

## Content of Raffinose Oligosaccharides and Sucrose in Various Plant Seeds

Tsung Min Kuo,\* Jody F. VanMiddlesworth, and Walter J. Wolf

Soluble saccharides in plant seeds, during dormancy and germination, were quantitated by high-performance liquid chromatography. Among seeds of 29 different crops examined, cotton, garden pea, soybean, cowpea, and alfalfa contained more than 50 mg of raffinose saccharides/g of defatted meal. Sucrose was predominant in peanut, soybean, sunflower, and garden pea. All Leguminosae species contained more stachyose than raffinose. However, seeds from cotton, sunflower, and Gramineae species contained more raffinose than stachyose. During seed germination, the major raffinose saccharide of mung bean, soybean, and cotton disappeared faster in the embryonic axes than in the cotyledons and no raffinose saccharides accumulated in the embryonic axes. Monosaccharides accumulated in the embryonic axes of soybeans and mung beans, but not obviously in the cotton tissues. The results suggest that raffinose saccharides present in cotyledons of dormant seeds were directly hydrolyzed during germination to produce sucrose, which was then transported to the embryonic axes for further metabolism.

The oligosaccharides of the raffinose family are widely distributed in higher plants (French, 1954; Kandler and Hopf, 1982; Dey, 1985). These galactosyl derivatives of sucrose accumulate in leaves during photosynthesis (Senser and Kandler, 1967) and in seeds during maturation (Amuti and Pollard, 1977). It is thought that the primary role of raffinose saccharides in mature seeds, vegetative organs, and leaves is to store or transport carbohydrates (Kandler and Hopf, 1982; Dey, 1985). The predominant form of raffinose saccharides, however, differs in various species.

The distribution of raffinose saccharides in 32 different dry seeds was surveyed by Amuti and Pollard (1977), who found these sugars in all seeds but sesame, tobacco, tomato, and castor bean. Quantitative data for raffinose saccharides were presented only for soybeans. In several plant species, the levels of raffinose saccharides in seeds have been shown to decrease during germination (Pazur et al., 1962; Aman, 1979; Reddy and Salunkhe, 1980; Doman et al., 1982; Abdullah et al., 1984; Ologhobo and Fetuga, 1986). Nevertheless, detailed information regarding the mobilization of these oligosaccharides in seed components during germination is limited (Abrahamsen and Sudia, 1966; Hsu et al., 1973; Wahab and Burris, 1975).

Accordingly, the objectives of this study were (1) to select a suitable high-performance liquid chromatography (HPLC) procedure and use this method to quantitate soluble saccharides in various plant seeds and (2) to investigate the degradative process of raffinose saccharides during seed germination. Our results have provided quantitative information concerning the oligosaccharide content in mature seeds of 29 different crops, and they suggest that raffinose saccharides are hydrolyzed directly in cotyledons during germination rather than being transported to embryonic axes for metabolism.

### MATERIALS AND METHODS

**Seed Sources and Chemicals.** Dry seeds of cotton, corn, rice, rye, oat, sorghum, triticale, and broad bean were obtained from colleagues at our Research Center. Tobacco seeds were harvested from plants grown in a greenhouse in 1984. Barley and wheat were obtained from Andris Kleinhofs, Washington State University, Pullman, WA, and peanuts from Edith Conkerton, Southern Regional

Research Center, USDA-ARS. Cotton seeds used for germination experiments were obtained from Lawrence Burdett, Delta & Pine Land Co., Casa Grande, AZ. Other seeds were purchased from Kelly Seed Co., Peoria, IL. Ultrapure sugar standards were acquired from Pfanstiehl Laboratories (Waukegan, IL). Preswollen microgranular DEAE-cellulose was obtained from either Whatman (Clifton, NJ) or Sigma (St. Louis, MO), and Uniflo membrane (0.2  $\mu$ m), from Schleicher & Schuell (Keene, NH). Other reagents were of analytical grade.

**Sample Preparation.** Seeds were ground to fine meals in a coffee mill (Varco Inc., Bellville, NJ; type 228). The meals were defatted with hexane for 4 h in a Soxhlet extractor and then air-dried. The defatting step was necessary to remove interfering lipids present in large amounts in oilseed crops, such as soybean, cotton, safflower, sunflower, and peanut. However, for comparative purposes, all plant seeds were defatted for HPLC analyses.

