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Occurrence of a Novel Galactopinitol and its Changes with Other Non-Reducing Sugars during Development of *Leucaena leucocephala* Seeds[1]

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A new cyclitol which is abundant in the late developmental stages of leucaena (*Leucaena leucocephala* (Lam.) de Wit) seeds was identified by HPLC, NMR, and GC-MS as *O*-α-D-galactopyranosyl-(1 → 1)-3-*O*-methyl-D-*chiro*-inositol, a new galactopinitol. This galactopinitol was initially detected midway through seed development and increased to 10.2 mg (g DW)$^{-1}$, but decreased in mature seeds to its about a half. Stachyose content increased greatly and remained the most abundant of the soluble sugars in mature seeds (25.6 mg (g DW)$^{-1}$). Artificial drying at 73% relative humidity of 70 DPA immature seeds induced the accumulation of raffinose, stachyose, galactopinitol and galactinol, but the total amounts of these sugars were only about half of those found in mature seeds. Seed germination decreased following an initial increase after 8 d artificial drying to a moisture content of 24%, and this dehydration damage probably is because of underdevelopment of seed tissue. Galactopinitol changes in a similar fashion to the oligosaccharides during the late developmental stage and dehydration experiment, implying that galactopinitol may play a role in desiccation tolerance of leucaena seeds.

Key words: Desiccation tolerance — Galactopinitol — *Leucaena leucocephala* — Oligosaccharides.

Sucrose and the raffinose family of oligosaccharides (raffinose, stachyose and verbascose) accumulate in developing or mature seeds, especially those of the legume family (Amuti and Pollard 1977). Several studies have demonstrated a relationship between desiccation tolerance and soluble sugar content during maturation of soybean seeds (Blackman et al. 1992, Saravitz et al. 1987), maize kernels (Chen and Burris 1990), and *Brassica campestris* seeds (Leprince et al. 1990). A mass ratio of 0.3 of raffinose to sucrose afforded better protection against desiccation than did a high content of raffinose alone (Caffrey et al. 1988). In immature soybean seeds at 34 DAF, slow drying in vitro increased stachyose and raffinose and decreased sucrose, and induced desiccation tolerance (Blackman et al. 1992). Therefore, the non-reducing oligosaccharides may be important in conferring protection against desiccation-induced damage in orthodox (desiccation-tolerant) seeds.

In addition to sucrose and oligosaccharides, galactosyl derivatives of cyclitols have been found to be abundant in seeds of legume and other species (Beveridge et al. 1977, Schweizer et al. 1978, Horbowicz and Obendorf 1994). Here we present data on a new cyclitol found in the seeds of *L. leucocephala*, a leguminous tree, and changes in sugars contents during development of leucaena seeds. The effects of drying on the germination rate of immature seeds and changes in their sugar content were also examined.

Materials and Methods

Seed collection—Seeds of Hawaiian Giant *L. leucocephala* (Brewbaker 1980), a variety characterized as a tall, single-trunk tree, were collected from 10-y-old trees at Wu Lai (24°52′N, 121°33′E), Taiwan, in 1993. To determine sugar content during seed development stages, pods were harvested at 42, 51, 61, 70, 80, 86, 92, 97, and 104 (dry pod) d post-anthesis, and stored at −70°C until used. Dry weight, moisture content and germination of seeds from each DPA period were analyzed before being stored at −70°C.

Drying treatment of immature seeds—Developing pods harvested at 70 DPA were first sterilized with 2% (v/v) sodium hypochlorite for 15 min followed by several washes with sterilized water before treatment. Seeds were extracted from the pods in a laminar flow cabinet and then dried in a closed desiccator equilibrated with a saturated solution of sodium acetate (73% RH at 25°C). Three replicates of thirty seeds each were withdrawn every other day for 8 d and then tested for germination. Meanwhile, seeds were stored at −70°C for sugar determination at a later time. For the germination test, seeds were mixed with moistened sphagnum in small sealable polyethylene bags (0.05 mm thickness). Moisture content of the sphagnum was four times of the dry mass. Germination was carried out in an incubator at a constant temperature of 22°C in 12 h fluorescent light (80–100 μE $m^{-2} s^{-1}$). Seeds were considered germinated when radicles had protruded at least 5 mm. Results are expressed as percentage germinated after 21 d.

