



OCCURRENCE OF PINITOL IN DEVELOPING SOYBEAN SEED TISSUES

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Abstract—Pinitol (1D-3O-methyl-*chiro*-inositol) was found to be a major cyclitol present in developing soybean (*Glycine max*) seed tissues. Pinitol was isolated by HPLC and identified via a combination of gas-liquid chromatography and mass spectral fragmentation comparisons with authentic pinitol. Changes in the concentration of pinitol were compared to those of *myo*-inositol, starch and raffinose saccharides in seed coats, cotyledons and embryonic axes of field-grown soybeans during seed development. In all three tissues, as seeds were progressing toward maturity, there were strong correlations between the decrease of *myo*-inositol and starch and the increase of raffinose saccharides. Pinitol was correlated negatively with raffinose saccharides in both seed coats and axes, but this correlation was insignificant in cotyledons. Pinitol concentration in all tissues fluctuated more than other components during seed development, and it decreased sharply as axes turned yellow and rapidly accumulated raffinose saccharides. The results suggest that pinitol may represent a transient component of the carbon pool in soybean seed tissues during development toward physiological maturity. Published by Elsevier Science Ltd

INTRODUCTION

D-Pinitol (1D-3O-methyl-*chiro*-inositol) and *myo*-inositol are major cyclitols in plants. In soybeans, pinitol has been found in various tissues, including leaf blades, petioles, stems, roots, nodules and seeds [1–7]. Mature, dry soybean seeds contain 6–17% of soluble carbohydrates [8], predominantly sucrose and raffinose saccharides (raffinose, stachyose and trace amounts of verbascose), and 0.6–0.9% pinitol [3]. In an extensive survey for the presence of cyclitols in various dry seeds, Horbowicz and Obendorf [7] found that D-pinitol was the predominant cyclitol in Chip-pewa 64 soybeans at about 0.34 and 0.37% in the axis and cotyledon, respectively, followed by *myo*-inositol (0.08% in axes, 0.02% in cotyledons) and *chiro*-inositol (0.05% in axes, 0.02% in cotyledons). Although pinitol is the major cyclitol in soybean seeds, it is not as abundant as *myo*-inositol in most other plant seeds. *myo*-Inositol is present in all dry seeds examined [7].

In the final stages of soybean seed development, carbon partitioning shifts from the production of protein and oil to the synthesis of soluble carbohydrates, particularly two raffinose saccharides, stachyose and raffinose [9–12]. The initial synthesis of raffinose saccharides begins with a galactosyl transfer from galactinol to sucrose to form raffinose [13]. Galactinol is formed by transferring the galactose moiety from UDP-galactose to *myo*-inositol as catalysed by galactinol synthase [14]. The role of *myo*-inositol is thus well established, aside from other complex metabolic functions [15], to serve as a key cofactor in the biosynthesis of raffinose saccharides [13, 16, 17]. Pinitol is formed directly by the methylation of *myo*-inositol, and subsequent epimerization of sequoyitol [18] or D-ononitol [19]. Therefore, metabolism of pinitol may also directly affect the accumulation of raffinose saccharides in plant tissues. Despite the potentially important interactions among pinitol, *myo*-inositol and raffinose saccharides, however, there is very limited information available on the occurrence of these cyclitols in relation to the accumulation of raffinose saccharides during seed development.

In a previous study, we reported that the level of *myo*-inositol in developing soybean seeds increased

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linearly during seed fill and then decreased substantially as seeds rapidly accumulated raffinose saccharides [12]. However, we failed to recognize the presence of pinitol. The objectives of this work were to determine the distribution of pinitol, along with *myo*-inositol, in different seed tissues during development. Since starch accumulated in immature seeds was thought to serve as a storage reserve also for the formation of stachyose [9], analysis of starch was included to establish the potential relationships with changes in the concentration of pinitol, *myo*-inositol and raffinose saccharides in different developing seed tissues. This basic information should be valuable for future research directed to elucidate the metabolism and function of these cyclitols in the formation of raffinose saccharides in developing soybean seeds.