**Germination Procedure.** Seeds (30-50 g) of soybean, mung bean, and cotton were washed in 0.5% sodium hypochlorite for 20 min at room temperature (23 °C), rinsed thoroughly with tap water, and imbibed in deionized water for 6 h. Seeds of uniform size were selected and placed on four layers of wetted filter paper in a Petri dish (100  $\times$  15 mm). Germination of seeds took place in a dark, environmental chamber at 27 °C, and seeds and filter paper were rinsed once daily with fresh deionized water. Six dishes were set up for each type of seed. Each day, seeds from one dish were rinsed with deionized water and separated into embryonic axes and cotyledons. These tissues were lyophilized and defatted as described above.

**Extraction and Cleanup.** To 0.3-1.0 g of defatted meal in a 50-mL Erlenmeyer flask were added 50 mg/g of meal of mannitol and ribitol (internal standards, each in a stock solution of 50 mg/mL) and a preheated, aqueous solution of 80% ethanol (at a ratio of 30 mL/g of meal), and the flask was sealed. The extraction was carried out for 30 min in a Lab-Line (Melrose, IL) Orbit-Enviro shaker maintained at 72-75 °C. At the end of extraction, the meal was allowed to settle prior to transferring 4 mL of extract into a 10-mL screw-capped centrifuge tube. After the mixture was centrifuged at 10000g for 5 min at 10 °C, 4 mL of clear supernatant was taken to dryness with a Haake-Buchler (Saddle Brook, NJ) vortex evaporator set at 40 °C. The dry extract was stored at -20 °C for further treatments or processed immediately. The dry extract was dissolved in 1.5 mL of double-deionized H<sub>2</sub>O, vortexed, and

Northern Regional Research Center, U.S. Department of Agriculture-Agricultural Research Service, Peoria, Illinois 61604.

passed through a 1.5-mL preswollen DEAE-cellulose minicolumn, equilibrated and later eluted with H<sub>2</sub>O to remove anionic substances from the sample. A total of 3.5 mL of eluent was collected for each sample and filtered through a Uniflo membrane prior to HPLC analysis. Up to 24 samples could be processed in a single batch.

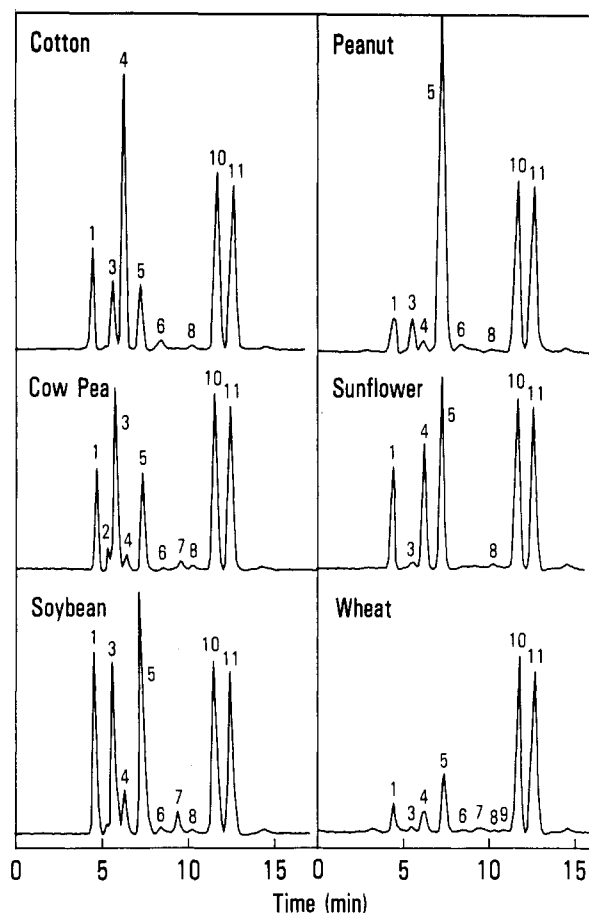
**HPLC Analysis.** Aliquots (20  $\mu$ L) were subjected to HPLC analysis with a Spectra-Physics (San Jose, CA) SP8780XR autosampler and a SP8700XR pump. The separation of sugars was achieved by a Sugar-Pak I column (Waters Associates, Milford, MA) connected to a guard column (cation cartridge; Pierce, Rockford, IL). The main column was maintained at 90 °C by means of a Bio-Rad (Richmond, CA) column heater. The elution was monitored by a Waters Model 401 refractometer, and the mobile phase was 0.1 mM CaNa<sub>2</sub>EDTA/H<sub>2</sub>O at 0.5 mL/min. The data were stored in a ModComp computer system (Modular Computer Systems, Ft. Lauderdale, FL) for sugar identification, integration of elution peak areas, and quantitation of sugars with proper response factors. The quantitation of sugars was based on the amount of the internal standard (mannitol) added to the sample.

