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*The Open Plant Science Journal,* **2013,** *7,* **10-16**



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# Are Raffinose and Stachyose Unloaded from Soybean Seed Coats to Developing Embryos?

Suzanne M. Kosina[1], Steven R. Schnebly[2] and Ralph L. Obendorf *[,1]

*[1]Seed Biology, Department of Crop and Soil Sciences, Cornell University, Ithaca, NY 14853, USA; [2]Pioneer Hi-Bred, A DuPont Business, 810 Sugar Grove Ave., Hwy44, Dallas Center, IA 50063, USA*

**Abstract:** During soybean [*Glycine max* (L.) Merrill] seed development, seed coat tissues contain sucrose, *myo*-inositol, D-*chiro*-inositol, D-pinitol and low concentrations of galactinol. Low concentrations of fagopyritol B1, galactopinitols, and raffinose also accumulate in seed coats during mid-maturation and stachyose accumulates late in maturation. Traces of raffinose can be detected in cotyledons of young seeds (24 days after pollination) and infrequently in seed coat cup exudates at mid-seed fill. On gas chromatograms, questionable peaks corresponding to the retention time of raffinose may be observed in seed coat cup exudates. To determine if raffinose and stachyose can be unloaded from seed coats into the free space surrounding developing seeds, soybean stem-leaf-pod explants from plants with low-raffinose, low-stachyose seeds (LRS) or normal raffinose and stachyose seeds (CHECK) were fed solutions containing 10 mM raffinose or 10 mM stachyose via the cut stem for 3 days. Raffinose was present in leaf, pod and seed coat tissues after feeding raffinose or stachyose to explants. Small amounts of raffinose were unloaded into seed coat cups. Stachyose accumulated in leaf and pod tissues after feeding stachyose to explants, but stachyose was detected in only one of the 32 seed coat exudates assayed. Soybean seed coats unloaded raffinose in very small amounts that may explain the presence of trace amounts of raffinose in embryo tissues of young seeds.

**Keywords:** *Glycine max* (L.) Merrill, stem-leaf-pod explants, raffinose, seed coat cup unloading, seed development, stachyose

## INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] leaf tissues contain the free cyclitols D-pinitol (1D-3-*O*-methyl-*chiro*-inositol), *myo*-inositol, D-*chiro*-inositol and D-ononitol (1D-4-*O*-methyl-*myo*-inositol), the non-reducing sugar sucrose, and the reducing sugars maltose, fructose, and glucose [1-3]. The raffinose family oligosaccharides (raffinose, stachyose, and verbascose; RFO), galactinol, galactopinitol A, fagopyritol B1, or their higher galactosyl oligomers generally are not detected in leaf blade tissues but are present in mature seeds. D-Pinitol concentrations in leaves commonly are variable among sampling dates and typically increase with plant water stress, upper positions on the plant, or increasing plant age [1, 2]. *myo*-Inositol, D-*chiro*-inositol, and D-ononitol typically decline as leaves age [3] from plant growth stage R2 (full bloom) to R6 (full seed) [4]. D-Ononitol, an intermediate in the conversion of *myo*-inositol to D-pinitol [5], is present in low amounts in younger leaves from plants at growth stages R2 and R3 (beginning pod) [3], but decreases, or may not be detected, in samples from older leaves from plants at growth stage R6 [1, 3]. Composition of soluble carbohydrates in leaf extracts differed little among four soybean lines [3] with low raffinose and stachyose (LRS) seeds expressing the mutant *stc1* phenotype [6-8], low raffinose, stachyose and phytin (LRSP1, LRSP2) seeds expressing the mutant *mips* phenotype [6, 7], or normal raffinose, stachyose and phytin (CHECK) seeds expressing the *Stc1* and *Mips* phenotype.