Moisture content analysis—Moisture content of seeds was determined by cutting them into small pieces and oven drying for 17 h at 103°C (International Seed Testing Association 1993). All moisture contents are expressed on a fresh weight basis.

Extraction of sugars—Sugars were extracted according to the method of Blackman et al. (1992). Frozen samples of 10 seeds (20–30 seeds for 42 and 51 DPA) stored at −70°C were weighed and

Abbreviations: DPA, days post-anthesis; DQF-COSY, double-quantum filtered correlation spectroscopy; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry; TFA, trifluoroacetic acid.

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homogenized in a centrifuge tube with a Polytron (Kinematica AG, Switzerland) in 4 ml 75% ethanol containing 400 μg melezitose (Sigma) as an internal standard. The homogenate was incubated at 75°C for 30 min and centrifuged at 12,000 × *g* for 10 min. The pellet was re-extracted one additional time with 2 ml 50% ethanol and centrifuged again as above. The combined supernatants were taken to dryness in SpeedVac (Savant Instruments, Farmingdale, NY), resuspended in 1 ml distilled water, and deionized on a column containing 1 ml polyvinylpolypyrolidone (PVPP), 1 ml Amberlite IRA-94 anion exchanger, and 1 ml Dowex-50W cation exchanger (all from Sigma). The neutral fraction was eluted with 4 ml distilled water, then taken to dryness, and resuspended in 400 μl double-distilled water for HPLC and GC analyses.

HPLC—The chromatographic system consisted of a Beckman programmable solvent pump, refractive index detector (Beckman Instruments, CA), an Eppendorf CH-30 column heater (P.J. Cobert Asso., St. Louis, MO), and Beckman System Gold chromatography software was used. The column used was a Rezex RNM-Oligosaccharide (Phenomenex, CA) with double-distilled water as solvent. The flow rate was 0.4 ml min^{-1} and column temperature was 75°C. Individual sugars were identified by comparison with retention times of sugar standards.

Three unknown glycosides, unknowns 1, 2, and 3, found in leucaena seeds were purified by HPLC using three columns: Rezex RNM-oligosaccharide, CHO carbohydrate (Interaction Chemicals, CA), and C_{18}-reversed phase (Merck, Germany). Each purified unknown sugar was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 100°C, neutralized with 10 M NaOH and then filtered through a column consisting of 1 ml Dowex-50W cation exchanger and 1 ml Amberlite IRA-94 anion exchanger. The eluate was dried in a SpeedVac and analyzed by HPLC on a Rezex RNM-Oligosaccharide column.

Mass spectroscopy—FAB-MS spectra were obtained using a JEOL SX-102A double-focusing mass spectrometer of reversed geometry (JEOL, Japan). The FAB gun was operated at 6 kV using xenon as the ionizing gas. Samples were dissolved in 10 μl dichloromethane, and a 1 μl sample solution was mixed with 1 μl matrix (3-nitrobenzyl alcohol) on a FAB probe tip for subsequent analysis. A Hewlett-Packard model 5890 series II gas chromatograph was coupled to the mass spectrometer. The derivatized sample was analyzed by GC/MS in the electron-impact-ionization mode. Column (30 m, 0.32 mm ID, 0.25 μm film thickness; J & W DB5) temperature was kept at 100°C for 1 min and then increased to 275°C at a rate of 5°C min^{-1}. Helium was used as the carrier gas.

NMR spectroscopy—Samples were dissolved in 300 μl high-purity D_2O under N_2 and sealed in NMR tubes. NMR experiments were performed on either a Bruker AM-300WB-FT-NMR or a Bruker AM-400-FT-NMR spectrometer. DQF-COSY and HOHAHA spectra were recorded in the phase-sensitive mode with the time-proportion phase incrementation (TPPI) method. A typical two-dimensional spectrum was recorded at 300K with 600(t1) × 2,048(t2) data points. For the HOHAHA experiment, a mixing time of 120 ms was used. After zero filling to a 2K × 2K matrix, sine-bell square by $\pi/2$ functions were applied to both dimensions prior to Fourier transformation.