## RESULTS AND DISCUSSION

A number of HPLC studies using a Ca<sup>2+</sup>-loaded cation-exchange resin for the analysis of sugars in plant tissues have been reported (Pharr and Sox, 1984; Boersig and Negm, 1985; Hughes and Lindsay, 1985; Picha, 1985; Wight et al., 1986). However, a detailed description of the chromatographic technique was not available in the summer of 1985 when we initiated the search for a suitable HPLC method. We first evaluated an organic acid column (Bio-Rad, Aminex HPX-87H) and found that it had a relatively high base line for analyzing soybean sugar extracts similar to that shown for peanuts (Conkerton et al., 1983). Subsequently, we found that Sugar-Pak I gave an excellent base line and the combination of simplicity and speed; it used water containing a low concentration of EDTA as mobile phase, resolved sugars in the order of decreasing dp in distinctive peaks of similar bandwidth, and completed the chromatography within 15 min.

**HPLC Separation of Sugars.** Figure 1 shows representative chromatographs for the soluble sugars in mature seeds as separated by a Sugar-Pak I column. The elution pattern of soluble sugars was characteristic for different plant seeds. For instance, sucrose (peak 5) was predominant in peanuts; raffinose (peak 4) was prominent in cotton and sunflower, and the latter also contained large amounts of sucrose; stachyose (peak 3) was prominent in cowpea and soybean, but the latter also contained a relatively large peak of sucrose; wheat contained small detectable amounts of stachyose, raffinose, sucrose, glucose (peak 7), galactose (peak 8), and fructose (peak 9). Peak 2 shown in cowpea was tentatively designated as verbascose based on its retention time; verbascose is the major raffinose saccharide present in mung bean (Aman, 1979). Peaks 1 and 6 were not identified. Since this column eluted sugars in the order of decreasing degree of polymerization (dp), peak 1 presumably contained oligomers larger than dp 5. Therefore, under the described experimental conditions, soluble sugars of up to dp 5 can be adequately resolved by a Sugar-Pak I column, which is packed with Ca<sup>2+</sup>-loaded cation-exchange resin.

The incorporation of an internal standard has been shown to greatly simplify the quantitation of sugars in plant seeds (Black and Bagley, 1978; Conkerton et al., 1983). Ribitol has been a useful internal standard for analyzing peanut carbohydrates (Conkerton et al., 1983). When both mannitol and ribitol were added to the sample



**Figure 1.** HPLC analysis of soluble sugars in various plant seeds: 1, unknown; 2, verbascose; 3, stachyose; 4, raffinose; 5, sucrose; 6, unknown; 7, glucose; 8, galactose; 9, fructose; 10, ribitol (internal standard); 11, mannitol (internal standard).

during sugar extraction, they were separated completely from each other and from other major soluble sugars in seeds (Figure 1). Their peak areas were always within  $\pm 5\%$  of each other when both sugar alcohols were added to the samples during preparation. This indicates that either mannitol or ribitol may be employed as a suitable internal standard. However, we found in a chromatographic analysis that ribitol was unresolved from myo-inositol, an important metabolite involved in the biosyntheses of raffinose saccharides, cell wall polysaccharides, and phytic acid in plants (Loewus, 1969; Tanner, 1969). Consequently, mannitol is the preferred internal standard when myo-inositol is to be quantitated. Because the peak areas for ribitol and mannitol were nearly constant (Figure 1), myo-inositol was either absent or present in only trace amounts in these mature dry seeds.

