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ALTERATIONS IN $\delta^{13}C$ VALUES OF SEEDLING CELLULOSE ASSOCIATED WITH RESPIRATION DURING GERMINATION

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Key Word Index—*Zea mays*; *Triticum aestivum*; Gramineae; *Cucurbita moschata*; Cucurbitaceae; cellulose; $\delta^{13}C$; lipid; respiration; starch; uncoupler.

Abstract—$\delta^{13}C$ values of heterotrophically synthesized cellulose of roots from germinating seeds treated with a respiratory uncoupler were compared with those produced by untreated seed. Seeds or grains having starch as their principal storage substrate synthesized cellulose enriched in ^{13}C relative to their substrate. Those having lipids as their principal storage substrate synthesized cellulose depleted in ^{13}C relative to their substrate. The respiratory uncoupler decreased respiration and caused lower ^{13}C abundance in synthesized cellulose relative to the controls for starch-storing seeds. Decreased respiration, however, caused an increase in ^{13}C of synthesized cellulose from lipid-storing seeds. The observed isotopic enrichment of cellulose with increase in respiration in the starch-storing seeds is consistent with the hypothesis that stems and roots of plants are isotopically enriched relative to leaves because of respiration.

INTRODUCTION

Organic matter is often depleted in ^{13}C relative to environmental inorganic carbon sources. It has been known that carbon isotopic fractionations occur during photosynthetic processes, specifically during the ribulose bisphosphate carboxylase reaction [1]. However, less is known about isotopic effects of metabolic processes after carbon fixation. Although stable carbon isotopic fractionations during metabolic processes may be small when compared with those occurring during photosynthesis, they may lead to significant variations in $\delta^{13}C$ values among different plant tissues. For example, as early as the 1950s, Craig [2] measured carbon isotopes in plants and showed that wood was enriched in ^{13}C relative to leaves. Subsequently, similar observations indicate that in general, leaves of plants are depleted in ^{13}C relative to other parts of the same plant: leaves, stems and roots of tomato [3]; leaves and tubers of potatoes [4]; leaves and woody tissues of tropical species [5]; and leaves and roots of *ca* 20 tropical species [6]. These differences in carbon isotope composition between parts of a plant do not seem to be explained by variation in chemical composition, because the discrepancies between leaves and wood are found to be significant even when cellulose is purified from these tissues [7].

A hypothesis for the difference in $\delta^{13}C$ values between stem and leaves has been proposed by Francey *et al.* [8] using Farquhar's diffusion model [9]. They suggested that young leaves during their growth have a lower stomatal diffusion resistance leading to a depletion in carbon-13, but as they mature and export carbohydrates their stomatal resistance increases leading to an increase in carbon-13 concentration of exported carbohydrates. Their hypothesis, however, may not be consistent with previous observations, where leaf maturation actually leads to a decrease in stomatal resistance [10]. On the other hand, Leavitt and Long [7] proposed that the difference in $\delta^{13}C$ values between stem and leaves is brought about by the effect of respiration. Respiration will favour the release of ^{12}C, thus, leading to ^{13}C enrichment of the remaining carbohydrate pool.

In order to test this hypothesis we measured the effect of respiration on $\delta^{13}C$ values of heterotrophically synthesized cellulose. This was done by germinating seeds or grains in the dark with and without an uncoupler of oxidation phosphorylation. Although an uncoupler may initially increase respiration, their long term effect is to decrease respiration. The values of the heterotrophically synthesized cellulose were compared with its carbon source, starch in the case of corn and wheat, and lipid in the case of squash.

RESULTS

Carbon isotope fractionation during dark germination

All species in this study showed clear differences in $\delta^{13}C$ values between root cellulose extracted from the

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Table 1. Mean $\delta^{13}C$ (‰) values of the root cellulose, of the dark-germinated seedlings, of the respective substrates stored in the seeds or grains of barley (*Hordeum vulgare* L.), castor bean (*Ricinus communis* L.), corn (*Zea mays* L.), squash (*Cucurbita moschata* L.), peanut (*Arachis hypogaea* L.) and wheat (*Triticum aestivum* L.)

Species	$\delta^{13}C$‰ (s.e., *n*)* Root cellulose (seedlings)	$\delta^{13}C$‰ (s.e., *n*) Starch or lipids (seeds or grains)	$\delta^{13}C$‰ Δ(root − seed)
Starch-storing			
Wheat	−25.35 (0.09, 9)	−26.56 (−, 1)	+1.22†
Barley	−26.50 (0.07, 5)	−27.76 (−, 1)	+1.26†
Corn	−10.61 (0.04, 4)	−11.40 (0.01, 2)	+0.80*
Mean (s.e., *n*)			+1.09 (0.15, 3)
Lipid-storing			
Castor bean	−29.10 (0.27, 5)	−28.40 (−, 1)	−0.70
Peanut	−30.24 (0.31, 3)	−28.90 (−, 1)	−1.34*
Squash	−26.40 (0.20, 4)	−25.22 (0.01, 2)	−1.18*
Mean (s.e., *n*)			−1.07 (0.19, 3)