Seed coats from mature soybean seeds may have small amounts of sucrose, raffinose, stachyose, galactinol, galactopinitols, fagopyritols, and the free cyclitols *myo*-inositol, D-pinitol, and D-*chiro*-inositol [9]. Sucrose, *myo*-inositol, D-pinitol, and D-*chiro*-inositol in seed coat tissues decrease while galactopinitols, fagopyritols, and stachyose increase in seed coat tissues during seed maturation and desiccation [10]. Early in seed formation (24 days after flowering) traces of galactinol and raffinose may be observed in embryo tissues [10]; typically these compounds accumulate later during seed maturation and desiccation [10, 11]. The use of seed coat cups, formed by surgically removing the immature embryo from immature soybean seed forming an empty seed coat, has been a useful technique to study compounds unloaded from the seed coat into the apoplastic space surrounding the embryo [3, 12-16]. Sucrose (90% of C), amides (glutamine, 52% of N; asparagine, 19% of N) and amino acids are the most abundant compounds unloaded [13, 14]. D-Pinitol, D-*chiro*-inositol, *myo*-inositol, sucrose, maltose, fructose, and glucose are unloaded from seed coat cups *in planta* [3, 15]. In contrast to leaf extracts in which D-pinitol is double the concentration of sucrose [3], the rate of sucrose unloading from seed coat cups is much greater than the rate of D-pinitol unloading [3, 15]. Galactinol, raffinose, stachyose, verbascose, galactopinitols and fagopyritols are not detected in seed coat cup exudates. On gas chromatograms, questionable peaks (close to

*Address correspondence to this author Department of Crop and Soil Sciences, 617 Bradfield Hall, Cornell University, Ithaca, New York, USA 14853-1901; Tel: +1-607-227-9313; Fax: 1-607-255-2644;
Email: rlo1@cornell.edu

1874-2947/13 

background) corresponding to the retention time of raffinose are observed occasionally. Often, these occurrences are considered errors, most likely caused by inadvertent contamination from the surgically removed embryo tissues during the preparation of the seed coat cups [14].

The small amounts of raffinose and stachyose in seed coats of maturing seeds, the occasional detection of traces of galactinol and raffinose, and rarely galactopinitol or fagopyritol in young embryo tissues and the questionable peaks corresponding to the retention time of raffinose in seed coat cup exudates prompted us to test whether raffinose and/or stachyose can be unloaded from soybean seed coats to the developing embryo. Soybean stem-leaf-pod explants fed solutions containing free cyclitols have been used to demonstrate the accumulation of cyclitols and galactosyl cyclitols in seed coat and embryo tissues [15], whereas the unloading of sucrose, *myo*-inositol, D-pinitol, and D-*chiro*-inositol in seed coat cup exudates has been demonstrated [16]. The explant seed coat cups [16] were prepared as described for seed coat cup unloading *in planta* [3, 12-15]. In the current study, explant seed coat cups were used to evaluate raffinose and stachyose unloading. To assess the distribution of raffinose and stachyose and other soluble carbohydrate metabolites in maternal and embryo tissues, samples of leaf blade, pod wall, seed coat, cotyledon, and axis tissues from explants were analyzed in addition to unloaded compounds from seed coats. The compositions of the seed coat cup exudates and the maternal and embryonic tissues were determined after feeding solutions containing raffinose or stachyose to stem-leaf-pod explants from soybean plants with low raffinose and stachyose (LRS) seeds expressing the mutant *stc1* phenotype (reduced raffinose synthase activity [7]) or plants with normal raffinose and stachyose (CHECK) seeds expressing the *Stc1* phenotype (normal raffinose synthase activity [7]). Seed embryos on explants of the LRS line should have minimal *de novo* synthesis of raffinose compared to seed embryos on explants of the CHECK line. Therefore, a detection of raffinose accumulation in cotyledon tissues during feeding of LRS explants is more likely to be from a maternal or exogenously fed source rather than synthesized endogenously in the embryo.

## MATERIALS AND METHODOLOGY

### Plant Materials

Seeds for each of two proprietary soybean [*Glycine max* (L.) Merrill] lines with low raffinose and stachyose (LRS) seeds expressing the mutant *stc1* phenotype (reduced raffinose synthase activity [7]) and normal raffinose and stachyose (CHECK) seeds expressing the *Stc1* phenotype (normal raffinose synthase activity [7]) were provided by Steve Schnebly, Pioneer Hi-Bred. Both are advanced breeding lines in related, but not isogenic, Group II maturity agronomic backgrounds developed by traditional breeding. The *stc1* allele in the breeding line utilized in this study has been described [6-8].

