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RESEARCH PAPER

# Influence of carbon to nitrogen ratios on soybean somatic embryo (cv. Jack) growth and composition

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## Abstract

**Soybean [*Glycine max* (L.) Merr.] seed are valued for their protein and oil content. Soybean somatic embryos cultured in Soybean Histodifferentiation and Maturation (SHaM) medium were examined for their suitability as a model system for developing an understanding of assimilate partitioning and metabolic control points for protein and oil biosynthesis in soybean seed. This report describes the growth dynamics and compositional changes of SHaM embryos in response to change in the carbon to nitrogen ratio of the medium. It was postulated that at media compositions that were sufficient to support maximal growth rates, changes in the C:N ratio are likely to influence the partitioning of resources between the various storage products, especially protein and oil. As postulated, at steady-state growth rates, embryo protein content was strongly correlated with decreasing C:N ratios and increasing glutamine consumption rates. However, oil content remained relatively unchanged across the C:N ratio range tested, and resources were instead directed towards the starch and residual biomass (estimated by mass balance) pools in response to increasing C:N ratios. Protein and oil were inversely related only at concentrations of sucrose in the medium <88 mM, where carbon limited growth and no starch was found to accumulate in the tissues. These observations and the high reproducibility in the data indicate that SHaM embryos are an ideal model system for the application of metabolic flux analysis studies designed to test hypotheses regarding assimilate partitioning in developing soybean seeds.**

**Key words:** Cell growth, glutamine, *Glycine max*, protein, oil, soluble sugars, somatic embryogenesis, starch, sucrose.

## Introduction

Soybean [*Glycine max* (L.) Merr.] seed are valued for their protein and oil content, and, when processed, provide the largest global source of vegetable protein for animal feed. Soybean seeds have a very high protein content (40%), but uniquely they are also high in oil (20%) (Sinclair and de Wit, 1975). The conundrum is that the oil and protein contents of soybean seeds are inversely related (Hartwig and Kilen, 1991); specifically a 1% reduction in oil content will lead to a 2% increase in protein content (Clemente and Cahoon, 2009). Although carbon shifts between protein and oil content, the underlying metabolic control mechanisms are not fully understood, and strong metabolic links between oil and storage protein synthesis are not apparent (Schwender *et al.*, 2003). The overall challenge is to increase the oil content of soybeans at the expense of other seed components (starch, soluble sugars, and cell wall material), while maintaining protein content at a level that the market demands, namely a dehulled, defatted meal with 47.5–49% protein (http://www.soymeal.org/composition). Biotechnology and metabolic engineering hold promise to accomplish this challenging task (Lu *et al.*, 2011), but, in order to make progress, the control points of carbon partitioning which dictate the inverse relationship between protein and oil contents in developing soybean seed need to be fully understood.

The genome sequence of soybean is available for the application of functional genomics to characterize the genetic basis of important traits (Schmutz *et al.*, 2010). Metabolic pathways for soybean seed components and their regulation

at the molecular level should provide important information for improvement of seed quality by genetic engineering (Nishizawa *et al.*, 2010). However, due to the long life cycle of soybeans, using developing seed (zygotic embryos) as an experimental system is neither an efficient nor a practical approach for screening the large number of genes (by knockout, overexpression, or underexpression) implicated in seed quality. Approximately 11 months are required to generate seed bearing transgenic plants, and several more generations are required to obtain homozygous plants capable of generating zygotic embryos for study. One response to the limitation of zygotic embryos to support discovery research programmes aimed at modifying seed composition has been the development of soybean somatic embryo model systems. Somatic embryos are the target tissue for one commonly used method of soybean genetic transformation (Finer and McMullen, 1991). For certain transgenic seed-specific traits, such as modified fatty acid profiles, somatic embryos have been shown to provide an excellent 'preview' of the ultimate composition of mature soybean seed, within 10 weeks of the initial transformation (Kinney, 1996). Despite the success of somatic embryo systems for the development of transgenic soybeans with modified seed quality, the systems have proven to be much more challenging when applied to evaluate transgenic approaches aimed at quantitatively increasing the total oil or protein content of soybean seed. Soybean somatic embryos accumulate seed-specific storage proteins, such as β-conglycinin and glycinin, and oils in the form of triacylglycerides (TAGs) in a manner similar to that observed during seed development (Schmidt *et al.*, 2005; Nishizawa and Ishimoto, 2009). However, heterogeneity in embryo development and composition within cultures results in such extreme variation in oil and protein content that transgenic approaches to change them are very difficult to detect within the natural variation of the system. A breakthrough occurred with the introduction of a somatic embryo model system based on Soybean Histodifferentiation and Maturation (SHaM) medium (Schmidt *et al.*, 2005). SHaM embryos show greater developmental uniformity, have compositions that are more seed like (Schmidt *et al.*, 2005), and have proven to be an excellent system for testing genes that lead to oil content increases in mature seed (Meyer *et al.*, 2012).

SHaM embryos are now a proven system for the evaluation of transgenic approaches to improve soybean quality; however, it has yet to be determined whether they provide a suitable test system for developing an understanding of the biochemical and physiological mechanisms underlying resource partitioning in developing soybean seed. Although studies of assimilate partitioning between storage products under different C:N ratios have been performed in zygotic soybean embryos (Saravitz and Raper, 1995; Hayati *et al.*, 1996; Pipolo *et al.*, 2004; Allen and Young, 2013), such studies have not been performed with somatic embryos. In this report, an in-depth study of the growth dynamics and compositional changes of SHaM embryos in response to changes in the C:N ratio of the medium is described, with two goals in mind. The first goal was to test a working hypothesis that at constant growth rates, changes in the relative proportions of sucrose (carbon source) and glutamine (nitrogen source) supply to the developing tissue will influence partitioning of resources between the protein and oil pools. The second goal was to identify which sets of C:N ratios in the SHaM media were best suited for future metabolic flux mapping studies designed to probe the metabolic control points that determine resource partitioning between protein and oil. The SHaM results under conditions of excess C and N illustrate that: (i) the protein content of the embryos increased from ~16% to 40% dry weight (Dwt) as the C:N mole ratio decreased; (ii) the protein content of the embryos indicated a strong positive correlation with the glutamine consumption rate, $R^2$=0.68; (iii) starch content was linearly correlated to increased C:N ratios, $R^2$=0.57, as was the residual biomass fraction, estimated by mass balance, $R^2$=0.77; and (iv) oil synthesis appeared to be saturated, as oil content was independent of C:N ratios. The data indicate that the partitioning of assimilates into protein was highly dependent on the supply of reduced organic nitrogen in the media. Furthermore, starch in SHaM embryos appears to represent a sink for excess carbon after the biosynthesis of other storage products, such as protein. The presented data show clear relationships between storage products and growth under carbon-limited regimes, as well as under conditions of excess carbon and nitrogen supply.