**Soluble Oligosaccharide Content of Plant Seeds.** As a necessary first step in the study of metabolic controls of raffinose saccharides in plant seeds, quantitative information is needed to select proper seed sources for physiological and biochemical investigations. In this study, we determined soluble oligosaccharides in seeds of 29 different crops by HPLC. The results are shown in Table I, which lists the species within each plant family in the order of decreasing content of total raffinose saccharides. The average standard deviations (percent of each measured quantity) for verbascose, stachyose, raffinose, and sucrose were 5.8, 8.1, 24.0, and 8.7, respectively, among seeds in the Leguminosae family; those for stachyose, raffinose, and sucrose were 1.5, 5.4, and 6.7, respectively, in the Cucurbitaceae family; those for raffinose and sucrose in seeds of the Gramineae family were 12.5 and 7.9, respectively.

Table I. Distribution (mg/g of Defatted Meal) of Soluble Oligosaccharides in Dry Seeds of Various Plants<sup>a</sup>

seed	raffinose sugars				sucrose	total sugar
	verbascose	stachyose	raffinose	total		
Malvaceae						
cotton ( <i>Gossypium herbaceum</i> ) cv. Deltapine 61	tr	23.6	69.1	92.7	16.4	109.1
Leguminosae						
garden pea ( <i>Pisum sativum</i> ) cv. Little Marvel	19.1	32.3	11.6	63.0	62.3	125.3
soybean ( <i>Glycine max</i> ) cv. Williams 82	tr	43.4	12.6	56.0	64.2	120.2
cv. Amsoy 71	tr	41.0	11.6	52.6	72.7	125.3
cowpea ( <i>Vigna sinensis</i> ) <sup>b</sup>	3.6	46.4	3.7	53.7	25.9	79.6
alfalfa ( <i>Medicago sativa</i> ) <sup>b</sup>	tr	39.5	13.5	53.0	22.7	75.7
mung bean ( <i>Phaseolus aureus</i> ) cv. Berken	26.6	16.7	3.9	47.2	13.9	61.1
lima bean ( <i>Phaseolus limensis</i> ) cv. Fordhook	tr	30.3	6.9	37.2	36.0	73.2
green bean ( <i>Phaseolus vulgaris</i> ) cv. Top Crop	tr	34.3	2.5	36.8	19.4	56.2
cv. Blue Lake	tr	28.1	2.2	30.3	29.0	59.3
red kidney bean ( <i>Phaseolus vulgaris</i> ) <sup>b</sup>	tr	31.6	3.1	34.7	21.5	56.2
pole bean ( <i>Phaseolus vulgaris</i> ) cv. Kentucky Wonder	tr	26.2	4.3	30.5	26.7	57.2
broad bean ( <i>Vicia faba</i> ) <sup>b</sup>	11.4	10.7	2.3	24.4	20.7	45.1
peanut ( <i>Arachis hypogaea</i> ) cv. Florunner	tr	9.9	3.3	13.2	81.0	94.2
Compositae						
sunflower ( <i>Helianthus annuus</i> ) <sup>b</sup>	—	1.4	30.9	32.3	65.0	97.3
safflower ( <i>Carthamus tinctorius</i> ) <sup>b</sup>	—	—	5.2	5.2	18.6	23.8
Cucurbitaceae						
pumpkin ( <i>Cucurbita pepo</i> ) cv. Jack O'Lantern	—	16.3	6.5	22.8	28.8	51.6
cucumber ( <i>Cucumis sativus</i> ) cv. Marketeer	—	10.8	9.2	20.0	16.2	36.2
squash ( <i>Cucurbita maxima</i> ) cv. Table King	—	11.8	7.8	19.6	34.4	54.0
Solanaceae						
tobacco ( <i>Nicotiana tabacum</i> ) cv. Dpl	—	tr	7.3	7.3	26.8	34.1
Gramineae						
barley ( <i>Hodeum vulgare</i> ) cv. Himalaya	—	tr	7.9	7.9	14.2	22.1
cv. Steptoe	—	tr	6.3	6.3	11.8	18.1
<i>Triticale</i> cv. Fas Gro 204	—	tr	7.2	7.2	8.4	15.6
rye ( <i>Secale cereale</i> ) cv. Balbo	—	tr	7.1	7.1	11.5	18.6
wheat ( <i>Triticum aestivum</i> ) cv. Chinese Spring	—	tr	7.0	7.0	13.8	20.8
corn ( <i>Zea mays</i> ) P-3737 hybrid	—	—	3.1	3.1	14.2	17.3
OH 43 inbred	—	—	2.1	2.1	15.0	17.1
oat ( <i>Avena sativa</i> ) cv. Dal	—	tr	2.6	2.6	8.8	11.4
sorghum ( <i>Sorghum vulgare</i> ) cv. WAC 694	—	tr	tr	tr	8.4	8.4
rice ( <i>Oryza sativa</i> ) cv. Blue Bell	—	—	—	—	5.6	5.6
Chenopodiaceae						
spinach ( <i>Spinacia oleracea</i> ) cv. Giant Noble	—	—	4.8	4.8	6.7	11.5
beet ( <i>Beta vulgaris</i> ) cv. Early Egyptian	—	—	3.7	3.7	3.4	7.1
Euphorbiaceae						
castor bean ( <i>Ricinus communis</i> ) <sup>b</sup>	tr	1.9	1.9	3.8	35.1	38.9