### Explant Feeding

Stem-leaf-pod explants [15, 16] were excised from the central part of the main stem of greenhouse grown plants at growth stage R5 (beginning seed) [4]. Each explant included one internode (stem), one node, one leaf, and one pod with three immature seeds (280–300 mg fresh weight each; about 35 days after pollination; at mid-seed fill before accumulation of RFOs, fagopyritols, and galactopinitols). The cut stem of one explant was placed in a 125-mL Erlenmeyer flask containing a 100-mL solution of 10 mM raffinose or 10 mM stachyose or a control solution without raffinose or stachyose; all solutions contained 10 mM sucrose, 10 mM asparagine, and 10 μM kinetin. Each solution was fed to the cut stem of explants for 3 days at 25°C under light (300 μmol $m^{-2}$ $s^{-1}$ PAR, fluorescent). The experiment used two explants per treatment for each line.

### Seed Coat Cup Unloading

To assess unloading after 3 days of feeding, the immature embryo was surgically removed from the central seed forming an empty seed coat cup [12, 14, 15] on explants [16]. Empty seed coat cups were rinsed two times with distilled water to remove cotyledon, axis, pod, and seed coat residues remaining after the excision process [14]. Because buffers, salts and mannitol [12] interfered with the derivatization and analysis of soluble carbohydrates by gas chromatography [3], unloaded compounds were collected in water [3]. Unloaded compounds were collected over four sequential 30-minute periods in 200 μL of distilled water [12] which was replaced at each 30-minute time point. An equal volume of ethanol and 50 μg of phenyl α-D-glucoside as internal standard were added to each sample. Exudates were passed through a 10,000 MW cutoff filter (Nanosep 10K Omega, Pall Life Sciences, Ann Arbor, Michigan, USA) by centrifugation at 14,000 x *g* for 20 min.

### Plant Tissue Collection and Soluble Carbohydrate Extraction

After collection of seed coat cup unloading samples, three 1-$cm^2$ leaf punches, a section of pod wall (about 1 $cm^2$) adjacent to the proximal seed, and the axis, cotyledons, and seed coat of the proximal seed from the same pod used for the seed coat cup were harvested for analysis of soluble carbohydrates. Distal seed and pod tissues were used to estimate the water concentrations for calculating sample dry weights. All tissues were frozen in liquid nitrogen and pulverized to a fine powder with a mortar and pestle. Frozen powder of leaf, axis or seed coat tissues were extracted with 1000 μL (leaf), 800 μL (axis), or 1100 μL (seed coat) of ethanol:water (1:1, v/v) containing 100 μg of phenyl α-D-glucoside as internal standard using a ground glass homogenizer. Cotyledons were extracted in 2300 μL of ethanol:water (1:1, v/v) containing 300 μg of phenyl α-D-glucoside. Extracts were centrifuged at 14,000 x *g* for 20 min. Supernatants (500 μL) were passed through 10,000 MW cutoff filters (Nanosep 10K Omega, Pall Life Sciences, Ann Arbor, Michigan, USA) by centrifugation (14,000 x *g*).

### Analysis of Soluble Carbohydrates

Filtrates from plant tissues (200 μL for leaf, pod wall, cotyledon and seed coat extracts; 400 μL for axis extracts) and seed coat cup exudates were dried in silylation vials under a stream of nitrogen gas and stored overnight above

**Table 1. Leaf soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatments | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | soluble carbohydrate (μg $cm^{-2}$) | | | | | |
| D-Pinitol | 13.73 a | 15.59 a | 20.46 a | 12.52 a | 15.2 ± 1.6 | 64.3 ± 7.4 |
| D-*chiro*-Inositol | 1.32 a | 2.55 a | 1.85 a | 2.68 a | 2.1 ± 0.3 | 7.6 ± 0.8 |
| *myo*-Inositol* | 1.43 a | 1.28 a | 3.04 a* | 4.83 a* | 2.6 ± 0.6 | 8.9 ± 2.3 |
| Sucrose* | 21.52 a | 29.57 a | 60.18 a* | 56.86 a* | 42 ± 7 | 68 ± 17 |
| Galactinol | 0.03 a | 0.03 a | 0.10 a | 0.07 a | 0.05± 0.01 | 0.4 ± 0.2 |
| Raffinose | 0.81 b | 0.71 b | 10.96 a | 12.12 a | 6.1 ± 2.1 | 1.5 ± 1.0 |
| Stachyose | 0.00 b | 0.15 b | 116.70 a | 92.73 a | 52 ± 20 | 0.0 ± 0.0 |
| Verbascose | 0.00 b | 0.00 b | 3.00 a | 2.80 a | 1.5 ± 0.6 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of leaf punches from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for three days. For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. * = pooled means (CHECK and LRS) for explants fed stachyose are significantly greater ($P < 0.05$) than pooled means (CHECK and LRS) for explants fed raffinose after applying Student's t-test. The treatment grand mean ± SE (n=8, pooled across the four experimental treatments) and the control mean ± SE (n=2, pooled across the controls) are shown in columns on the right.