Permethylation and analysis of sugars—After deionization by PVPP, Dowex 50W, and Amberlite IRA-94 column as described above, the dried sugars were methylated using NaOH and dimethyl sulphoxide (DMSO) method (Ciucanu and Kerek 1984, Karlsson et al. 1989), and quantified by GC. For methylation, the dried sugars were dissolved in 100 μl DMSO (using as little as possible) for 5 min, 0.2 g powdered NaOH and 100 μl iodomethane added, and then stirred for 1 h. The reaction was stopped by the addition of 1 ml water and 1 ml dichloromethane. The water phase was removed after centrifugation, and the dichloromethane phase was washed three times with 2 ml water. The dichloromethane phase was dried under a stream of nitrogen gas. The methylated sugars were dissolved in 200 μl dichloromethane before GC analysis.

GC analysis was carried out on a Hewlett-Packard 5890 series II fitted with a 30 m DB5MS (J&W Scientific) column (0.25 mm ID) and flame ionization detector. Prepurified nitrogen was used as the carrier gas at a linear gas velocity of 1.5 ml per min. Detector gases were 30 ml min^{-1} H_2, 400 ml min^{-1} air, and 25 ml min^{-1} N_2. The injector was operated at 275°C and FID at 350°C. The initial column temperature was set at 140°C for 5 min, followed by a 4°C min^{-1} increase to 240°C for 21 min, and finally a 10°C increase per min up to 320°C for 26 min. Sample sugars were identified by comparison with retention times of known standards, and quantified by comparing the peak area with a known quantity of sugar after correction by the recovery of internal standard. Galactopinitol and galactinol were quantified by comparing with a known quantity of sucrose. Peak areas were computed by HP-3365 ChemStation.

Results

Structures of unknown sugars—HPLC chromatogram of seed sugars is shown in Fig. 1A. The seed chromatogram shows three unknown compounds. TFA hydrolysis of unknown 1 resulted in galactose and unknown 3 (Fig. 1B). Unknown 3 extracted from seeds was not hydrolyzed by TFA. Further support was provided by GC analysis of methylated unknown 3 and methylated TFA hydrolysate of unknown 1. TFA hydrolysis of unknown 2 resulted in galactose and *myo*-inositol (Fig. 1C). Therefore, unknown 2 appears to be galactinol.

Unknown 3 was acetylated according to the method of Dell and Tiller (1986) and analyzed by FAB-MS. The molecular ion (m/z 405) (spectrum not shown) suggested that unknown 3 was an inositol with one methylated hydroxyl group. With the help of 2D NMR (DQF-COSY), the ^1H NMR spectrum of unknown 3 was assigned as follows: 3.31 (1H, t, J=9.7 Hz, 4-H), 3.48 (3H, s, MeO), 3.62 (1H, t, J=9.7 Hz, 3-H), 3.73 (1H, dd, J=9.9 and 2.8 Hz, 2-H), 3.78 (1H, dd, J=9.9 and 2.8 Hz, 5-H), 3.95–3.98 (2H, m, 6-H and 1-H). The NMR spectrum was the same as the spectrum of pinitol which was reported by Ley and Sternfeld (1989). Thus, unknown 3 was assigned as pinitol (1D-3-*O*-methyl-*chiro*-inositol).