RESULTS AND DISCUSSION

Isolation and identification of putative pinitol

The practical advantages of using high-performance liquid chromatography (HPLC) for the separation and analysis of cyclitols and their derivatives were discussed by Loewus [20]. We have used Sugar-Pak I* column (a Ca^{2+} -loaded cation-exchange resin) in a HPLC system for routine analyses of soluble carbohydrates in plant seeds. However, we began to question the identity of an eluted component, previously thought to be glucose (Fig. 1, peak 7), because of the following observations. This component was constantly present in soybean seeds in the mature, dry stage [21], during seed development [12], and at similar levels in cotyledons during early germination [22]. For unambiguous identification we isolated it from a commercial soy flour, in order to circumvent the tedious grinding and defatting steps needed to process mature Williams 82 seeds, which also had very limited supply from the experiment. Nutrisoy 7B was chosen because it was processed from commercial soybeans (derived from a cross of 'Williams' $\times$ 'Essex') that shared a common genetic background with Williams 82. The soluble carbohydrate fraction of both Nutrisoy 7B and Williams 82 exhibited a very similar, if not identical, HPLC profile (Fig. 1). The same HPLC procedure successfully used to isolate sorbitol in germinating soybean axes [22] was also applied to isolate this component. In the quantitation of carbohydrate components in seed tissue extracts, however, an evaporative light scattering detector (ELSD) was chosen over the previously used refractometer to monitor the HPLC elutant because the latter could not always

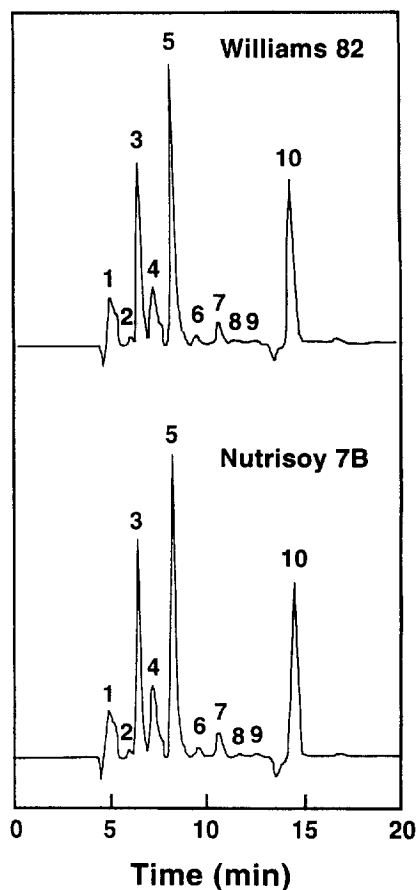


Fig. 1. HPLC separation of soluble carbohydrates extracted from mature soybean [*Glycine max* cv. Williams 82] seeds (top) and Nutrisoy 7B (bottom), as monitored by a differential refractometer. The eluted components were identified, based on the retention time of authentic standards. Mannitol was added to each sample as an internal standard. 1, unknown; 2, verbascose; 3, stachyose; 4, raffinose; 5, sucrose; 6, galactinol; 7, pinitol, glucose; 8, galactose; 9, fructose; 10, mannitol. During quantitation of soluble carbohydrates using an ELSD monitor, *myo*-inositol was eluted in the area between fructose and mannitol.

provide a smooth baseline, especially in the vicinity of *myo*-inositol elution (between peaks 9 and 10, Fig. 1).