**Table 2. Pod wall soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatments | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | soluble carbohydrate (μg $g^{-1}$ DW) | | | | | |
| D-Pinitol | 42.45 a | 39.79 a | 37.98 a | 38.91 a | 39.8 ± 1.4 | 26.0 ± 7.5 |
| D-*chiro*-Inositol* | 0.53 a | 1.11 a* | 0.52 a | 1.16 a* | 0.8 ± 0.1 | 1.3 ± 0.9 |
| *myo*-Inositol | 0.38 a | 0.50 a | 0.30 a | 0.44 a | 0.4 ± 0.04 | 0.2 ± 0.02 |
| Sucrose | 3.35 a | 3.23 a | 2.70 a | 3.35 a | 3.2 ± 0.5 | 3.1 ± 2.2 |
| Galactinol | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Raffinose | 0.17 a | 0.51 a | 0.56 a | 0.65 a | 0.5 ± 0.1 | 0.0 ± 0.0 |
| Stachyose | 0.00 b | 0.13 b | 3.74 ab | 5.97 a | 2.5 ± 1.0 | 0.0 ± 0.0 |
| Verbascose | 0.00 a | 0.00 a | 0.00 a | 0.45 a | 0.1 ± 0.1 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of pod wall tissue from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for 3 days. For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. * = pooled means (across raffinose and stachyose feeding treatments) for LRS are significantly greater ($P < 0.05$) than pooled means (across raffinose and stachyose feeding treatments) for CHECK after applying Student's t-test. The treatment grand mean ± SE (n=8, pooled across the four experimental treatments) and the control mean ± SE (n=2, pooled across the controls) are shown in columns on the right.

$P_2O_5$ in desiccators to remove traces of water. Soluble carbohydrate dry residues were derivatized with trimethylsilylimidazole:pyridine (1:1, v/v; 200 μL) for 45 min at 80°C and analyzed by high resolution gas chromatography [11, 15]. Values below the level of detection were presented as zero. Soluble carbohydrate values are compared as μg per g dry weight for seed parts and pod wall, μg per $cm^2$ for leaf tissues, and μg per hour for seed coat cup exudates. Statistical analyses (ANOVA) were performed using a square root transformation of the response to correct for non-constant residual variance. Soluble carbohydrate values from explants fed raffinose and stachyose are represented by the mean of two replicates (n = 2 for tissue parts; n = 8 for seed coat cup exudates, 2 explants by 4 unloading periods, mean of 120 min). For each soluble carbohydrate, means not connected by the same letter are significantly different ($P < 0.05$) between feeding treatments and lines after applying a Tukey correction for multiple comparisons (JMP Statistical Discovery Software, SAS Institute Inc., Cary, NC, USA). Due to a limited number of explants, control treatments were conducted in a separate experiment using the same procedures (n = 2); therefore, a direct statistical comparison to raffinose and stachyose feeding treatments was not valid. For explants fed a control solution for 3 days, values are represented by the pooled mean ± the standard error of the mean (S.E.) of