## Materials and methods

### *Plant material and culture conditions*

Cultures of proliferative soybean somatic embryos cv. Jack were provided by DuPont Pioneer Hi-Bred and were maintained, by weekly subculture, in SB196 medium. SB196 medium is a modified form of MSD20 medium (Walker and Parrott, 2001), and contained FN Lite Halides, FN Lite P, B, Mo, Murashige and Skoog (MS) sulphate, MS Fe EDTA, B5 vitamins, $KNO_3$, L-asparagine, and 29 mM sucrose. In addition, the SB196 medium contained a moderately high concentration of synthetic auxin, 2, 4-dichlorophenoxyacetic acid (10 mg $l^{-1}$). Clusters of globular-stage embryos (~10 mg) were used to initiate cultures by dropping them into 250 ml Erlenmeyer flasks containing 35 ml of SB228 liquid medium (Schmidt *et al.*, 2005). The SB228 medium contained FN Lite macro salts, MS micro salts, B5 vitamins, $CaCl_2$, L-methionine, 88 mM sucrose, 30 mM glutamine, and 165 mM sorbitol. Embryos were induced, maintained, and matured under a light intensity of 35–50 µmol photons $m^{-2}$ $s^{-1}$, provided by cool-white fluorescent bulbs, and a 16 h photoperiod. Cultures were maintained at 26°C and were continuously shaken at 130 rpm for 2 weeks to build biomass prior to experimentation.

After 2 weeks in culture, working in a laminar flow hood, clusters of SHaM embryos collected from 20 flasks derived from the same subculture cycle were separated into batches of ~40–50 randomly chosen, uniformly sized embryos, with an initial fresh weight of 1.00 g (measured and recorded to a precision of 0.01g), unless stated otherwise. These were placed into 250 ml Erlenmeyer flasks containing 35 ml of SHaM medium with the different sucrose and glutamine concentrations to be tested (Table 1). The medium in each flask was exchanged every 3 d, unless stated otherwise. Cultures were harvested after 6 d by pouring the contents of each culture into a sieve. The harvested embryos were rinsed with 100 ml of distilled water, blotted dry, weighed (fresh weight determination), and frozen in liquid nitrogen prior to lyophilization at –50 °C and 0.0158 mbar for 72 h. The lyophilized embryos were weighed (Dwt determination) and were finely ground using a Geno/Grinder® (SPEX

SamplePrep, Metuchen, NJ, USA) prior to further analysis.

Initial studies testing the influence of medium composition [ranges of sucrose concentration at fixed glutamine (30 mM); ranges of glutamine concentration at fixed sucrose (88 mM); the influence of inorganic nitrogen (ammonium nitrate)] on SHaM embryo growth and composition were performed as described above except that the cultures were initiated with 2.00 g (measured and recorded to a precision of 0.01 g) of uniformly sized embryos. The cultures were harvested after 7 d and the medium was not exchanged during the experimental culture period.

### *Relative growth rate*

The growth rates of the SHaM embryos were normalized based on their relative linear growth rates. Growth was expressed as the dry mass added during a given growth interval, per unit of original dry mass at the beginning of the growth interval. The relative growth rate ($d^{-1}$) was calculated using the following equation:

$$\mu_{relative} = \frac{\left[\frac{X_{dayn} - X_{day0}}{t}\right]}{X_{day0}}$$

where, $X_{dayn}$ is the tissue Dwt at final harvest, $X_{day0}$ is the initial Dwt of the culture (see below), and $t$ is the culture duration. To determine the initial Dwt of the culture, three 1.00 g fresh weight batches of representative embryos were prepared at the same time as the experimental cultures were being initiated (see above). The embryos were placed into pre-weighed 50 ml centrifuge tubes and were frozen in liquid nitrogen prior to lyophilization, as described above. After lyophilization, the embryo Dwt was determined and used in the calculations described above.

### *Lipid and protein extraction*

Approximately 100 mg of dry powdered embryo sample was extracted with 1 ml of *n*-hexane at 40 °C for 1 h; the extracts were centrifuged at high speed (13 200 rpm) for 10 min at room temperature. The process was repeated five times and, after each extraction, the solvent containing the lipids was pooled into a pre-weighed glass tube (~4.8 ml final volume) and dried for 4 d in a hood at room temperature. The mass of lipids after solvent evaporation was measured gravimetrically. The remaining hexane-extracted biomass was dried and further extracted for protein in 600 µl of 200 mM phosphate buffer (pH 7.2) containing 14 mM β-mercaptoethanol at 4 °C for 20 min to dissolve and suspend the protein into solution. The extracts were centrifuged at high speed at 4 °C for 15 min and the supernatant was transferred into a microcentrifuge tube. The extraction was performed on the pellet twice more with 400 µl of the buffer (per extraction) and the supernatants were pooled with that from the initial extraction (~1.2 ml final volume). Protein contents of the extracts were measured by Bradford assay (Bradford, 1976). The soluble protein extraction also removed soluble sugars from the samples. Aliquots of the protein extract were therefore mixed with 100% ethanol at a ratio of 1 ml of protein extract/1 ml of 100% ethanol, vortex mixed, and centrifuged for 2 min at high speed. The supernatants were run on a high-pressure liquid chromatograph for analysis of soluble sugars (see below).

### *Residual soluble sugar and starch extraction*

Residual soluble sugars in the defatted/deproteinated biomass pellets were extracted into 1 ml of 80% aqueous ethanol in a water bath sonicator (Fisher Scientific FS110H Ultrasonic Cleaner) at 60 °C for 20 min. The extraction was repeated four times and, after each extraction, the solvent containing the soluble sugars was pooled into a pre-weighed glass tube (~4.8 ml final volume) and dried for 3 d in a 40 °C oven, and the soluble sugar content in these fractions was measured gravimetrically. To obtain the total soluble sugar content for the tissue, the results of high-pressure liquid chromatography (HPLC) analysis for soluble sugars in the protein extracts were added to those obtained for the 80% ethanol extracts. The remaining pellets from each sample were placed in 1.5 ml of distilled deionized water, covered with foil, and autoclaved (liquid cycle at 121 °C and a pressure of 15 psi) for 30 min prior to starch digestion and extraction. Starch was digested in 1.5 ml of 100 mM citrate buffer (pH 5.0) containing amyloglucosidase at a ratio of 0.025 mg enzyme:1 mg of tissue Dwt (~2 µl of amyloglucosidase or 0.6 U of enzyme per sample). Samples were incubated overnight in a 30 °C water bath. Starch content was quantified using a Glucose Assay Kit (Sigma, St Louis, MO, USA).

### *Residual biomass fraction estimation*

The percentage of the residual biomass fraction was estimated by subtraction of the measured sum of the biomass; that is, protein (by combustion analysis)+oil+starch+soluble sugar+ash and an estimation of the DNA/RNA content (see below), from the original mass of tissue extracted. Ash content was determined by thermogravimetric analysis as described below. DNA/RNA content was assumed to be 5% on a Dwt basis (Stephanopoulos *et al.*, 1998).