In searching for proper references to use for identification, our attention was drawn to the reports that a substantial quantity of pinitol was also present in mature, dry soybean seeds [3] as well as soy flakes [23, 24]. Subsequent HPLC analyses revealed that the isolated material co-migrated with authentic pinitol and had a retention time similar to glucose. To ascertain the identity of pinitol, the isolated material was assayed enzymically for glucose and also subjected to GC-MS analyses. The isolated peak material showed high purity on GC (Fig. 2, top), and it contained <1.2% (w/w) glucose as analysed by the glucose oxidase assay as described in the Experimental. Moreover, the peracetylated compound had identical GC elution time and mass spectrum to those of the authentic pinitol peracetylated derivative (Fig. 2), but

*Names are necessary to report factually on available data, however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

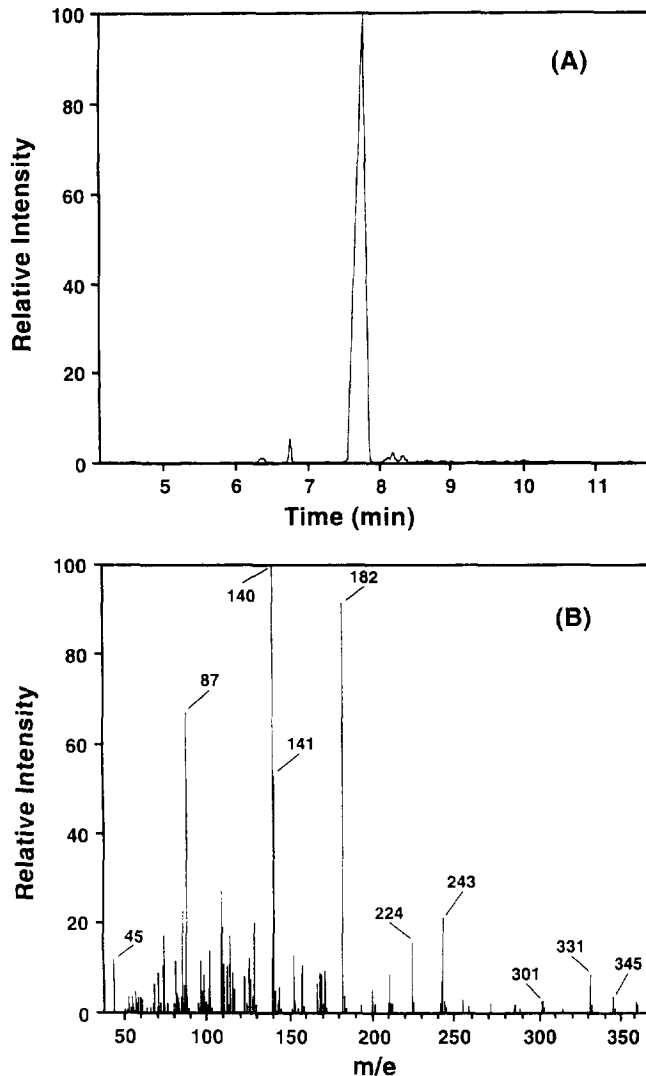


Fig. 2. GC elution (A) and mass spectrum (B) of acetylated pinitol isolated from Nutrisoy 7B. The authentic pinitol had same GC retention time and mass spectrum. Using the same GC-MS procedure, pinitol was identified to be a major component in the soluble carbohydrate fraction of seed coats, cotyledons, and embryonic axes of developing soybeans, each having the same mass spectrum and relative retention time as that of authentic pinitol.

different from the peracetylated glucose. HPLC and GC-MS analyses also showed that the isolated compound was not quebrachitol or sequoyitol, isomers of pinitol (data not shown). GC-MS analyses also confirmed the HPLC analysis that substantial amounts of pinitol present in the soluble carbohydrate fractions of different seed tissues during linear seed fill stage (R5.5) and at the beginning of maturity stage (R6.5). All samples examined in this study contained only trace or small amounts of glucose as analysed enzymically (0.9–4.1, 0.1–0.4 and 0.1–0.6 mg g dry wt⁻¹ for seed coats, cotyledons, and axes, respectively). Thus, pinitol is not only present in mature seeds (R7), but also in immature seed tissues during development. Although pinitol in soybean flakes and plant parts has been identified via GC-MS by its trimethylsilylated derivatives [1, 4, 24], the basic procedure as described by Seymour *et al.* [25] that was used in this study has

proven to be an useful alternative for analysing pinitol and *myo*-inositol in soybean seed tissues. Under the specified GC conditions [22], the methyl silicone capillary column was able to provide a good separation for peracetylated pinitol, *myo*-inositol and glucose.

