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Carbon Partitioning into Cell Wall Structural Carbohydrates by Following ^{13}C Label in Switchgrass

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***Abstract*—Carbon isotope ratio analyses of stover tissue from both the lowland (Kanlow) and the upland (Summer) cultivars of switchgrass indicated that the value of Kanlow was less negative (-12.7 per mil.) than the upland variety Summer (-13.1). Preliminary observations on the carbon isotope ratio of cellulose from switchgrass showed a less negative elta ^{13}C value (-12.6) than the leaf carbon isotope ratio (a surrogate for non-structural carbohydrates) which showed a value of -13.1. Preliminary results from ^{13}C pulse labeling of greenhouse grown switchgrass plants indicated an active partitioning of carbon in their stem tissue over a period of 6 weeks as compared to leaves. Results from further analyses of carbon partitioning into hemicelluloses, celluloses and lignins from stem tissues also indicated an active partitioning of carbon into lignin as the plants mature. Further evidence for an active lignin biosynthesis was also provided by a gradual increase in one of the key enzymes in lignin biosynthesis, cinnamyl alcohol dehydrogenase., in the stem tissue while the same enzyme showed a gradual decrease in its activity in the leaf.**

Results from this study appear to suggest that an increased carbon partitioning into cellulosic biomass, may necessitate a comprehensive approach to reduce carbon partitioning into the lignin biosynthetic pathway in these plants.

Keywords-Switchgrass, carbon isotope ratio mass spectrometry, cinnamyl alcohol dehydrogenase, cellulose, lignin.

I. INTRODUCTION

Switchgrass (*Panicum virgatum L.*).has drawn a considerable research effort in the area of renewable energy production due to its ability to produce significant amounts of biomass when grown on marginal land. Using *in vitro* dry matter digestibility (IVDMD) as a selection criterion to improve its forage quality, Vogel et al., [3] have reported that amongst switchgrass populations differing in *in vitro* dry matter digestibility there were only modest variations in cell wall hemicellulose and cellulose concentration but a significant change in lignin concentrations.

The use of stable carbon isotopes in agricultural and ecological research has become more frequent in recent years. Since ^{13}C is a naturally occurring stable isotope, there are no regulatory problems, environmental hazards and/or health risks associated with its use. These advantages make it an ideal tool for studying carbon metabolism in production field environments. As a tracer, ^{13}C offers great potential for generally defining source-sink relationships in terms of which plant sources are net exporters of carbon and how such products are mobilized to various parts. Thus far only a few studies have been carried out to assess carbon allocation following pulse-labeling of plants with $^{13}CO_2$.

The purpose of this study is two-fold: 1) to follow carbon partitioning into the cell wall structural carbohydrates of switchgrass plants , and 2) to determine the relative activity of cinnamyl alcohol dehydrogenase, a key enzyme in the lignin biosynthetic pathway, at various stages of plants' growth.

II. MATERIALS AND METHODS

A. Plants

1) Field grown plants: Tissue material from Kanlow, a lowland variety and Summer, an upland variety, of switchgrass were provided by Dr. Gautam Sarath.

2) Grenhouse grown plants: Kanlow plants were grown in 2L pots containing standard greenhouse mixture with 4 plants/pot. Plants were grown at a daytime temperature of 83-88°F and 73-78°F nighttime temperature. Plants had 16 hours of daylight at 800 µmols m^{-2} s^{-1}.

3) Harvesting tissues: Both stover and leaf tissue samples were harvested weekly from both control and labeled plants. A portion of these tissues were frozen immediately in liquid nitrogen and stored at -20°C for enzyme assays. The rest of the tissue samples were dried at 60°C in an oven for 72 hours for carbon isotope ratio mass spectrometry.

4) Carbon Isotope Ratio (CIR) Mass Spectrometry: CIR analysis of tissue samples were performed following the procedures as in [1]. The ^{13}C contents of the samples are given by

$$^{13}C = (R_{[sample]} / R_{[standard]} - 1)*1000$$
$$\text{where } R = {}^{13}CO_2 / {}^{12}CO_2.$$

5) Pulse Labeling with $^{13}CO_2$: For isotope abundance studies, 5 week old greenhouse grown Kanlow plants were enclosed in a leak-proof plexiglass chamber and labeled

with 99% $^{13}CO_2$. Uptake of $^{13}CO_2$ by plants was monitored by Li-COR Li 6262 Infrared Gas Analyzer.

6) ^{13}C enrichment (atom % excess) determination: The ^{13}C enrichment (atom % excess) in the sample was calculated as the difference in ^{13}C atom% between the sample and its corresponding control tissue. The amount of labeled carbon (A) in the sample was calculated by: ^{13}C atom % in the sample - ^{13}C atom % in the control * amount of C in the sample.

7) % Carbon Content Determination: The amount of carbon (% total C) in both stover and leaf tissue samples were determined by analyzing them through a Costech (Model ECS 4010) elemental analyzer.

8) Fractionation of Cell wall structural carbohydrates: Fractionation of cell wall structural carbohydrates into hemi-cellulose, cellulose and lignin/ash fractions was performed by the sequential detergent fiber analysis procedure of as in [2].

B. Cinnamyl Alcohol Dehydrogenase (CAD) activity assay

1) Preparation of Crude Extract: Previously ground in liquid nitrogen and frozen at -20°C stover and leaf tissue samples were extracted in a cold extraction buffer containing 250 mM Tris-HCl, pH 7.5, 100mM Na ascorbate, 10% PVPP, 5% ehtylene glycol, 2% PEG, 0.1% ME, and protease inhibitor cocktail. Crude extract was filtered through two layers of cheesecloth, centrifuged at 27,000g for 15 min and desalted on a PD-10 column.