### *Determination of ash content*

Ash content was determined by complete combustion of the samples in a thermo-gravimetric analyser (TGA 7) running the following temperature ramp program: ramp from room temperature to 100 °C and hold for 10 min; ramp to 200 °C at 10 °C $min^{-1}$; hold at 200 °C for 10 min; ramp to 400°C at 10°C $min^{-1}$; hold at 400 °C for 30 min; ramp to 700 °C at 10 °C $min^{-1}$; hold at 700 °C for 180 min; drop to, and hold at, room temperature until sample removal. The remaining residue in the crucible was taken as the ash content.

### *Sucrose and glutamine measurement, and elemental analysis*

Sucrose and glutamine consumption rates from the medium were determined by measuring the residual sucrose and glutamine in the culture medium at the initiation of the experiments, in the medium recovered at the 3 d exchange, and when the culture was harvested. Each sample of medium was filtered with a syringe filter (25 mm, 0.45 µm, Nylon, XPERTEX®) prior to injection into a high-pressure liquid chromatograph (Waters 1525 Binary Pump and 717$^{plus}$ Auto Sampler fitted with an Aminex HPX-87K column, and a Waters 2414 RI detector). Samples were eluted with a water mobile phase at 50 °C, at a flow rate of 0.35 ml $min^{-1}$; each run was 80 min. By running the separation for 80 min, both sucrose and glutamine were detected in the same HPLC run. The HPLC run also detected sorbitol in the liquid medium. The concentrations of soluble sugars from the protein extracts were analysed as above, except the runs were 30 min long to detect sucrose, glucose, and fructose. A series of sucrose (15, 44, 88, 146, 176, and 234 mM), glutamine (5, 10, 20, 30, 40, and 60 mM), and sorbitol (50, 100, 150, and 200 mM) standards were prepared and measured with the unknown samples. Standard curves were generated by plotting the area under the peaks versus their concentrations, and were used to compute the concentrations of the unknown samples. In the protein extracts, glucose and fructose concentrations were very low and calibration curves were independently derived for these sugars.

Sucrose and glutamine consumption rates were calculated using the following equation:

$$\frac{ds}{dt} = \frac{\left(S_{day6} - S_{day0}\right) \times V}{\left(t_{day6} - t_{day0}\right) \times \left(X_{day6} - X_{day0}\right)}$$

where $S$ is the medium sucrose or glutamine concentration at the

onset and end of the culture period, $V$ is the volume of the medium after 3 d or 6 d of culture, $t$ is time, and $X$ is the embryo Dwt.

The Dwt percentages of carbon, hydrogen, and nitrogen were measured by elemental analysis using the Perkin-Elmer Model 2400 Series II CHN&S Elemental analyzer (Chemical Instrument Facility, Chemistry Department at Iowa State University). In the determinations of biomass used throughout this study, total protein content was calculated from the elemental analysis (%N×6.25).

### *Determination of free amino acids*

Approximately 20 mg of dried ground embryo material was weighed into a microcentrifuge tube (to a precision of 0.1 mg) and 40 µl of norleucine (1 mg $ml^{-1}$ deionized water) was added as an internal standard followed by methanol:chloroform:water (700 µl:300 µl:450 µl). The samples were vortexed for 1 min and centrifuged at 15 000 rpm for 10 min at 4 °C. A 300 µl aliquot of the upper polar phase was transferred into a new 1.5 ml microcentrifuge tube and dried in a vacuum concentrator (Vacufuge Plus, Eppendorf, Hauppauge, NY, USA). After centrifugation at 15 000 rpm for 2 min, 30 µl of the solution was transferred into GC vials fitted with glass inserts. An equal volume (30 µl) of *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide containing 1% tert-butyldimethylchlorosilane (TBDMS; Sigma-Aldrich, St Louis, MO, USA) was added, and the vials were capped tightly and incubated at 80 °C for 30 min in a heated block. The TBDMS-derivatized amino acids were analysed by gas chromatography on an Agilent 6890 fitted with an HP-5MS (30m×0.25 mm i.d., 0.25 µm) column and a 5973 mass spectrometer detector. The temperature program was as follows: 70 °C for 1 min, ramp at 15 °C $min^{-1}$ to 130 °C followed by a ramp of 5 °C $min^{-1}$ to 300 °C, which was held for 1 min. Amino acid standards for 13 amino acids (0.25 µmol $ml^{-1}$ for each amino acid; Thermo Fisher Scientific) were diluted in series (0.25, 0.125, 0.063, 0.031, and 0.016 µmol $ml^{-1}$) with deionized water. The standard solutions were mixed with the internal standard solution (1 mg $ml^{-1}$ norleucine) at a 1:1 volume ratio, and were dried and derivatized, as described above, prior to GC-MS analysis. Based on the retention times and response factors for mass fragments of the amino acids and the internal standard, intracellular free amino acids were identified, and quantified. In order to estimate the influence of the sample matrix (proteins and salts) on the amino acid derivatization efficiency, three sets of samples were prepared with internal standards: (i) samples alone (extracted from cultures grown at high, medium, and low C:N ratios); (ii) the same samples spiked with the 13 amino acid standards each at a defined concentration of 0.1 µmol; and (iii) the 13 amino acid standards at the same concentrations as in (ii). Glutamate (Glu) and alanine (Ala) were the dominant amino acids present in the tissue extracts. The derivatization efficiency of Glu and Ala was 90.2±8.5% and 96.7±8.5%, respectively. These derivatization efficiency values were used to estimate the free amino acid content of the tissue.

**Table 1.** Concentrations of sucrose and glutamine in the medium and the mole ratios of carbon to nitrogen used.

| C mole of sucrose/N mole of glutamine (C:N mole ratio) | | | | |
|---|---|---|---|---|
| | Glutamine concentration (mM) | | | |
| **Sucrose concentration (mM)** | **20** | **37** | **50** | **60** |
| 88 | 26.40 | 14.27 | 10.56 | 8.80 |
| 117 | 35.10 | 18.97 | 14.04 | 11.70 |
| 146 | 43.80 | 23.68 | 17.52 | 14.60 |
| 176 | 52.80 | 28.54 | 21.12 | 17.60 |
| 234 | 70.20 | 37.95 | 28.08 | 23.40 |

### *Statistical analysis*

Data have been presented as the mean ±SE for each medium condition treatment ($n$=3). Initial sucrose and glutamine concentrations were converted to C:N mole ratios for each of the medium conditions (Table 1). Significant differences between the treatments were determined by analysis of variance (ANOVA), using the multiple comparison method of Tukey–Kramer's honestly significant difference (HSD). Least significant difference (LSD) values were calculated at the $\alpha$=0.05 probability level (JMP v. 8.0.2, SAS Institute Inc., Cary, NC, USA).