<sup>a</sup> Results are an average of at least three separate analyses. Key: tr, trace amount detected; —, not detected. <sup>b</sup> Cultivar not known.

The large standard deviation for the quantitation of raffinose in legumes was probably due to its relatively small quantity among oligosaccharides present in the seeds. The major oligosaccharides extracted from defatted meal with

hot 80% ethanol were sucrose and various types of raffinose saccharides; more than 50 mg/g of defatted meal were found in peanut, soybean, sunflower, and garden pea. Among these 29 different crops, raffinose was present in

**Table II. Changes in Soluble Sugar Content (mg/g of Defatted Meal) in Mung Beans during Germination<sup>a</sup>**

days of germin	verbascose		stachyose		raffinose		sucrose		glucose		fructose	
	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb
0	35.0	nd	20.2	nd	4.2	nd	12.9	nd	0.0	nd	0.0	nd
1	13.9	4.6	14.5	13.1	4.2	b	29.1	26.1	0.0	0.0	0.0	9.3
2	2.5	0.0	6.3	10.6	0.0	b	27.7	46.1	0.0	30.3	0.0	67.5
3	0.5	0.0	2.6	0.0	0.6	b	42.7	51.0	2.7	60.1	0.0	102.6
4	0.0	0.0	0.0	0.0	1.4	b	50.2	54.9	3.3	65.1	0.0	115.8
5	0.0	0.0	0.8	0.0	1.3	b	49.5	51.2	5.0	57.8	0.6	109.3

<sup>a</sup> Results are an average of two separate experiments. Key: Cot, cotyledon; Emb, embryonic axis; nd, not determined. <sup>b</sup> Could not quantitate due to an interfering substance.

**Table III. Changes in Soluble Sugar Content (mg/g of Defatted Meal) in Soybeans during Germination<sup>a</sup>**

days of germin	stachyose		raffinose		sucrose		glucose		fructose	
	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb
0	50.5	109.0	12.9	19.4	68.3	51.5	0.0	0.0	0.0	0.5
1	50.3	47.5	12.8	9.6	68.3	38.6	0.0	2.0	0.0	6.6
2	38.6	12.2	9.2	6.2	57.0	24.9	0.0	0.0	0.0	12.7
3	32.3	4.9	7.9	2.9	44.4	18.5	0.0	0.0	0.0	44.7
4	22.8	0.0	4.5	1.5	36.4	13.3	0.0	24.1	0.0	43.0
5	13.2	0.0	3.7	0.3	22.1	10.5	0.0	16.5	0.0	37.7

<sup>a</sup> Abbreviations as in Table II.

**Table IV. Changes in Soluble Sugar Content (mg/g of Defatted Meal) in Cotton Seeds during Germination<sup>a</sup>**

days of germin	stachyose		raffinose		sucrose		glucose		fructose	
	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb	Cot	Emb
0	22.9	27.1	69.6	78.5	17.0	22.2	0.2	0.0	0.0	0.0
1	24.9	20.3	52.9	29.1	11.7	15.4	0.0	0.0	0.0	0.0
2	23.0	20.3	39.5	21.0	10.5	15.9	0.0	0.0	0.0	0.0
3	19.0	25.1	23.0	11.7	13.6	11.9	0.0	0.0	0.0	0.0
4	18.1	19.4	19.5	6.8	11.4	9.4	0.0	1.9	0.0	1.6
5	14.8	19.0	10.8	2.4	8.7	5.8	3.3	1.4	0.0	3.3