**Table 3. Seed coat soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatments | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | soluble carbohydrate (μg $g^{-1}$ DW) | | | | | |
| D-Pinitol | 11.41 a | 12.51 a | 10.36 a | 6.38 a | 10.1 ± 1.3 | 10.4 ± 4.0 |
| D-*chiro*-Inositol | 2.25 a | 3.13 a | 2.45 a | 2.00 a | 2.4 ± 0.2 | 2.0 ± 0.5 |
| *myo*-Inositol | 6.98 a | 7.42 a | 6.57 a | 4.89 a | 6.5 ± 0.6 | 3.6 ± 0.8 |
| Sucrose | 257 a | 238 a | 236 a | 147 a | 219 ±22 | 45.3 ± 8.3 |
| Galactinol | 0.00 a | 0.34 a | 0.00 a | 0.42 a | 0.2 ± 0.1 | 0.0 ± 0.0 |
| Raffinose | 3.53 a | 2.14 a | 4.50 a | 2.34 a | 3.1 ± 0.7 | 0.0 ± 0.0 |
| Stachyose | 0.00 a | 0.00 a | 0.00 a | 0.61 a | 0.2 ± 0.2 | 0.0 ± 0.0 |
| Verbascose | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of seed coat tissue from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for 3 days. For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. The treatment grand mean ± SE (n=8, pooled across the four experimental treatments) and the control mean ± SE (n=2, pooled across the controls) are shown in columns on the right.

**Table 4. Seed coat cup unloaded soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatments | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | seed coat cup unloading rate (μg $h^{-1}$) | | | | | |
| D-Pinitol | 5.15 ab | 5.39 ab | 8.64 a | 4.85 b | 6.0 ± 0.5 | 7.5 ± 5.3 |
| D-*chiro*-Inositol | 0.46 a | 0.79 a | 1.06 a | 0.80 a | 0.8 ± 0.1 | 3.2 ± 0.4 |
| *myo*-Inositol | 2.43 b | 2.74 ab | 4.29 a | 2.21 b | 2.9 ± 0.3 | 3.0 ± 0.7 |
| Sucrose | 148 b | 283 a | 159 b | 194 b | 195 ±14 | 182 ±25 |
| Galactinol | 0.28 a | 0.09 a | 0.14 a | 0.15 a | 0.3 ± 0.1 | 0.0 ± 0.0 |
| Raffinose | 0.17 a | 0.32 a | 0.21 a | 0.19 a | 0.2 ± 0.1 | 0.0 ± 0.0 |
| Stachyose* | 0.00 a | 0.00 a | 0.00 a | 0.88 a | 0.2 ± 0.2 | 0.0 ± 0.0 |
| Verbascose | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of seed coat cup exudates from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for three days. For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. *Stachyose was detected in only one of the 32 seed coat cup exudates assayed. The treatment grand mean ± SE (n=32, pooled across the four experimental treatments) and the control mean ± SE (n=8, pooled across the controls) are shown in columns on the right.

CHECK and LRS explants. Also, values for the four experimental treatments (stachyose fed LRS, stachyose fed CHECK, raffinose fed LRS, and raffinose fed CHECK) have been pooled and are represented as the grand mean of treatments ± S.E. (n=8).

## RESULTS

The presence of sucrose, *myo*-inositol, D-pinitol, and D-*chiro*-inositol in soybean maternal and seed tissues is normal and expected. These compounds are used as substrates in seeds for the synthesis and accumulation of raffinose family oligosaccharides and galactosyl cyclitols. The natural unloading of sucrose, *myo*-inositol, D-pinitol, and D-*chiro*-inositol in seed coat cup exudates has been demonstrated both in planta [3, 15] and on stem-leaf-pod explants [16] and represents a functional seed coat cup. Explants not unloading sucrose, *myo*-inositol, D-pinitol, and D-*chiro*-inositol in seed coat cup exudates represent damaged or non-functional seed coat cups or explants and therefore were eliminated from analysis. Galactinol (the galactosyl donor for synthesis of raffinose, stachyose, and verbascose), raffinose, stachyose, and verbascose are compounds naturally formed and accumulated in soybean seeds [17, 18], but these compounds are not normally detected in seed coat cup exudates [3, 15, 16]. The detection of galactinol, raffinose, and stachyose in seed coat exudates represents new information.

