth and metabolism of switchgrass and other perennial species by isotopic enrichment methods under controlled conditions.

Author contribution BRE carried out plant cultivation experiments with assistance of JMcG, DTR, CSR, RS, and HO'N. Photosynthesis assays were carried out by BRE. MF and GB carried out characterization by NMR, FTIR, and other methods at Georgia Tech under supervision of AJR. BD supplied switchgrass seeds and coordinated the research project.

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Conflict of interest The authors declare no conflict of interest.

References

Alexandrova KS, Denchev PD, Conger BV (1996) Micropropagation of switchgrass by node culture. Crop Sci 36:109–1711

Bali G, Foston MB, O'Neill HM, Evans BR, He J (2013) The effect of deuteration on the structure of bacterial cellulose. Carbohydrate Res 374:82–88

Bhatia CR, Smith HH (1968) Adaptation and growth response of *Arabidopsis thaliana* to deuterium. Planta 80:176–184

Bouton JH (2007) Molecular breeding of switchgrass for use as a biofuel crop. Curr Opin Genet Develop 17:1–6

Bouton J (2008) Improvement of switchgrass as a bioenergy Crop. In: Vermerris W (ed) Genetic improvement of bioenergy crops, Chapter 11, Springer Science + Business Media, LLC, pp. 295–308

Brown RH (1999), Agronomic aspects of C4 plants. In: Sage RF and Monson RK (eds) The biology of C4 plants, Chapter 14, Academic Press, San Diego, pp. 133–164

Conger BV (2002) Development of in vitro systems for switchgrass (*Panicum virgatum*). Final Report for 1992–2002. ORNL/SUB-02-11XSY161/01. (Available online at http://bioenergy.ornl.gov/pdfs/ornlsub_02_11xsy16101.pdf). Verified 19 September 2013

David K, Ragauskas AJ (2010) Switchgrass as an energy crop for biofuel production: a review of its ligno-cellulosic chemical properties. Energy Environ Sci 3:1182–1190

Dengler NG, Nelson T (1999) Leaf structure and development in C4 plants. In: Sage RF, Monson RK (eds) The biology of C4 plants, Chapter 5, Academic Press, San Diego, pp. 133–164

Douglas J, Lemunyon J, Wynia R, Salon P (2009) Planting and managing switchgrass as a biomass energy crop. USDA NRCS Plant Materials Program, September 2000, Technical Note No. 3

Evans BR, Bali G, Reeves D, O'Neill H, Sun Q, Shah R, Ragauskas AJ (2014) Effect of D_2O on growth properties and chemical structure of annual ryegrass (*Lolium multiflorum*). J Agric Food Chem 62(12):2592–2604

Foston M, Ragauskas AJ (2010) Changes in lignocellulosic supramolecular and ultrastructure during dilute acid pretreatment of Populus and switchgrass. Biomass Bioenergy 34:1885–1895

Foston M, Ragauskas AJ (2012) Biomass characterization: recent Progress in understanding biomass recalcitrance. Indus Biotechnol 8:191–208

Foston MB, McGaughey J, O'Neill H, Evans BR, Ragauskas AJ (2012) Deuterium incorporation in biomass cell wall components by NMR analysis. Analyst 137:1090–1093

Grusak MA (2000) Intrinsic stable isotope labeling of plants for nutritional investigations in humans. J Nutr Biochem 71:1555–1562

Katz JJ (1960) Chemical and biological studies with deuterium. Am Sci 48:544–580

Katz JJ, Crespi H (1966) Deuterated organisms: cultivation and uses. Science 151:1187–1194

Langan P, Evans BR, Foston M, Heller WT, O'Neill HM, Petridis L, Pingali SV, Ragauskas AJ, Smith JC, Davison B (2012) Neutron Technologies for Bioenergy Research. Indus Biotechnol 8(4):209–216

Lewis GN (1933) The biology of heavy water. Science 79:151–153

Parrish DJ, Casler MD, Monti A (2012) The evolution of switchgrass as an energy crop. In: Monti A (ed) Switchgrass a valuable biomass crop for energy, Chapter 1, Springer London, London, pp 1–28

Perkins SJ (1981) Estimation of deuteration levels in whole cells and cellular proteins by 1H n.m.r. spectroscopy and neutron scattering. Biochem J 199:163–170

Pingali SV, Urban VS, Heller WT, McGaughey J, O'Neill HM, Foston M, Myles DA, Ragauskas AJ, Evans BR (2010a) SANS study of cellulose extracted from switchgrass. Acta Crystallographica D D66:1189–1193

Pingali SV, Urban VS, Heller WT, McGaughey J, O'Neill HM, Foston M, Myles DA, Ragauskas A, Evans BR (2010b) Breakdown of cell wall nanostructure in dilute acid pretreated biomass. Biomacromolecules 11:2329–2335

Pratt R, Curry J (1937) Growth of roots in deuterium oxide. Am J Botany 24(7):412–416

Putzbach K, Krucker M, Albert K, Grusak MA, Tang F, Dolnkowski GG (2005) Structure determination of partially deuterated carotenoids from intrinsically labeled vegetables by HPLC-MS and 1H NMR. J Agric Food Chem 53:671–677

Shibabe S, Yoda K (1984a) Transport of calcium, germanium, and rubidium ions in rice seedlings in deuterium oxide. Radioisotopes 33:606–610

Shibabe S, Yoda K (1984b) Hydrogen isotope effect on transport of potassium ion in rice seedlings equilibrated with deuterium oxide. Radioisotopes 33:675–678

Shibabe S, Yoda K (1985) Water and potassium ion absorption by deuterium resistant winter rye seedlings. Radioisotopes 34:266–269

Siegel SM, Halpern LA, Giumaro C (1964) Germination and seedling growth of winter rye in deuterium oxide. Nature 201:1244–1245

Tang G, Qin J, Dolnikowski GG, Russell RM, Grusak MA (2009) Golden rice is an effective source of vitamin A. American J Clinical Nutrition 89:1776–1783

Zegada-Lizarazu W, Wullschleger SD, Nair SS (2012) Crop physiology. In: Monti A (ed) Switchgrass: a valuable biomass crop for energy, Chapter 3, Springer London, London, pp 55–86