The ^1HNMR spectrum of unknown 1, composed of galactose and pinitol, was assigned by DQF-COSY and HOHAHA spectra. The ^1HNMR was assigned as follows: 3.37 (1H, dd, J=9.3 Hz, 4-H), 3.57 (3H, s, MeO), 3.71 (2H, d, J=6.3 Hz, 6′-H), 3.76 (1H, dd, J=9.3 Hz, 3-H), 3.78–3.84 (3H, m, 2-H, 5-H, 2′-H), 3.92 (1H, dd, J=3.3 and 10.3 Hz, 3′-H), 4.02 (1H, d, J=3.3 Hz, 4′-H), 4.04 (1H, t, 6-H), 4.16–4.20 (2H, m, 5′-H, 1-H), 5.08(1H, d, J=

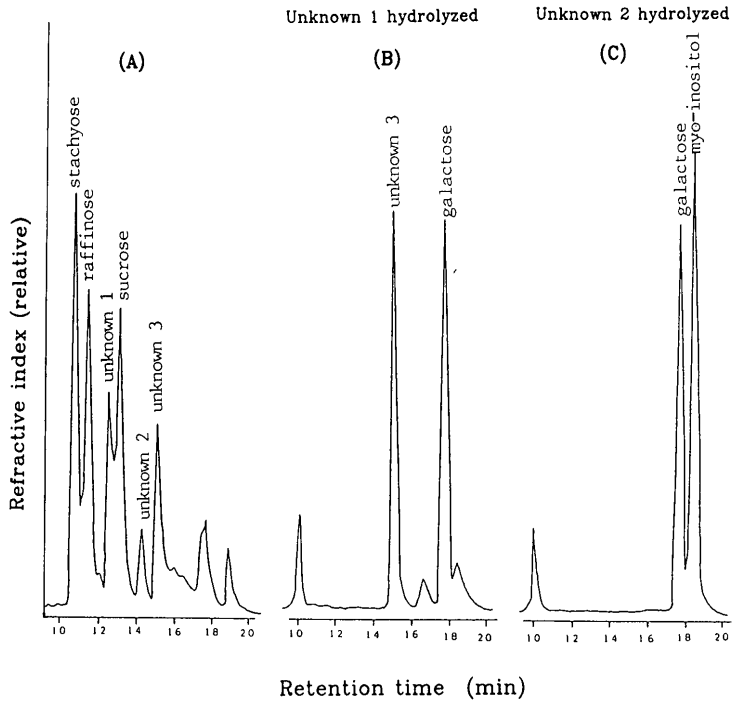

Fig. 1 HPLC chromatograms of (A) neutral sugars extracted from late maturation stage leucaena seeds; (B) purified unknown 1 hydrolyzed with TFA; and (C) purified unknown 2 hydrolyzed with TFA. A refractive index detector and Rezex RNM-Oligosaccharide column (300 mm × 7.8 mm i.d.) were used with ddH_2O. The flow rate was 0.4 ml min^{-1} and column temperature was at 75°C.

3.9 Hz, 1′-H). Based on the magnitude of $^3J_{H1',H2'}$ in this sugar (3.9 Hz), the anomeric configuration was assigned to be α-linked. The point of attachment of the galactosyl residue to the pinitol was established with the help of GC/MS and NOE experiments. According to the method of Schweizer and Horman (1981), unknown 1 was derivatized with iodoethane followed by acid hydrolysis and acetylation. The significant ions at m/z 233, 186, 172, 116 and 102 in EI spectrum (not shown) suggest that the acetylated galactosyl residue is connected to either 5 or 1 position of pinitol. More evidence was provided by NOE experiment. Irradiation of 1′-H (at $\delta=5.08$) caused a 16% NOE enhancement of 1-H (at $\delta=4.17$) and an enhancement of 2′-H (at $\delta=3.83$). Therefore, the galactosyl residue must link to the 1 position of pinitol. We did not distinguish between D-pinitol and L-pinitol but probably reasonable to assume that D-pinitol is contained in plants. The data of ^{13}CNMR ($CDCl_3$, 400 MHz) of 62.2, 63.4, 70.1, 70.8, 71.7, 71.9, 72.3, 73.2, 73.4, 73.6, 78.3, 85.2, 98.3 revealed thirteen carbons on this unknown 1. As a result of these analyses, unknown 1 was assigned as *O*-α-D-galactopyranosyl-(1 → 1)-3-*O*-methyl-D-*chiro*-inositol (Fig. 2), a type of galactopinitol.