## Results

### *Determining the carbon-limited regime for SHaM embryos*

Typical medium concentrations of the carbon and nitrogen sources for zygotic embryos cultured *in vitro* are designed to mimic the conditions experienced by developing seed on the plant. The actual concentrations of sucrose and amino acids at the apoplastic interface between the maternal and filial tissues are extremely difficult to measure with any degree of certainly, and reported values lie in the range of 3.4–200 mM sucrose and 10–44 mM amino acids (Hsu *et al.*, 1984; Gifford and Thorne, 1985; Saravitz and Raper, 1995; Pipolo *et al.*, 2004; Schmidt *et al.*, 2005). Therefore, in this study, a broad range of sucrose and glutamine concentrations was used to better define the influence of the carbon and nitrogen source concentrations on embryo growth and composition. The regime where the sucrose was not limiting growth was determined from experiments performed at a fixed glutamine concentration (30 mM) and sucrose concentrations ranging from 0 to 234 mM. The highest sucrose concentration in the medium tested was slightly high when compared with the measured sucrose concentrations reported for the soybean seed apoplast, 150–200 mM (Gifford and Thorne, 1985), but was lower than the upper range of sucrose concentrations (73–292 mM) used by Thompson *et al.* (1977) for the *in vitro* culture of soybean zygotic embryos. As shown in Fig. 1A, the slope of the relative growth rate ($d^{-1}$) versus sucrose concentration at sucrose concentrations <88 mM was steeper than at higher concentrations (Fig. 1A). This indicated that carbon was limiting below ~88 mM sucrose, an observation that was supported by a total absence of starch in the embryos cultured at sucrose concentrations <88 mM (Fig. 1B). However, at the lowest medium sucrose concentrations tested (0 and 44 mM), the soluble sugar content of the SHaM embryos was significantly greater than that in embryos cultured in media with higher sucrose concentrations (Fig. 1C). SHaM embryos with the highest soluble sugar concentrations (i.e. those cultured in the 0–44 mM sucrose range) were darker green than those cultured at higher sucrose concentrations (data not shown). It is speculated that under the limiting sucrose regime, the SHaM embryos may be capable of autotrophic synthesis of sugars via photosynthesis. It is apparent that photosynthetic synthesis of sugars did not result in starch accumulation in these tissues.

In a complementary study, the influence of changing the glutamine concentration (from 0 to 37mM) at a fixed sucrose

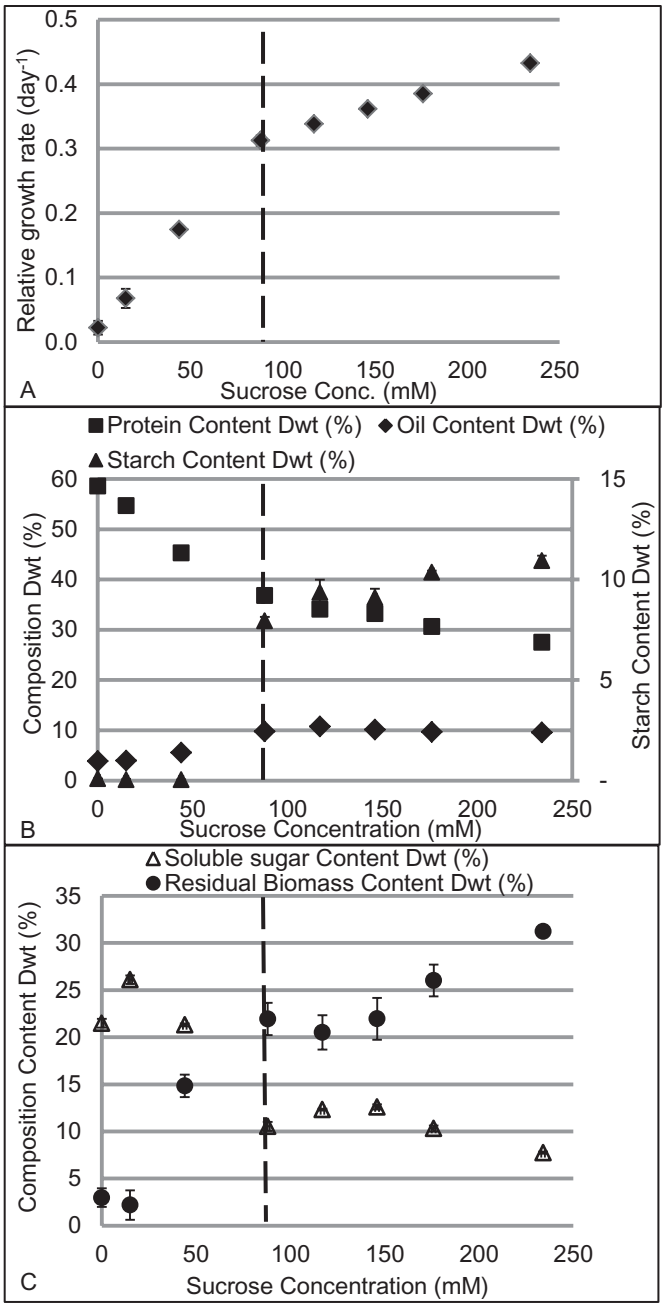


**Fig. 1.** Relative growth rate ($d^{-1}$) (A) and compositional analysis of: protein, oil, and starch (B), soluble sugars, and residual biomass content (C) in soybean somatic embryos cv. Jack as a function of the initial sucrose concentrations in the SHaM medium; note that the medium was not changed during the 7 d culture period. Initial sucrose concentrations ranged from 0 to 234 mM. The initial concentration of glutamine was 30 mM. Below 88 mM sucrose (indicated by a dashed line), SHaM embryos were carbon limited, and starch was not detected in these cultures at harvest.

concentration (88 mM) was investigated. Under these conditions, relative growth rates were unaffected across the range of glutamine concentrations tested (Supplementary Fig. S1A available at *JXB* online). Glutamine concentrations also had little influence on the starch, oil, and soluble sugar contents of the embryos, which all remained relatively constant across the glutamine concentration range (Supplementary Fig. S1B, C). In contrast, protein contents were positively correlated to increasing glutamine concentrations in the media (Supplementary Fig. S1B) and the residual biomass fraction showed a negative correlation (Supplementary Fig. S1C).

### *Linear relative growth rate of SHaM embryos*

Having established a sucrose concentration below which culture growth was significantly reduced, the influence of a range of carbon (sucrose) to nitrogen (glutamine) (C:N) mole ratios on the partitioning of resources between protein and oil was investigated. The protocol was also slightly modified in that cultures were initiated with only 1 g of embryonic tissue (compared with 2 g in the initial studies) so as to reduce the degree of resource depletion during the culture period; the culture solutions were also changed after 3 d and cultures were harvested after 6 d. Sucrose concentrations from 88 mM to 234 mM, in combination with glutamine concentrations of 20– 60 mM, were selected for further experimentation. Table 1 shows the ratio of moles of C, provided by sucrose, to the moles of N, provided by glutamine in the initial SHaM medium.