Students' *t*-test was made with Statview program for the samples having more than two duplicates and with the Sokal and Rohlf method [15] for the samples having a single specimen, s.e.: standard error and *n*: the number of duplicates.
* ≤ 0.05.
† ≤ 0.01.

dark-germinating seedlings and the respective substrates stored in seeds or grains. These results are consistent with our previous observations (unpublished data, Luo and Sternberg), which are summarized in Table 1. The tested species may be separated into two groups. One group corresponding to the starch-storage species had cellulose enriched in carbon-13 relative to its substrate. The other group corresponding to the lipid-storage species had cellulose depleted in carbon-13 relative to its substrate. On average, seedling cellulose of starch-storage species had $\delta^{13}C$ values *ca* 1 ml^{-1} higher than their main substrate. The tendency is reversed in lipid-storage species, with cellulose having $\delta^{13}C$ values *ca* 1 ml^{-1} lower in the seedling cellulose relative to the substrate.

The uncoupler (CCCP) of oxidative phosphorylation had significantly inhibitive effects on both respiration and seedling growth of all three species. The relative mass growth of seedling increased exponentially with an increase in respiration (Fig. 1, using the definition for these parameters given in the Experimental) with no significant differences between two starch-storing species, corn (C_4) and wheat (C_3). However, squash seedlings had a noticeably low respiration and growth rate.

The treatment with an uncoupler had a significant effect on the carbon isotopic composition of heterotrophically synthesized cellulose. For example, $\delta^{13}C$ values of seedling cellulose from uncoupler-treated seeds were on average 0.33‰ ($P < 0.05$) and 0.34‰ ($P < 0.005$) lower than those of normally grown wheat and corn, respectively. Conversely, the value was *ca* 0.29‰ ($P < 0.1$, $n = 5$) higher in cellulose from uncoupler-treated squash seeds. $\delta^{13}C$ values of root cellulose of seedlings correlated with respiration (Fig. 2). We used root tissue here because

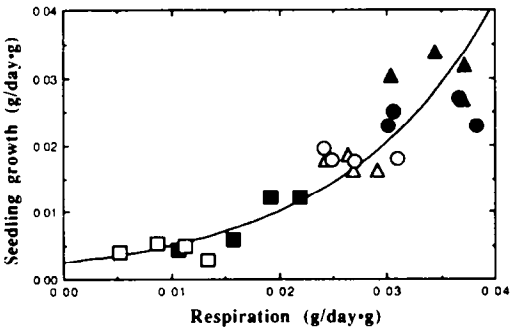

Fig. 1. Growth rate (g day^{-1} g^{-1}, g of shoots and roots dry weight per day per g of initial mass weight) of corn (*Zea mays* L.) [circle], squash (*Cucurbita moschata* L.) [square], and wheat (*Triticum aestivum* L.) [triangle] as a function of the corresponding respiration rate (g day^{-1} g^{-1}, g of lost mass via respiration per day − g of initial mass weight). Open symbols indicate uncoupler treatments. Each point represents a single sample. $Y = 0.0025 \times 10^{30.42x}$, $n = 24$, $r^2 = 0.866$.

CCCP being relatively insoluble in water may not have been transported to the upward growing shoot, whereas the root remains in contact with the uncoupler throughout the experiment. $\Delta\delta^{13}C_{(root-seed)}$ values indicate differences in all $\delta^{13}C$ values of root cellulose relative to $\delta^{13}C$ values of the corresponding storage substrate. The $\Delta\delta^{13}C_{(root-seed)}$ values increased in both corn and wheat, but squash tended to decline with an increase in respiration, suggesting that the alterations of $\delta^{13}C$ values in a product would depend on the metabolism of a particular substrate during respiration.

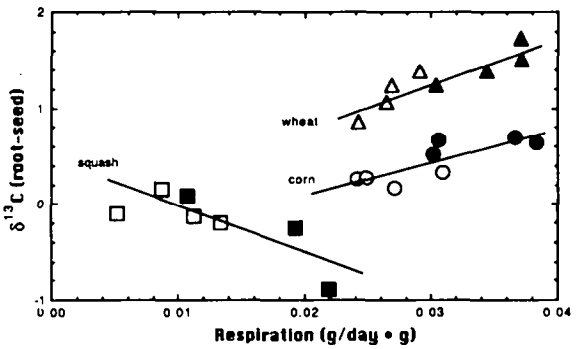

Fig. 2. Difference between the carbon isotope composition of the root cellulose and substrate stored in seed for corn (circle, $r^2=0.672$, $P\leqslant 0.01$), squash (square, $r^2=0.632$, $P\leqslant 0.02$), and wheat (triangle, $r^2=0.810$, $P\leqslant 0.001$) as a function of respiration rate (g day^{-1} g^{-1}). Open symbols indicate uncoupler treatments. Each point represents a single measurement.