Changes in levels of soluble carbohydrates and starch in soybean seed tissues during development

The concentrations of examined soluble carbohydrates including pinitol, *myo*-inositol and raffinose saccharides, and of starch in three different tissues of five growth stages of soybean seeds are given in Table 1. Both pinitol and *myo*-inositol accumulated in all three tissues at each growth stage with concentrations generally higher in seed coats and cotyledons than in axes. These two cyclitols were present as major non-structural carbohydrates in all seed tissues as imma-

Table 1. Cyclitol, starch, and raffinose saccharide concentrations in the seed coat, cotyledon and embryonic axis of soybeans grown from linear seed fill to physiological maturity. The description of five growth stages of field-grown Williams 82 seeds is given in the experimental. The concentration of each measured component is expressed as mg g⁻¹ dry wt seed tissue. Values are the means of triplicate samplings of seed coats and cotyledons, and duplicate samplings for embryonic axes

	Growth stage	Seed coat	Cotyledon	Axis
Pinitol	R5.5	20.9 ^b	22.7 ^a	17.6 ^c
	R6	18.5 ^a	13.3 ^b	14.3 ^b
	R6.2	6.8 ^a	4.1 ^b	0.4 ^c
	R6.5	12.3 ^b	27.9 ^a	10.4 ^c
	R7	9.4 ^a	2.3 ^b	3.5 ^b
<i>myo</i> -Inositol	R5.5	9.4 ^c	15.5 ^a	12.3 ^b
	R6	5.9 ^c	8.0 ^a	6.9 ^b
	R6.2	3.2 ^a	3.3 ^a	0.3 ^b
	R6.5	2.9 ^b	3.8 ^a	1.0 ^c
	R7	0.6 ^a	0.1 ^b	0 ^b
Starch	R5.5	3.1 ^c	18.0 ^b	21.7 ^a
	R6	1.7 ^c	16.9 ^b	23.0 ^a
	R6.2	0.8 ^c	15.4 ^a	11.2 ^b
	R6.5	0.5 ^b	12.4 ^a	11.3 ^a
	R7	0.2	1.8	1.5
Raffinose saccharide	R5.5	0.5	0	0
	R6	0 ^b	0 ^b	2.7 ^a
	R6.2	2.6 ^b	1.0 ^b	78.4 ^a
	R6.5	2.5 ^c	10.7 ^b	66.1 ^a
	R7	4.2 ^c	59.0 ^b	148.4 ^a

^{a,b,c}Different letters within a row indicate significant ($P < 0.05$) differences among seed tissues. Those without letters do not differ significantly.

ture seeds were fully developed into the full seed stage (R6) (Table 1). The level of *myo*-inositol in all tissues declined gradually as seeds grew toward maturity, and at the physiological maturity stage (R7) it was barely detectable in the seed coats and cotyledons and absent in the axes. The level of pinitol showed a sharp decrease at R6.2 and then an increase at R6.5 in all tissues as the full seeds began the maturing process. The fluctuation of pinitol levels seemed dependable because: (1) standard deviations for all replicated samples of R6.5 as analyzed by HPLC were relatively small and in similar ranges for those of R6.2 and R7; (2) a repeated HPLC analysis using a Waters 410 refractometer to replace a laser light-scattering mass detector showed similar results as (1); and (3) since only small amounts of glucose were present in the samples of R6.2 to R7 (0.9–4.1, 0.1–0.4 and 0.1–0.3 mg g dry wt⁻¹ for seed coats, cotyledons and axes, respectively), they could not largely affect the quantitation of pinitol. However, reasons for the transient increase in pinitol levels at R6.5 remain unknown. Perhaps the increase was for the seed to respond to the onset of maturation process in which full seeds turn yellow and begin dehydration toward physiological maturity. The transient increase in pinitol levels was concomitant with a substantial decrease in sucrose levels in all three tissues (Kuo, unpublished