<sup>a</sup> Abbreviations as in Table II.

all but rice. Stachyose was detected in all seeds but safflower, spinach, beet, corn, and rice. Verbascose could only be accurately measured in mung bean, garden pea, broad bean, and cowpea. Cotton, garden pea, soybean, cowpea, and alfalfa contained more than 50 mg of raffinose saccharides/g of the defatted meals. Almost all Leguminosae species contained stachyose as a major raffinose saccharide, while all Gramineae species contained more raffinose than stachyose. Mung bean and broad bean contained verbascose as a major raffinose saccharide. It is not clear how the preferential accumulation of a specific raffinose saccharide is biochemically regulated in different seeds. Our results are, in general, consistent with those qualitatively determined by Amuti and Pollard (1977), but we have also found low levels of raffinose saccharides present in tobacco seed and castor bean. We further show that the relative levels of raffinose saccharides and sucrose present in six legume seeds are similar to those reported by Sosulski et al. (1982) who used gas-liquid chromatography and mass spectroscopy. However, the contents of sucrose in lima bean and verbascose in pea were relatively high in their studies.

**Metabolism of Oligosaccharides in Seeds during Germination.** We have shown that the HPLC procedure described above (Figure 1) is able to resolve raffinose saccharides, sucrose, glucose, fructose, and galactose. Therefore, this method is suitable for the analysis of metabolic products of sucrose and raffinose saccharides during seed germination. We chose to study mung bean, soybean, and cotton, because each of these species contained a different major raffinose saccharide (Table I) (namely, verbascose in mung bean, stachyose in soybean, and raffinose in cotton) that might have different kinetics of metabolism. Sucrose levels progressively decreased in

both soybeans and cotton seeds but increased in mung beans (Tables II-IV). The accumulation of sucrose in mung beans during germination might be related to the mobilization of a large amount of reserve starch present in the cotyledons. Both fructose and smaller amounts of glucose accumulated in the embryonic axes of mung bean and soybean, but the accumulation of monosaccharides in cotton embryonic axes was minimal. Either a very low amount of monosaccharide or none was detected in cotyledons. These results suggest that embryonic axes are the major site for the breakdown of sucrose and that the metabolism of sucrose and monosaccharides is characteristic of different species. Our results for the soluble sugar content in soybean seed components during germination were similar to those obtained by Hsu et al. (1973) and Wahab and Burris (1975) except that we observed a smaller accumulation of glucose in embryonic axes during germination. This low amount of glucose present in embryonic axes might indicate that glucose was rapidly metabolized for new growth, perhaps leading to the formation of UDP glucose (Rodaway et al., 1979).

As noted by the growth of embryonic axes and the decrease in dry weight of cotyledons, mung beans germinated faster than soybeans, which in turn germinated much faster than cotton seeds. Therefore, the rate of germination corresponded well with both the rate of fructose accumulation in the embryonic axes and the disappearance rate of the major raffinose saccharide, such as stachyose in soybeans, from both seed components (Tables II-IV). The raffinose saccharide content in the imbibed seeds of mung bean and soybean showed an increase in comparison with those obtained for the dormant seeds (Table I). This is probably due to some synthesis of oligosaccharides in the early stage of germination (Wahab and Burris, 1975;

Dey, 1985). In all cases, the raffinose saccharides present in both cotyledon and embryonic axis disappeared during germination, but at a faster rate in the embryonic axis. This was especially true for the major raffinose saccharide in the seeds. There was no accumulation of raffinose saccharides in embryonic axes. These results suggest that, during germination, raffinose saccharides present in cotyledons were first hydrolyzed to produce sucrose, which was then transported to the embryonic axes for further metabolism.

In summary, we have evaluated a simple and suitable HPLC procedure for the separation and quantitation of soluble sugars in plant seeds in dormancy and during germination. The amounts of raffinose saccharides and sucrose vary considerably in many common edible seeds, and this information will be useful to food chemists and nutritionists. Analysis of soluble sugars in germinated seeds has provided some insight into the metabolism of oligosaccharides in cotyledons and embryonic axes. A better understanding of the mobilization of raffinose saccharides during seed germination requires an extensive analysis of the associated metabolic enzymes in seed components, such as  $\alpha$ -galactosidase, invertase, and sucrose synthase. Such an analysis is in progress.

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