The linear relative growth rates of SHaM embryos for 20 different C:N mole ratios are shown in Fig. 2. The average relative growth rate under all media conditions for SHaM embryos was $0.61 \pm 0.07$ $d^{-1}$ during the 6 d culture period. The lowest relative growth rate, $0.44 \pm 0.01$ $d^{-1}$, was at a C:N mole ratio in the medium of 26.40. This low relative growth rate was at a medium condition of 88 mM sucrose and 20 mM glutamine, which is consistent with the fact that these conditions are at the boundary of the carbon- and nitrogen-limited regime. The relative growth rate data indicate that for the most part, the SHaM embryos were not under carbon or nitrogen limitation.

### *Effect of sucrose and glutamine supplements on biomass composition*

The protein content of the embryos increased from ~16% to 40% Dwt as the C:N mole ratio decreased from 70.20 to 8.80, with the regression coefficient $R^2$=0.79 (Supplementary Fig. S2A at *JXB* online). This result is consistent with the role of glutamine as the main reduced nitrogen source available for protein biosynthesis. The base medium used for these studies contained 28 mM inorganic nitrogen, in the form of potassium nitrate. To test whether the inorganic nitrogen could influence SHaM growth and composition, an experiment was performed in which the glutamine was replaced with ammonium nitrate at 5, 15, 30, and 40 mM; the sucrose concentration was fixed at 88 mM. The relative growth rate of the embryos was not influenced by the inorganic nitrogen concentration (Supplementary Fig. S3A at *JXB* online). However, in contrast to the results where nitrogen was supplied in the form of glutamine, inorganic nitrogen concentrations had little influence on embryo composition (Supplementary Fig. S3B, C). Notably, protein contents were unaffected.

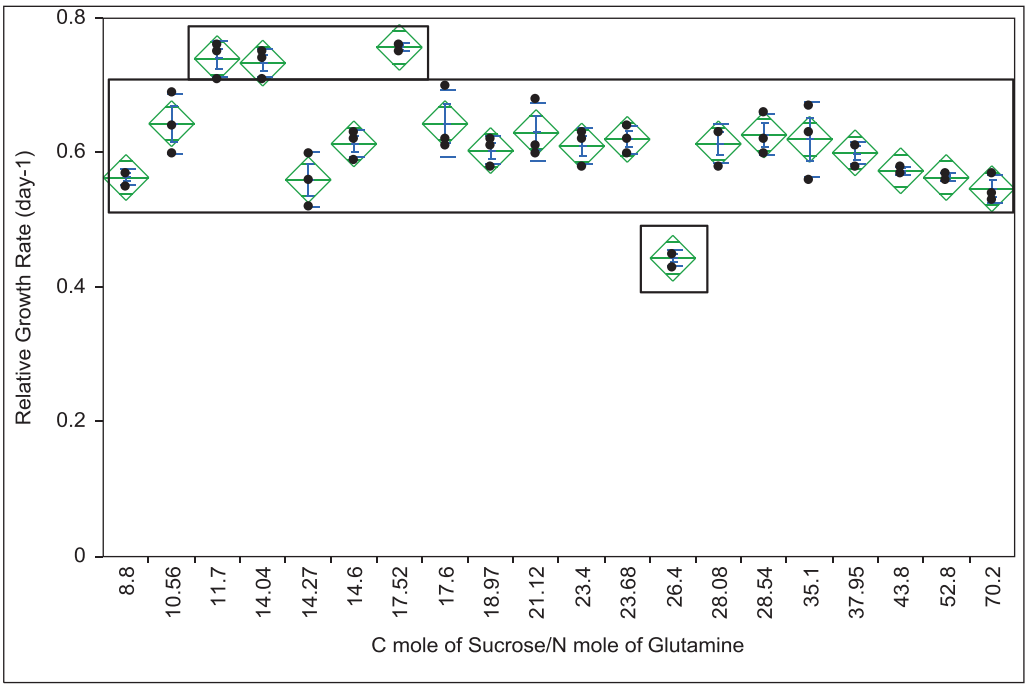


**Fig. 2.** The relative growth rates ($d^{-1}$) of soybean somatic embryos cv. Jack with different initial carbon to nitrogen (C:N) mole ratios. The embryos were cultured in SHaM medium for 6 d with a medium change on day 3. Error bars represent the standard error ($n$=3). The diamonds show the confidence limits ($\alpha$=0.05) for each mean (determined by analysis of variance (ANOVA) of the three biological replicates under each medium condition). Tukey–Kramer's honestly significant difference analysis shows that the relative growth rates, within each box, did not differ significantly at the $\alpha$=0.05 level.

Bradford assays were performed on a selection of the samples, as an independent estimate of protein content. The results, along with the protein content estimates from elemental analysis of the same tissues, are given in Table 2. The results of the Bradford assay (which only measures soluble proteins) were only 40–50% of those determined by elemental analysis, but the trends, namely the increases in protein content in response to decreasing C:N ratios, were equally apparent. One concern when using combustion analysis for determining protein content is that the method reports total nitrogen content for a sample and, as such, includes both total protein and any other nitrogen-containing molecules, both organic and inorganic. Because the treatments involved supplying some of the cultures with very high levels of glutamine in the medium, it was important to determine the contribution of endogenous free amino acid pools to the overall measurement of tissue nitrogen content. Analysis of the free amino acid pools show that the embryos contained significant levels of both glutamate (Glu) and alanine (Ala). All other amino acids detected were at concentrations that were two orders of magnitude lower (data not shown). Estimates of the contributions of the Glu and Ala pools to the total tissue percentage N measurements are given in Table 3. The data show that total protein contents were overestimated by ~20 ± 5%.

The influence of sucrose and glutamine concentrations in the medium on the oil content of SHaM embryos is shown in Fig. 3A. The oil content across the C:N ratios tested in these experiments was 5–8%, on a Dwt basis. The oil content of SHaM embryos therefore appears to have no correlation to the medium C:N mole ratios, and is therefore in stark contrast to protein biosynthesis. To examine whether the oil and protein content of SHaM embryos were inversely related across the C:N ratios tested, the oil and protein contents were plotted against each other (Supplementary Fig. S4 at *JXB* online). Although the slope from a linear fit was –1.82, which indicates that a 1% reduction in oil content would lead to a 1.8% increase in protein content, similar to the rule of thumb reported in Clemente and Cahoon (2009), the correlation coefficient was $R^2$=0.13 (Supplementary Fig. S4) and is therefore insignificant in the system used here.