Changes in seed moisture content, germination percentage and constituents during embryogenesis—The time from flowering to seed maturity in a dry and dark brown pod was approximately 104 d. Seed weights and moisture contents were determined after 42 DPA, when the seed was visible. Percent seed moisture declined from 76% to 20% while seed dry weight increased to 35 mg per seed during seed development (Fig. 3A). Fifty-three percent of freshly harvested seed at 70 DPA germinated. The highest germination percentage was measured at 97 DPA, and then declined slightly during the final stages. Developing seeds were green until 87 DPA, then appeared light yellow and

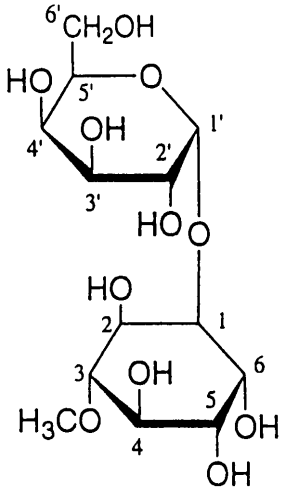

Fig. 2 Structure of *O*-α-D-galactopyranosyl-(1 → 1)-3-*O*-methyl-D-*chiro*-inositol.

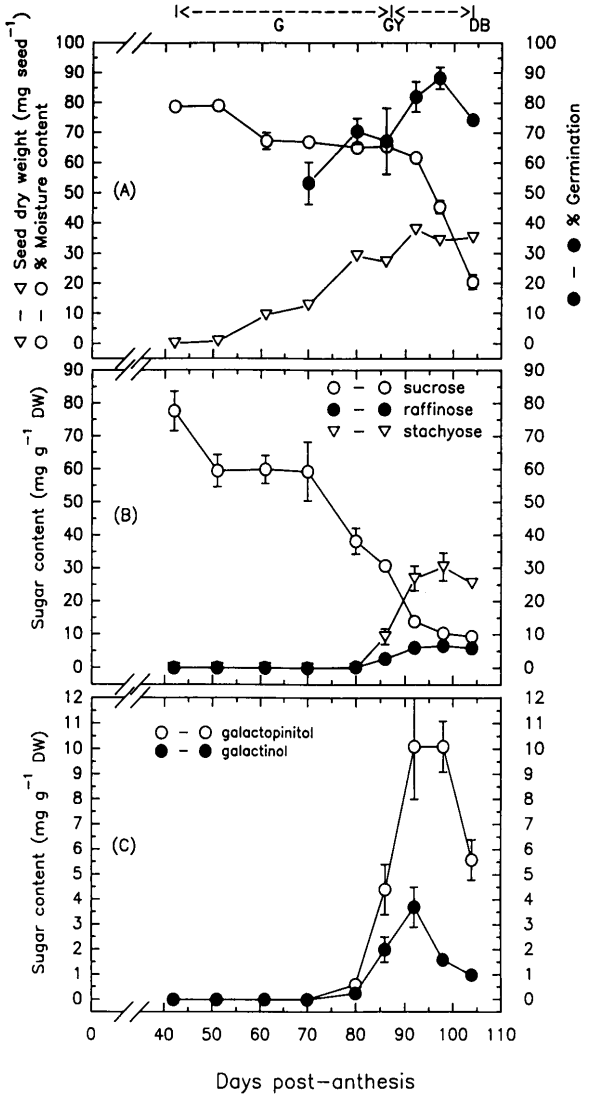

Fig. 3 Changes in (A) moisture content, seed dry weight, and germination percentage; (B) sucrose, raffinose and stachyose contents; and (C) galactopinitol and galactinol contents in developing leucaena seeds. Data are the means of at least three replicates. Vertical bars are ±SE. Seed color: G=green; GY=green–yellow; DB=dark brown.