Seed biomass growth rates on soybean stem-leaf-pod explants are comparable to those on intact plants, demonstrating the validity of using stem-leaf-pod explants in this study. *In planta* seed growth rates are 5 mg dry weight

**Table 5. Cotyledon soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatments | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | soluble carbohydrate (μg $g^{-1}$ DW) | | | | | |
| D-Pinitol* | 11.15 a* | 9.99 a* | 7.08 a | 7.80 a | 9.0 ± 0.7 | 11.3 ± 0.9 |
| D-*chiro*-Inositol | 2.38 a | 2.12 a | 1.86 a | 1.69 a | 2.0 ± 0.2 | 3.4 ± 1.0 |
| *myo*-Inositol | 6.86 a | 7.85 a | 6.72 a | 6.89 a | 7.0 ± 0.3 | 3.2 ± 0.1 |
| Sucrose | 175 a | 166 a | 235 a | 214 a | 198 ±18 | 60 ± 31 |
| Galactinol | 0.11 a | 0.12 a | 0.16 a | 0.00 b | 0.09 ± 0.02 | 0.0 ± 0.0 |
| Raffinose | 0.15 ab | 0.00 b | 0.17 a | 0.00 b | 0.08 ± 0.03 | 0.0 ± 0.0 |
| Stachyose | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Verbascose | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of cotyledon tissue from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for three days For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. * = pooled means (CHECK and LRS) for explants fed raffinose are significantly greater ($P < 0.05$) than pooled means (CHECK and LRS) for explants fed stachyose after applying Student's t-test. The treatment grand mean ± SE (n=8, pooled across the four experimental treatments) and the control mean ± SE (n=2, pooled across the controls) are shown in columns on the right.

**Table 6. Axis soluble carbohydrates**

| Soluble carbohydrate | Explants fed raffinose | | Explants fed stachyose | | Treatment | Control |
|---|---|---|---|---|---|---|
| | CHECK | LRS | CHECK | LRS | Mean ± SE | Mean ± SE |
| | | | | | | |
| | soluble carbohydrate (μg $g^{-1}$ DW) | | | | | |
| D-Pinitol | 14.84 a | 19.19 a | 23.96 a | 20.30 a | 19.6 ± 1.6 | 21.1 ± 7.3 |
| D-*chiro*-Inositol | 1.93 a | 2.46 a | 4.24 a | 2.93 a | 2.9 ± 0.4 | 6.8 ± 2.9 |
| *myo*-Inositol | 6.34 a | 8.47 a | 6.55 a | 6.64 a | 7.0 ± 0.4 | 6.4 ± 3.3 |
| Sucrose | 425 a | 476 a | 436 a | 297 a | 408 ±29 | 250 ± 118 |
| Galactinol | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Raffinose | 0.35 a | 0.00 a | 0.00 a | 0.00 a | 0.08 ± 0.08 | 0.0 ± 0.0 |
| Stachyose | 4.73 a | 0.00 a | 0.00 a | 0.00 a | 1.2 ± 1.2 | 0.0 ± 0.0 |
| Verbascose | 0.00 a | 0.00 a | 0.00 a | 0.00 a | 0.0 ± 0.0 | 0.0 ± 0.0 |

Soluble carbohydrate analysis of axis tissue from soybean leaf-stem-pod explants of CHECK and LRS lines after feeding raffinose, stachyose or control solution for three days. For comparisons between columns (four feeding treatments) within a row, means not linked by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple means comparisons. The treatment grand mean ± SE (n=8, pooled across the four experimental treatments) and the control mean ± SE (n=2, pooled across the controls) are shown in columns on the right.

per seed per day in the field and 8 mg dry weight per seed per day in the greenhouse [19]. In soybean stem-leaf-pod explants, two cotyledons accumulated 40 to 70 mg dry weight during 7 days of feeding with four different treatment solutions (5-10 mg dry weight per seed per day) plus a drying period [15], representing seed growth rates that are comparable to those on intact plants of the same cultivar.

Raffinose was detected in leaf blade, pod wall, and seed coat tissues and seed coat cup exudates after feeding raffinose or stachyose to both LRS and CHECK stem-leaf-pod explants (Tables **1**-**4)**. Except in leaf tissues, raffinose was not detected in any of these tissues after feeding control solutions without raffinose or stachyose (Tables **1**-**4**). Stachyose was detected in leaf blade and pod wall tissues for LRS explants fed raffinose and for CHECK and LRS explants fed stachyose (Tables **1** and **2**); stachyose was not detected in the maternal tissues of CHECK explants fed raffinose (Tables **1**-**3**). Stachyose was present in the seed coat of LRS explants fed stachyose (Table **3**), and the stachyose concentration was significantly higher in LRS pod wall following stachyose feeding *versus* raffinose feeding (Table **2**). Verbascose was