The starch contents in SHaM embryos in response to medium composition are shown in Fig. 3A. The data indicated that there was a linear correlation between starch content and C:N mole ratios, with the linear correlation coefficient $R^2$=0.57 (Supplementary Fig. S2B at *JXB* online). Moreover, the level of starch in the SHaM embryos decreased as the glutamine concentration increased at the four higher

**Table 2.** Measurement of protein content, via elemental analysis and Bradford assay, of soybean somatic embryos cv. Jack cultured under a selection of C:N mole ratios.

| C:N mole ratio | Elemental analysis | Bradford assay |
|---|---|---|
| 8.80 | 41.2±0.5 | 16.4±1.1 |
| 23.68 | 29.6±0.7 | 13.4±0.8 |
| 52.80 | 21.5±0.3 | 11.4±0.4 |

**Table 3.** Estimates of the apparent total nitrogen content of soybean somatic embryos cv. Jack cultured under a selection of C:N mole ratios, contributed by endogenous free amino acids. Estimates of their possible contributions to the total protein contents measured by combustion analysis are given.

| C:N mole ratio | % N from alanine (Ala) | % N from glutamate (Gln) | Apparent contribution to protein content [% N (Ala+Gln)×6.25] | Total protein via elemental analysis |
|---|---|---|---|---|
| 8.80 | 0.41±0.11 | 1.33±0.04 | 10.9±0.6 | 41.2±0.5 |
| 14.04 | 0.47±0.01 | 0.81±0.08 | 8.0±0.4 | 34.5±0.4 |
| 23.68 | 0.27±0.01 | 0.80±0.06 | 6.7±0.4 | 29.6±0.7 |
| 28.54 | 0.25±0.02 | 0.73±0.05 | 6.1±0.3 | 27.0±0.3 |
| 35.10 | 0.16±0.01 | 0.32±0.04 | 3.0±0.3 | 22.3±0.2 |
| 70.20 | 0.10±0.01 | 0.32±0.02 | 2.6±0.2 | 20.4±0.1 |

sucrose concentrations tested (117–234 mM; data not shown). As shown above, on a Dwt percentage basis, protein content decreased with increasing media C:N mole ratios (Fig. 3A). Therefore, the starch and protein contents of SHaM embryos were inversely related, with a linear correlation coefficient, $R^2$=0.59 (data not shown).

The response of endogenous soluble sugar pools to medium sucrose and glutamine concentrations is shown in Fig. 3B. The total soluble sugar content (from the ethanol and protein extractions combined) ranged between 14% and 20% on a Dwt basis. There was no discernible correlation between the soluble sugars in the SHaM embryos and the C:N mole ratios in the medium (Fig. 3B).

### *Closing the mass balance: residual biomass of SHaM embryos*

To complete the biomass composition balance, the residual biomass fraction was estimated based on mass balance (see the Materials and methods). The percentage residual biomass fraction was calculated by subtracting the sum of the biomass composition [protein (by elemental analysis)+oil+starch+soluble sugar+ash+DNA/RNA] from the mass of tissue extracted. The residual biomass fraction increased as the C:N mole ratio increased, with the linear correlation coefficient $R^2$=0.76, as shown in Supplementary Fig. S2C at *JXB* online. Protein and residual biomass of SHaM embryos were inversely related, with a linear correlation coefficient, $R^2$=0.91 (Supplementary Fig. S2D). Preliminary studies showed that the lipid and soluble sugar extractions were exhaustive. Therefore, it can be stated with confidence that little residual oil, soluble sugar, or protein (measured by combustion analysis) led to errors in the calculation of the residual biomass fraction.

### *Glutamine and sucrose uptake rate of SHaM embryos*

The 3D graph of the glutamine consumption rate provides a clear indication that the SHaM embryos consumed more glutamine, as glutamine concentrations in the medium increased from 20 mM to 60 mM, and the concentration of sucrose in the medium decreased from 234 mM to 88 mM (Supplementary Fig. S5 at *JXB* online). Measurements of endogenous free amino acids pools show clearly that the majority of the glutamine taken up by the embryos was converted to protein. The protein content of the SHaM embryos indicated a strong positive correlation with the glutamine consumption rate, $R^2$=0.68 (Supplementary Fig. S6).

The sucrose consumption rate was more complex. No significant correlation between the sucrose consumption rate

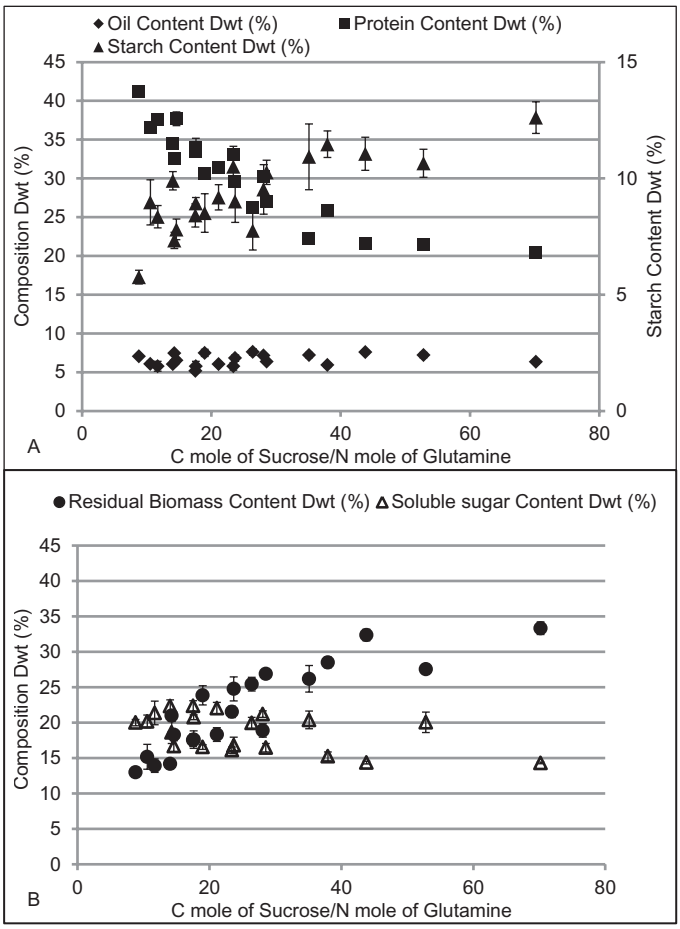


**Fig. 3.** Protein, oil, and starch (A), and soluble sugars and residual biomass (B) contents all expressed on a dry weight basis, for soybean somatic embryos cv. Jack cultured in SHaM medium with different initial carbon to nitrogen (C:N) mole ratios for 6 d. The medium was replaced after 3 d of culture.

and the C:N mole ratio in the medium was observed (data not shown). In the present studies, the medium was replaced every 3 d to ensure that the nutrient supplies were constant for carbon and nitrogen sources throughout the experimental period. The percentages of sucrose depletion under the 20 media conditions were examined for day 3 and day 6 cultures. The data indicated that after 3 d and 6 d of culture, the percentage of sucrose depletion were 19.2 ± 8.5 and 29.0 ± 9.7, respectively (data not shown).