data). This is analogous to the drought-stressed pigeonpea leaves in which pinitol is accumulated in the tissue corresponding to a decrease of sucrose, starch, *myo*-inositol and ononitol [26]. Analyses of enzyme activities associated with these non-structural carbohydrates [26] as well as some associated transcriptional products [27, 28] are needed to provide a better insight into the metabolism of cyclitols in developing soybean seeds. The sharp decrease of pinitol at R6.2 as embryonic axes turned yellow coincided with the beginning of a rapid accumulation of raffinose saccharides in the axis and, to a lesser degree, of the rapid increase in the cotyledon (Table 1). The concentration of raffinose saccharides was highest in the embryonic axis, followed by cotyledon and seed coat, whereas starch concentration was similar in the cotyledon and embryonic axis and also the lowest in the seed coat (Table 1). Starch concentration declined sharply in all three tissues during maturation from R6 through R7.

The possible interrelationships among the concentrations of soluble carbohydrates and starch in three seed tissues and at five growth stages of seed development are shown by statistical analyses (Table 2). The concentration of pinitol, *myo*-inositol and starch in all three tissues was negatively correlated with the seed development toward maturity, except

Table 2. Correlation matrix of characteristics examined in developing soybean seed coats, cotyledons and embryonic axes

	Growth stage	Pinitol	<i>myo</i> -Inositol	Starch
Seed coats (<i>N</i> = 15)				
Pinitol	-0.77**			
<i>myo</i> -inositol	-0.96**	0.86**		
Starch	-0.94**	0.85**	0.98**	
Raffinose saccharide	0.90**	-0.85**	-0.87**	-0.82**
Cotyledons (<i>N</i> = 15)				
Pinitol	-0.37			
<i>myo</i> -inositol	-0.93**	0.55*		
Starch	-0.88**	0.47	0.74**	
Raffinose saccharide	0.80**	-0.48	-0.63**	-0.97**
Embryonic axes (<i>N</i> = 10)				
Pinitol	-0.70*			
<i>myo</i> -inositol	-0.89**	0.88**		
Starch	-0.93**	0.79**	0.83**	
Raffinose saccharide	0.93**	-0.82**	-0.83**	-0.99**

* **Significant at the probability levels of 0.05 and 0.01, respectively.

that pinitol in cotyledons the correlation was insignificant. The increase of raffinose saccharide concentration in all three tissues was highly correlated with the progress of seed development. This increase was also highly correlated with the decrease of pinitol, *myo*-inositol and starch concentrations in all three tissues, except that the negative correlation was insignificant with pinitol in the cotyledon. The relationship between raffinose saccharides and starch concentration in whole soybean seeds has been demonstrated for other soybean cultivars [9, 29]. A transient starch accumulation also was associated with oligosaccharide depletion in germinating embryonic axes of soybean [30] and in soy suspension cells derived from hypocotyl [31]. It was theorized that during initial germination, the transient starch pool represented a mechanism to regulate the concentration of osmotically active substances (soluble sugars) to allow for mobilization of other reserves such as lipid and protein [30, 31]. Similarly, starch could represent a secondary metabolite pool in developing soybean seeds. This secondary carbon source could be converted into seed reserves (oligosaccharides, lipid, protein) as the import of photosynthates to the seed decreases toward maturity [9].