detected only after stachyose feeding in LRS and CHECK leaf tissue (Table **1**) and in LRS pod wall (Table **2**); verbascose was not detected in the seed coat nor unloaded from the seed coat in either line (Tables **3** and **4**). Interestingly, the raffinose, stachyose, and verbascose concentrations in both CHECK and LRS leaf tissues were significantly higher after stachyose feeding than after raffinose feeding (Table **1**). For all three of these analyzed RFOs, unloading was consistent with the presence of the compound in the seed coat but not in other maternal tissues. To check for accumulation of raffinose or stachyose in embryo tissues, cotyledon and axis samples from the immature proximal seeds were assayed from the same pod sampled for seed coat cup exudates in the middle seed position. Stachyose was not detected in CHECK or LRS cotyledons of the proximal seed after feeding stachyose or raffinose to explants, but raffinose was detected in CHECK cotyledons after both raffinose and stachyose feedings (Table **5**). Although raffinose and stachyose were detected in CHECK axes after feeding raffinose to explants, they were detected in only one of the replications and the amounts were not statistically different from samples with no detectable raffinose (Table **6**). Seed coat cup unloading of a specific compound was not always consistent with the detectable presence of that compound in immature fresh embryo tissues.

D-Pinitol, D-*chiro*-inositol, *myo*-inositol and sucrose were present in leaf, pod wall, seed coat, cotyledon, and axis tissues and also unloaded by seed coat cups of stem-leaf-pod explants of both lines and all feeding treatments (Tables **1**-**6**). For control treatments, galactinol was only detected in the leaf punch tissue whereas for feeding treatments, galactinol was detected in leaf punch tissue, LRS seed coat, cotyledons (except in LRS cotyledons after stachyose feeding), and in seed coat cup unloading exudates (Tables **1**-**6**). Leaves of explants fed stachyose had higher concentrations of *myo*-inositol and sucrose than leaves of explants fed raffinose when data were pooled across lines (Table **1**). D-*chiro*-Inositol concentrations were higher in pod walls of LRS explants than CHECK explants when data were pooled across feeding treatments (Table **2**). The seed coat cup sucrose unloading rate was highest for LRS explants fed raffinose (Table **4**). Of explants fed stachyose, D-pinitol unloading was higher in CHECK than LRS, while *myo*-inositol unloading was higher in CHECK explants fed stachyose than in CHECK explants fed raffinose or LRS explants fed stachyose (Table **4**). D-Pinitol concentrations were higher in cotyledons of explants fed raffinose compared to explants fed stachyose when data were pooled across lines (Table **5**).

## DISCUSSION AND CONCLUSION

Soybean seeds accumulate about 15% of their dry weight as soluble carbohydrates in maturing embryo tissues (cotyledons, axis), primarily sucrose and α-galactosides of sucrose (RFO; raffinose, stachyose, verbascose) with smaller amounts of galactosyl cyclitols, α-galactosides of *myo*-inositol, D-pinitol, and D-*chiro*-inositol (galactinol, galactopinitols, fagopyritol B1, respectively) [9, 10]. Sucrose accumulates during seed growth while >70% of RFO accumulate after physiological maturity (maximum seed dry weight) during the desiccation phase of seed maturation [10]. However, traces of raffinose have been detected in immature embryos (24 days after flowering) of young soybean seeds [10]. RFO are proposed to be one factor contributing to desiccation tolerance in seeds [17]. Unfortunately, RFO are not efficiently utilized by humans and non-ruminant animals [6]. Therefore, raffinose and stachyose in soybean seeds has been reduced genetically by the mutant *stc1* gene to improve the feed value of soybean products [6, 7] without a reduction in field emergence or agronomic performance [8].

Sucrose is the major carbon source unloaded from seed coats to developing embryos [12, 13] while amides (glutamine, asparagine) and amino acids are the major nitrogen source unloaded from seed coats to developing embryos [13]. D-Pinitol, *myo*-inositol and D-*chiro*-inositol also are unloaded from seed coats to developing embryos [3, 14, 15] where they are stored primarily as their respective α-galactosides [9-11, 15, 17, 18]. Sucrose accumulates in seed coat tissues during early to mid-maturation, low concentrations of fagopyritol B1, galactopinitols, and raffinose accumulate in seed coat tissues during mid-maturation, and stachyose accumulates in seed coat tissues during late seed maturation [10]. The experiment described herein assayed for the presence of raffinose and stachyose in seed coat cup exudates after feeding solutions containing raffinose or stachyose to stem-leaf-pod explants.