DISCUSSION

The uncoupler concentration used here for seed germination was several hundred times higher than that used for microchondrial extracts [11], at which oxidative phosphorylation efficiency was below 20% of the control value. Such a low sensitivity of seedling tissues to the uncoupler may be related to its water-insoluble properties. Thus, the actual concentration of the uncoupler may be lower than that reported, because there was an undissolved residue in the treatment solution. The lower effects may also have been caused by amino acid residues of protein in tissue. For example, the CCCP-uncoupling of respiration can be prevented by cysteine [12]. Consequently, the uncoupling effect of oxidative phosphorylation could be weakened by small quantities of cysteine in the tissue.

An essential difference of this study from other investigations is that we completely avoided the effect of the photosynthetic carboxylation reaction on carbon isotopic composition of seedling organic matter. A similar observation was carried out by Smith [13] who measured $\delta^{13}C$ values of both respired CO_2 and organic matter of seedlings. However, exposure of these plants under a well-lighted laboratory bench 1 week after germination [13] might be a primary reason which led to lower differences in $\delta^{13}C$ values between respired CO_2 and organic matter among the species. Nevertheless, the four species (wheat, radish, peas and corn) had respired CO_2 with $\delta^{13}C$ values that were slightly more negative relative to those values of their respective seedling organic matter, while squash and castor bean had less negative $\delta^{13}C$ values in the respired CO_2 relative to seedling organic matter. Thus, both our measurements (Table 1 and Fig. 2) and Smith's [13] indicate that heterotrophically synthesized biomass in seedlings is slightly enriched in ^{13}C relative to its substrate in starch-storing seeds and slightly depleted relative to the substrate in lipid-storing seeds. The latter case involves the transformation of lipids into carbohydrates via gluconeogenesis, which may have isotopic fractionations leading to an overall enrichment in ^{13}C.

^{13}C enrichment of seedling root cellulose with an increase in respiration (Fig. 2) in corn and wheat may provide evidence to support the interpretation that differences between $\delta^{13}C$ values of stem or roots and leaves is brought about by respiration, since the primary substrate for cellulose synthesis in these tissues, as in starch-storing seeds, is a carbohydrate.

EXPERIMENTAL

Corn (*Zea mays* L.), squash (*Cucurbita moschata* L.) and wheat (*Triticum aestivum* L.) seeds or grains were, respectively, sterilized by soaking them under 70% EtOH for 5 min and then under 15% of commercial bleach for 10 min. After washing with pre-sterilized distilled H_2O, the seeds were germinated in the dark on an agar–H_2O medium in flasks at a constant temp. of 28° from 7 to 10 days depending on the growth rate of each species (corn, 7 days; squash, 9 days; wheat, 10 days).

Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, $ClC_6H_4NHN{=}C(CN)_2$) [Aldrich Chem. 85, 781–5] used in this study is an extremely effective uncoupler of oxidative phosphorylation in mitochondrial systems [11]. After flasks with agar–H_2O medium were sterilized, the uncoupler, dissolved in a small amount of 100% EtOH was added to treatment flasks which had been autoclaved, and immediately mixed well with agar–H_2O medium.

Seedlings were harvested and carefully sepd into 3 parts: roots, shoots and the remaining part of the seeds or grains which were dried under 80° for at least 36 hr and then weighed. Total respiration was then calcd by the following formula:

$$R_t = S-(G+S_r), \quad (1)$$

where R_t is total mass lost by respiration, S is dry weight of seeds or grain before germination, G is the total dry weight of shoot and root, and S_r is the weight of the remaining seeds or grains. Considering large differences in the rate of growth, the size of seeds or grains (initial weight of matter), and the duration of germination in the dark among the species, respiration for each species, is expressed as the specific respiration, i.e. g of respired mass per day per g of initial mass weight (g day^{-1} g^{-1}). Thus, specific respiration allows us to compare species having inherently different seed sizes and growth rates. Correspondingly, growth rate is measured here by the specific growth, that is g of shoot and root per day – g of initial mass weight of seeds (g day^{-1} g^{-1}).

Procedures for purification of cellulose and starch, and for purification of carbon dioxide from combustion of cellulose and starch all followed previous methods [14]. Lipids from the lipid-storing seeds were extracted by the method described in ref. [3].

All mass spectrometer analyses were performed on a VG–Isogas mass spectrometer (Dept. of Geology, University of Miami). Results are reported in δ values (‰),

where:

$$\delta(‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3, \quad (2)$$

and R is $^{13}C/^{12}C$ ratios of sample or standard (PDB), respectively.

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