## Discussion

In this study, SHaM embryos were cultured in medium with varying C:N mole ratios. To the authors' knowledge, this is the first reported study of resource partitioning between storage products under various C:N ratios for soybean somatic embryos. It was hypothesized that at constant growth rates, sucrose and glutamine in excess of that needed to support growth and maintenance metabolism would be directed to the synthesis of the storage products, protein, oil, and carbohydrates, and that the proportional distribution would be influenced by the C:N mole ratios of the medium. The information from this study also allowed the selection of a set of medium conditions to be used for future metabolic flux mapping studies that are designed to probe metabolic control points regulating the partitioning of storage reserves. Such data will allow direct comparisons to be made with the fluxes previously reported for soy zygotic embryos (Sriram *et al.*, 2004; Iyer *et al.*, 2008; Allen *et al.*, 2009), including soy zygotic embryos grown under different C:N ratios (Allen and Young, 2013), in order to assess the relevance of measurements made in this model system to processes occurring during seed development *in planta*.

For sucrose concentrations <88 mM, and a constant glutamine concentration of 30 mM [the recommended concentrations of these two components in standard SHaM medium (Schmidt *et al.*, 2005)], the SHaM embryos were carbon limited. The relative growth rates decreased sharply in media with sucrose levels <88 mM (Fig. 1A), but did not appear to be influenced by increases in the sucrose concentration above that amount, a result that is consistent with the observations of Egli and Bruening (2001) and Thompson *et al.* (1997) with soybean zygotic embryos. Interestingly, Gifford and Thorne (1985) measured the sucrose concentration in the tissue water of developing soybean cotyledons to fall in the 83–93 mM range during a diurnal cycle, a value that was significantly reduced (to 23 mM) only after defoliation of the mother plant the morning before the cotyledons were harvested. At sucrose concentrations in the medium below 88 mM, the SHaM embryos did not accumulate starch. From these results and previous studies on soybean embryos cultured *in vitro* (Hsu *et al.*, 1984; Saravitz and Raper, 1995; Gifford and Thorne, 1985; Pipolo *et al.*, 2004; Schmidt *et al.*, 2005), an experimental scheme for testing the influence of C:N mole ratios on resource partitioning in SHaM embryos was established. As postulated, under excess carbon and nitrogen in the liquid medium (concentrations ≥88 mM sucrose and 30 mM glutamine), the relative growth rates of the SHaM embryos were constant across the range of C:N mole ratios tested (8.80–70.20) during the 6 d culture period, indicating the presence of an optimal supply of carbon and nitrogen sources for growth (Fig. 2).

For a meaningful comparison of the growth rate results between somatic embryos and zygotic embryos, all growth data were expressed as relative growth rates (Fig. 2). Under optimal conditions, the average relative growth rate of SHaM embryos across the broad range of C:N mole ratios tested here was 0.61 ± 0.07 $d^{-1}$. This compared well with a value of 0.52 ± 0.10 $d^{-1}$ measured for zygotic embryos of the same cultivar cultured *in vitro* at 146 mM sucrose and 37 mM glutamine (unpublished data). Further, the SHaM embryo relative growth rates were comparable with those of two different cultivars of zygotic embryos: cv. Evans (cultured *in vitro* at 146 mM sucrose and 37 mM glutamine) with a value of 0.45 ± 0.07 $d^{-1}$ (Iyer *et al.*, 2008) and cv. Jack (cultured *in vitro* at C:N ratios from 6 to 91) with an average value of 0.67 ± 0.10 $d^{-1}$ (Allen and Young, 2013).

In addition to their roles in supporting growth and as the principal precursors for storage product synthesis (Thompson *et al.*, 1977; Haga and Sodek, 1987), sucrose and glutamine have also been shown to be signalling molecules triggering storage-associated processes in plant sink tissues (Thompson *et al.*, 1977; Haga and Sodek, 1987; Koch, 1996; Smeekens, 2000; Koch, 2004). The present data clearly show that the partitioning of assimilates into protein was strongly influenced by the C:N mole ratio in the medium, a relationship that had an $R^2$=0.79 (Fig. 3A; Supplementary Fig. S2A at *JXB* online) and was therefore highly dependent on the glutamine concentration in the media. Similar results have been reported in *in vitro* studies of soybean zygotic embryos, where increasing glutamine concentrations in the media resulted in increased protein contents (Saravitz and Raper, 1995; Pipolo *et al.*, 2004; Allen and Young, 2013). In the present study, the total protein content also correlated strongly with the glutamine consumption rate, with an $R^2$=0.68 (Supplementary Fig. S6). When inorganic nitrogen (ammonium nitrate) was used to replace glutamine, embryo growth was little influenced across the concentration range tested, but higher levels of inorganic nitrogen did not lead to increased protein contents (Supplementary Fig. S3). Taken together, these results indicate that protein biosynthesis in SHaM embryos is extremely dependent on the supply of reduced organic nitrogen in the medium. The conversion of glutamine to the other amino acids required for storage protein synthesis demands carbon skeletons derived from the metabolism of sucrose imported from the phloem (Miflin and Lea, 1977). In the case of glutamine, its amide-N group must be donated to a suitable carbon skeleton acceptor molecule, such as 2-oxoglutarate [a product of the tricarboxylic acid (TCA) cycle in the mitochondria], which can then be used in the formation of other amino acids (Weigelt *et al.*, 2008). The present data clearly show that as glutamine levels are increased (C:N mole ratios are decreased), protein synthesis draws carbon away from the non-structural (starch; Supplementary Fig. S2B) and structural (residual biomass; Supplementary Fig. S2C) fractions, but does not influence the pool sizes of soluble

sugars (data not shown). The negative correlation between the protein content and the structural carbohydrate pools is of significant interest as it is consistent with the observation of Somers and his colleagues who have demonstrated a negative relationship between the oil plus protein content and the fibre fraction in mature soybeans (Stombaugh *et al.*, 2000, 2003).

In a previous *in vitro* physiological study, Pipolo *et al.* (2004), using zygotic embryos from a different soybean cultivar (cv. Williams 82), have reported an inverse relationship between oil and protein contents in response to different glutamine concentrations (20, 40, 60, and 80 mM) in the medium, at a fixed sucrose concentration of 204 mM. The slope of the protein to oil content regression graph was –0.19, which, as the authors commented, was significantly less than the expected value of –0.82, predicted from theoretical calculations of the conversion of photosynthate to protein and oil mass. The authors conclude that the negative relationship between protein and oil in soybean seed is not simply a trade-off between the energy requirements for protein and oil synthesis, a conclusion that is consistent with the present findings where no clear relationship between protein and oil accumulation was apparent. In those studies where the sucrose concentration in the medium was >88 mM, oil levels remained in the range from 5% to 8% Dwt, and showed no correlation to C:N mole ratios (Fig. 3A). The oil levels observed in these experiments are in good agreement with the oil content of somatic embryos reported by other researchers (Schmidt *et al.*, 2005; Li *et al.*, 2010; He *et al.*, 2011). Although a linear regression line, derived from a plot of the oil and protein data, showing that a 1% reduction in oil content led to an apparent 1.82% increase in protein content, a value that was close to the 2% rule of thumb suggested for soybean by Clemente and Cahoon (2009), the correlation was very weak (Supplementary Fig. S4 at *JXB* online). Therefore, carbon that was in excess of that needed to synthesize protein, and for maintenance metabolism, did not go to oil synthesis but instead appeared to be directed to the starch and residual biomass pools. Therefore, in this model system, the capacity to synthesize oil appears to be fixed and is not influenced by the C:N mole ratios supplied. Examination of the data reported for soy zygotic embryos (Allen and Young, 2013) reveals that the oil content was static for the broad range of C:N ratios tested. This observation is in good agreement with the present results.