The close correlation between the decrease of *myo*-inositol and the increase of raffinose saccharides in soybean seed tissues during development agrees well with one of the established physiological roles of *myo*-inositol, which is to serve as a cofactor for the synthesis of raffinose saccharides [13, 16, 17]. The physiological role of its methyl derivative, pinitol, has not yet been established. One possible function of pinitol in plant leaves is in response to water or salinity stress [26, 32, 33]. The stress response is activated at both translational and transcriptional levels [26, 27]. It also involves a coordinated induction of multiple genes at

the transcriptional level to confer tolerance [28, 34]. Another suggestion is that pinitol and its galactosyl derivatives may be associated with seed desiccation tolerance in some species [7, 35]. In soybean leaves, pinitol was shown to accumulate as plants were subjected to high temperature stress [36], whereas in stems it might function as a transport assimilate translocated from lower to upper leaves of the plant [6]. In soybean root nodules, pinitol was related to nitrogen fixation [2] and metabolism of other soluble carbohydrates [37], and in mature seeds pinitol was postulated to play a role in the development of soil microorganisms [38]. Pinitol in soybean leaves also possesses anti-growth activity for the Boddie bollworm [39]. Based on the results shown here, that pinitol and its precursor *myo*-inositol were predominant soluble carbohydrates and their concentrations changed during seed development (Table 1), it is possible that pinitol may serve as a transient component directly related to the raffinose saccharide synthesis in the seeds. More investigations are needed to address the physiological role of pinitol in developing soybean seeds.

In conclusion, we have described a potential problem associated with the HPLC system used to quantify pinitol where small amounts of glucose are also present. However, this obstacle can easily be overcome by an enzymic assay for glucose. We have found that a substantial amount of pinitol is present in different tissues of developing soybean seeds. From the distribution of pinitol, *myo*-inositol, starch and raffinose saccharides as determined in different tissues, correlations were established among changes in the concentration of these components during seed development. While the concentration of *myo*-inositol in the seed tissue gradually decreased, much like starch, as seeds grew toward maturity, the concentration of pinitol changed more readily at different growth

stages. The rapid changes in the pinitol level occurred as the axes turned yellow, which coincided with the beginning of a rapid accumulation of raffinose saccharides in the seed tissue. The result suggests that pinitol is very active metabolically during seed development, and perhaps it is involved in the metabolism of raffinose saccharides.

EXPERIMENTAL

Plant material. Soybean plants [*Glycine max* (L.) Merr. cv. Williams 82] were grown in the field in rows spaced 0.30 m apart. At an average plant height of about 0.30 m, plants in each row were thinned to about 15 cm apart. Based on soil test results, potash (K_2O) (Cargill; Seymour, IN) was applied once to the soil at 242 kg ha^{-1} . Pre-emergence herbicides, Lasso Flowable (Monsanto, St Louis, MO) and Ambien (Union Carbide, Research Triangle Park, NC), were applied at 28 and 14 l, respectively, in a total of $2710 \text{ l H}_2\text{O ha}^{-1}$. Plants were watered biweekly with overhead sprinklers. Plants were also sprayed, as needed, with Liquid Sevin (Chevron Chemical, San Francisco, CA) at 5.3 ml l^{-1} to control Mexican bean beetles, and Malathion (pbl/Gordon, Kansas City, KS) and Pentac (Sandoz Crop Protection, Des Plaines, IL) at 1.8 ml l^{-1} to control spider mites. Developing seeds were harvested randomly from 35 to 55 days after flowering (DAF). Seeds from linear seed fill (35–40 DAF) through physiology maturity (50–55 DAF) were separated into four reproductive growth stages, R5.5, R6, R6.5 and R7, based on seed and pod morphology [12]. A new morphological growth stage (designated as R6.2 in this study), as the embryonic axis of green full seeds turned yellow at 40–45 DAF, was identified to correlate some physiological activities. Seeds from each stage were pooled, sepd into seed coat, cotyledon and embryonic axis, and immediately frozen with dry ice and stored at -85° . Tissues were lyophilized, ground to a uniform powder with a mortar and pestle, and stored at -20° until use. The average dry weight upon lyophilization, estimated from a minimum of 26 seeds, for seed coats, cotyledons, and axes was 7.9, 33.7 and 1.3 mg seed^{-1} , respectively, at R5.5; 13.4, 105.9 and 3.4 mg seed^{-1} , respectively at R6; 15.1, 171.2 and 5.0 mg seed^{-1} , respectively at R6.2; 17.4, 247.1 and 6.4 mg seed^{-1} , respectively at R6.5; 15.3, 217.9 and 5.8 mg seed^{-1} , respectively at R7. For the purpose of this study, the seed coat comprised all cell layers external to the cotyledons including epidermis, hypodermis, vascularized parenchymatous zone and endothelium [40].