There were few consistent differences across the tissue types between the lines and treatments for *myo*-inositol, D-pinitol, D-*chiro*-inositol, sucrose and galactinol. The differences may reflect the plant's response to uptaking high concentrations of raffinose and/or stachyose and may involve passive and active transport systems and effects on osmotic balance resulting in potentially altered transpiration and/or translocation. Plants fed control solutions had no detectable raffinose or stachyose in maternal tissues, seed coat cup exudates or embryo tissues (except for raffinose detection in leaf tissue). The results are consistent with the interpretation that raffinose and stachyose fed to the cut stem of soybean stem-leaf-pod explants may be transported via the transpiration stream to leaf blade or to pod wall tissues accounting for their accumulation in these maternal tissues. Whether or not stachyose can reach the seed coat and unload to the apoplastic space is uncertain given the low occurrence in these experiments (1/8 seed coats and 1/32 unloading periods, both occurring in LRS explants fed stachyose); raffinose, on the other hand, accumulated in the seed coat and was detected in seed coat cup unloading exudates. The presence of raffinose following stachyose feeding may indicate hydrolysis of stachyose is occurring early after uptake by explants. In the leaf tissue, the raffinose concentrations are actually higher after stachyose feeding than after raffinose feeding, whereas for pod wall, seed coat and unloading rates, the raffinose concentrations are the same after both raffinose and stachyose feeding treatments. In addition, the detection of raffinose in CHECK cotyledons after feeding either raffinose or stachyose and in CHECK axis after feeding raffinose supports the hypothesis that the trace amounts of raffinose detected in young embryos [10] may be due to the unloading of raffinose from the seed coat and not to embryo tissue contamination.

It is concluded that small amounts of raffinose may be unloaded from CHECK and LRS line soybean seed coats

with potential to be taken up by CHECK line embryos during seed development and maturation. Further, raffinose unloading may account for trace amounts of raffinose reported in embryo tissues from young soybean seeds whereas it does not appear that stachyose is as readily unloaded by seed coats or taken up by embryo tissues. The difference in raffinose uptake by CHECK and LRS embryos warrants further investigation.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

## ACKNOWLEDGEMENTS

This research was conducted as part of Multistate Projects W-1168 and W-2168 and supported in part by Morley and Hatch-Multistate Undergraduate Research Grants (S.M.K.), a President's Council for Cornell Women Undergraduate Research Grant (S.M.K.), and Cornell University Agricultural Experiment Station federal formula funds, Project No. NYC-125802 and NYC-125852, received from the National Institutes for Food and Agriculture (NIFA), U.S. Department of Agriculture. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the U.S. Department of Agriculture. Some of the results were included in a Biology Undergraduate Research Honors Thesis (Cornell University, May 2007) by S.M.K. A preliminary report has been published as a conference abstract: Obendorf RL, Kosina SM, Schnebly SR. Are raffinose and stachyose unloaded from soybean seed coats to developing embryos? Polish J Nat Sci 2008; (suppl. 5/2008): Book of Abstracts, 34 (ISSN 1643-9953). We gratefully acknowledge Françoise Vermeylen for advice on statistical analysis.

## ABBREVIATIONS

CHECK = Normal raffinose, stachyose, and phytin seed phenotype

LRS = Low raffinose and stachyose seed phenotype

LRSP1 and LRSP2 = Low raffinose, stachyose and phytin seed phenotype

*Mips* = Mutant form of *Mips* gene (Gm mI 1-PS-1A, AY038802)

MIPS = *myo*-Inositol-phosphate synthase (EC 5.5.1.4)

RFO = Raffinose family oligosaccharides

*stc1* = Mutant form of *Stc1* gene conveying reduced raffinose synthase (EC 2.4.1.82) activity in seeds

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Received: July 19, 2012 Revised: September 19, 2012 Accepted: October 24, 2012