The present data provide evidence for the physiological role of starch accumulation in SHaM embryos. As observed in the carbon-limited regime, under medium conditions containing <88 mM sucrose, there was no starch detected in the embryos at harvest (Fig. 1B). At sucrose concentrations >88 mM, starch did accumulate, but protein synthesis appeared to be a more competitive sink for the available carbon pool. Therefore, starch appears to represent a sink for excess carbon after the biosynthesis of other storage products, such as protein. However, the results also indicate that starch does not serve as a temporary carbon storage reserve that can be readily used for the biosynthesis of oil, and are therefore in contrast to what has been reported for developing rape seed (Norton and Harris, 1975).

Soluble sugars such as sucrose, glucose, fructose, myo-inositol, raffinose, and stachyose are accumulated in developing soybean seeds (Wilson, 1995) and somatic embryos (Chanprame *et al.*, 1998). In the present study, sucrose in the medium in excess of that needed for growth had no dramatic effect on the soluble sugar content (Fig. 3C). The data agree with field studies of zygotic embryos (Hymowitz *et al.*, 1972; Wilcox and Shibles, 2001), where soluble sugars had a weak interaction or were not associated with seed yield or protein and oil content. Nonetheless, under carbon-limited conditions (0–44 mM, at constant glutamine concentration; 30 mM), the low sucrose concentrations did result in accumulation of soluble sugars in the tissues, with levels rising to >25%, on a Dwt basis (Fig. 1C). In addition to the increased soluble sugar content, the colour of the embryos was observed to change from light to darker green (data not shown). One possible explanation for this is that under severe carbon limitation, embryos synthesized their own sugars via photosynthesis. Photosynthesis is active in early phases of soybean seed development (Borisjuk *et al.*, 2005). The data indicate that the soluble sugar pools are highly regulated in embryonic tissues.

The weak correlation between oil and protein content in embryos suggested that oil and protein biosynthesis was independent at sucrose concentrations in excess of 88 mM. These results are in contrast to the observations under sucrose limitation (at 30 mM glutamine), as both protein and oil were inversely related and there was a complete absence of starch (Fig. 1B). These results may be explained by the role that sucrose plays in developing seeds. Sucrose is the primary translocated carbohydrate, and it plays an essential role in carbon partition for the biosynthesis of protein and oil (Smith *et al.*, 1989). As mentioned above, the starch level is strongly dependent on the sucrose concentration in the medium. More importantly, the SHaM embryos used in this investigation clearly demonstrated that medium compositions (in a range that does not adversely influence growth) had a relatively neutral influence on oil content, whereas protein and starch content were strongly influenced by C:N mole ratios in the medium. Indeed, cytosolic amino acid and protein biosynthesis were shown to be independent from plastid fatty acid synthesis in *Brassica napus* in mid-storage phase despite the potential exchange of shared metabolic intermediates between the two pathways (Schwender and Ohlrogge, 2002).

Supplementary Table S1 at *JXB* online shows comparisons between a published study focused on the influence of C:N ratios on assimilate partition in soybean zygotic embryos (Allen and Young, 2013) and the present SHaM data. The data clearly show that SHaM embryos respond to changes in C:N ratios in a similar manner to zygotic embryos and provide further support for their relevance as a model system for developing soybean seed. Furthermore, the experimental data reported in this study were highly reproducible, indicating the metabolic uniformity and growth characteristics of soybean somatic embryos cultured in SHaM media. Therefore, SHaM embryos, which, based on their composition and transcript profiling data (not shown), represent zygotic embryos in the

early to mid-storage phase of development, are attractive as a means to test genotype–phenotype relationships in transgenic research programmes. They are therefore an ideal test bed for the application of metabolic flux analysis studies designed to test hypotheses regarding carbon partitioning.

### *Conclusion*

The results from *in vitro* culture demonstrated that the relative growth rate, sucrose and glutamine consumption rates, and biomass composition of SHaM embryos were dictated by the concentration of carbon and organic nitrogen sources in the medium. A current understanding of the biochemistry of legumes is that plants create a homeostatic state in the developing seed by providing a constant supply of carbon from photosynthesis in the source tissues. When the carbon supply to the developing zygote is in excess of that required to drive growth and maintenance of metabolism, it is used for the biosynthesis of storage reserves. Overall, the results of these experiments revealed that protein biosynthesis was sensitive to decreasing C:N ratios in the culture medium. Conversely, starch and residual biomass content increased with increasing C:N mole ratios. Embryo oil content, however, appeared to be insensitive to the culture conditions. A better understanding of how carbon flux between protein and oil synthesis is regulated might lead to the development of molecular strategies to improve soybean seed quality. Metabolic flux analysis is an ideal experimental tool for studying such interactions as it not only enables the flux into various pools to be estimated but it also brings insight into the contributions and interactions of the various metabolic compartments. The data presented here inform on the culture conditions that should provide the greatest insights into the metabolic control points of assimilate partitioning in SHaM embryos and, by extension, developing soybean seeds.

## Supplementary data

Supplementary data are available at *JXB* online

Figure S1. (A) Relative growth rate ($d^{-1}$), (B) compositional analysis of protein, oil, and starch, and (C) soluble sugar and residual biomass content of soybean somatic embryos cv. Jack as a function of the initial glutamine concentrations in the SHaM media.

Figure S2. Correlation between media C:N mole ratios and (A) protein content, (B) starch content, and (C) residual biomass content of soybean somatic embryos. (D) Relationship between protein and residual biomass contents of soybean somatic embryos.

Figure S3. (A) Relative growth rate ($d^{-1}$), (B) compositional analysis of protein, oil, and starch, and (C) soluble sugar and residual biomass content of soybean somatic embryos cv. Jack as a function of the initial ammonium nitrate concentrations in the SHaM media.

Figure S4. Correlation analysis between oil and protein contents of soybean somatic embryos cultured in media with a range of C:N mole ratios.

Figure S5. Three-dimensional graph of glutamine consumption rates of soybean somatic embryos cultured in media containing different glutamine and sucrose concentrations.

Figure S6. Linear fit of the glutamine consumption rate versus the protein content of soybean somatic embryos cultured in media with a range of C:N mole ratios.

Table S1. Biomass composition comparisons between soybean zygotic and SHaM embryos cultured in media with a range of C:N ratios.

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