Soluble carbohydrate and starch analyses. In this study the amount of each measured component was more properly expressed in mg g dry wt^{-1} of each separated seed part than based on the whole seed. Lyophilized soybean seed tissues were ground, defatted and extracted for soluble carbohydrates as described previously [12]. Each soluble carbohydrate, except glucose, in the extracts was quantitated by

HPLC in reference to pure standards and proper response factors as described previously [21]. Ultra-pure carbohydrate standards were acquired from Pfanstiehl Laboratories (Waukegan, IL). Pinitol was a gift from Dr L. Anderson, University of Wisconsin, Madison. Both main and guard columns for HPLC were maintained at 85° using H_2O as the mobile phase and a laser light-scattering mass detector, ELSD-MK II (Varex; Rockville, MD), for monitoring the elution. The concentration of pinitol was determined by subtracting the amount of contaminating glucose from the HPLC result for pinitol since glucose and pinitol were not well separated in this HPLC system. Glucose was assayed enzymically using a Sigma Diagnostic Kit (Cat. no. 510-A; St Louis, MO), with the colour produced within a linear range of glucose standards read at 540 nm [41] using a MR700 Microplate Reader (Dynatech; Chantilly, VA).

For starch analysis, the above defatted seed samples were extracted $\times 2$ in 80% hot aqueous ethanol. H_2O was added to its remaining sample (1 : 40, w/v), which was then sonicated for 5 min, followed by vacuum infiltration for 15 min. This step was repeated until all particulate matter was infiltrated. Starch was gelatinized and enzymically degraded to glucose [42]. Glucose produced was quantitated enzymically as described above. Corn starch was used as a standard under the same experimental conditions.

Pinitol isolation and identification. Pinitol was isolated from Nutrisoy 7B (Archer Daniels Midland; Decatur, IL) by a HPLC system used to isolate the soybean axis component [22] with the temp. of both main and guard columns maintained at 90° . In a typical experiment, 1 g of Nutrisoy 7B in a capped 125-ml Pyrex flask was extracted $\times 5$ with 50 ml hot 80% aqueous ethanol at 75° for 30 min with constant shaking. The extract after cooling down to room temperature was centrifuged at $11\,500 g$ and 10° for 10 min. The supernatant was dried to remove EtOH and the content was resuspended in water to dissolve carbohydrates. The solution was combined (total 25 ml) and passed through a column (2.5 cm dia.) of 2 ml CM52 (preswollen microgranular cation exchanger by Whatman; Maidstone, England) overlaid by 13 ml DE52 (the corresponding anion exchanger) to remove ionic substances. The column was equilibrated and eluted with water. A total of 50 ml was collected, filtered, lyophilized, and the solids were redissolved in 2 ml pure water. An aliquot of $20 \mu\text{l}$ of the concentrated sample was subjected to HPLC analysis in each case. An elution peak corresponding to the retention time of authentic pinitol was collected, lyophilized, and used without further purification for GC and GC-MS analyses. The yield was estimated to be about $7 \text{ mg g dry wt}^{-1}$ Nutrisoy 7B.

The isolated HPLC peak material and authentic standards, including glucose, *myo*-inositol, pinitol, quebrachitol (2-*O*-methyl-*chiro*-inositol), and sequoyitol (5-*O*-methyl-*myo*-inositol) were converted

into acetylated derivatives and subjected to GC-MS analysis as described previously [22].

Statistics. Differences in soluble carbohydrates and starch were analysed by least squares analysis of variance procedures. Sources of variation were growth stage and tissue plus their interaction. The interaction was significant ($P < 0.01$) in all cases. Differences between tissues within each growth stage and differences among growth stages for each tissue were determined by pair-wise *t*-tests of least squares means at $P < 0.05$.

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