2) CAD Assay: CAD was assayed spectrophotometrically at 340 nm. and at 25°C from the desalted crude extracts following the procedures as in [4]. The reaction mixture contained 100mM KH_2PO_4/Na_2HPO_4, pH 6.25, 200µM coniferaldehyde, 2mM NADPH and ~ 8 µg of protein.

III. Results And Discussion

A. Carbon Isotope Ratio determination studies using natural abundance

Stover carbon isotope ratio (CIR, ^{13}C, ‰) analyses of from both the lowland (KANLOW) and the upland (SUMMER) cultivars of switchgrass indicated that CIR of Kanlow was less negative (-12.67‰ ± 0.04 S.E., n= 15) than Summer (-13.06 ± 0.046 S.E.). The observed range in CIR values in both varieties is consistent with other C_4 grasses. Carbon isotope ratios are good indicators of whether the carbohydrates are of the structural or non-structural types. Preliminary observations on the CIR of cellulose (structural) from switchgrass showed a less negative ^{13}C value (-12.646 ± 0.109) than the leaf CIR (a surrogate for non-structural carbohydrates) which showed a ^{13}C value of -13.140 ± 0.187. Fractionation of cell wall structural carbohydrates into hemicelluloses, celluloses and lignin followed by their CIR determinations showed that both hemicellulose and cellulose fractions were not very different in their CIR values, whereas the CIR of lignin fraction however was more negative (Table I). This depletion in ^{13}C of lignin is not surprising, as it is due to the isotopic discrimination associated with its two amino acids precursors, phenylalanine and tyrosine.

TABLE I. Variations in carbon isotope ratios (± S.E.) in different cell wall fractions of Kanlow stover tissue

Tissue/Fraction	CIR, 13C, ‰
Stover	-13.14 ± 0.19
Hemicellulose	-12.36 ± 0.21
Cellulose	-12.65 ± 0.11
Lignin / Pectin	- 16.73 ±0.12

B. ^{13}C enrichment (atom % excess) determination studies

Stover tissues harvested on 52 and 81days after planting (DAP) from plants labeled with $^{13}CO_2$, showed an increase in ^{13}C amount in the lignin/ash fraction per mg lignin

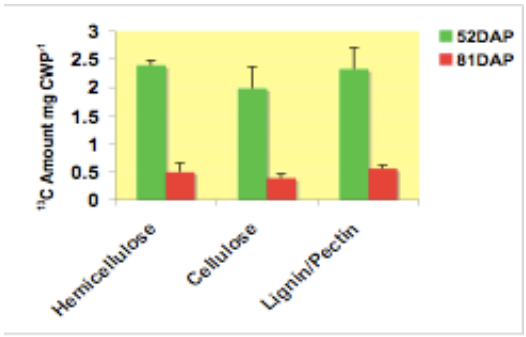

Figure 1. Partitioning of carbon (^{13}C Amount mg CWP^{-1}) into different cell wall polysaccharides (CWP) of ^{13}C labeled switchgrass plants.

when compared with either hemicellulose or cellulose fractions obtained from the tissues harvested on 81 DAP (Fig.1).

This suggests an active deposition of lignin in the stover tissue cell wall as plants mature.

C. Cinnamyl Alcohol Dehydrogenase (CAD) activity

A gradual increase in the relative activity of CAD was observed in the stover tissue extracts, while a gradual decrease in the leaf extracts of both Kanlow and Summer varieties of switchgrass plants over a 60 day growth period (Fig.2a and b). This supports the results from ^{13}C enrichment studies where the stover tissue lignin had an increased ^{13}C atom % enrichment than either cellulose or hemicellulose fractions.

Increased CAD activity (both in Kanlow and Summer varieties) and increased ^{13}C amount mg CWP^{-1} in lignin/ash fractions of the stover tissue in the Kanlow variety suggest an increased carbon partitioning into the lignin fraction, especially in "biomass abundant" growth stage in switchgrass. Down regulating lignin biosynthesis through conventional or transgenic means could result in increased cellulose content and thereby enhanced value as a feedstock of switchgrass biomass.

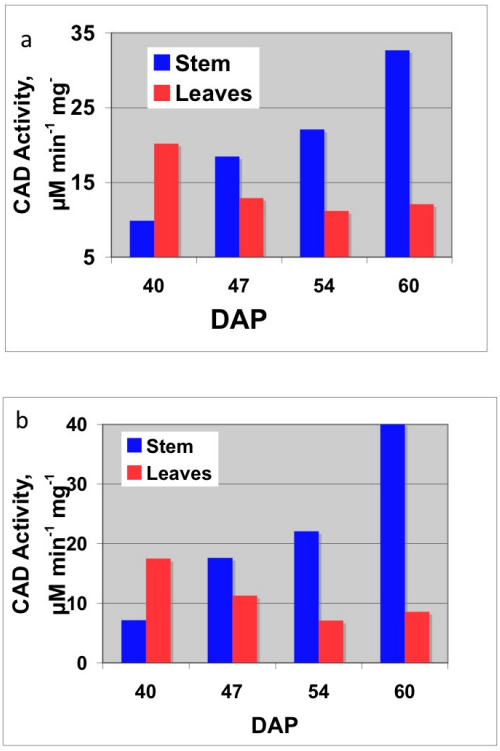

Figure 2. Cinnamyl Alcohol Dehydrogenase (CAD) enzyme activity in Kanlow (a) and Summer (b) cultivars of Switchgrass. Note the progressive increase in CAD activity in the stem and the gradual decline in the leaf tissue as the plants mature.

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