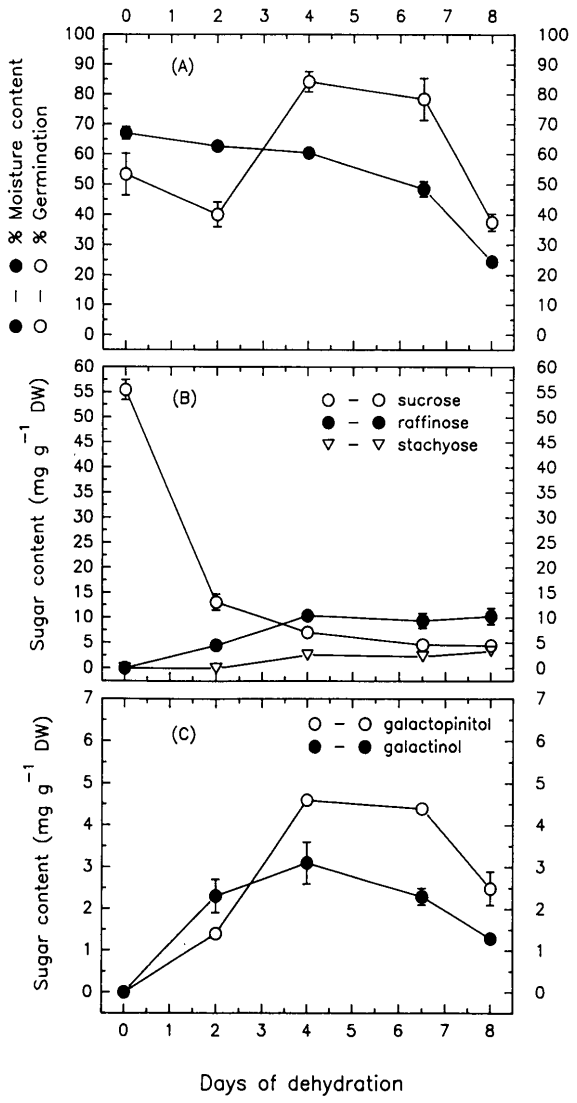

Fig. 4 Changes in (A) moisture content, germination percentage; (B) sucrose, raffinose and stachyose contents; (C) galactopinitol and galactinol contents in leucaena seeds harvested at 70 DPA, and dried at 73% RH and room temperature. Data are the means of at least three replicates. Vertical bars are ±SE.

progressively turned dark brown.

Soluble sugars in embryos of developing and mature seeds were analyzed. Sucrose content decreased greatly, whereas raffinose and stachyose were not detected until after 80 DPA (Fig. 3B). In subsequent days four times more stachyose accumulated than raffinose.

Using GC, galactopinitol and galactinol were separable with retention times of 30.0 and 30.3 min, respectively. Both galactopinitol and galactinol were undetectable until after 70 DPA (Fig. 3C). About three times more galactopinitol accumulated than galactinol at 92 DPA. Galactinol content declined first, followed by that of galactopinitol, which coincided with the increase in stachyose. Stachyose was the largest sugar component of mature seeds, followed in descending order by sucrose, raffinose, galactopinitol and galactinol.

Drying induces the accumulation of galactopinitol, galactinol, raffinose and stachyose—Fresh seeds harvested at 70 DPA, when there were no oligosaccharides other than sucrose present, were incubated at 73% RH and 25°C, and sampled every other day for 8 d. The short-term drying enhanced germination from 53% to 80% after 4 d, but germination then decreased to 37% after 8 d drying as moisture content gradually decreased from 67% to 24% (Fig. 4A).

Sucrose content declined rapidly, reaching about

4.5 mg $(g\ DW)^{-1}$ after 8 d drying, while raffinose and stachyose increased (Fig. 4B). Raffinose increased after 2 d drying but stachyose did not until 2 d later. Raffinose content was about twice as high as sucrose, and three times higher than stachyose at 8 d. Because raffinose accumulates but not stachyose, the conversion of raffinose to stachyose may be blocked at this stage.

During dehydration of immature seeds, both galactopinitol and galactinol increased (Fig. 4C). Galactopinitol content was slightly lower than galactinol after 2 d drying, but increased more rapidly than galactinol by 2 d later and maintained a higher content during dehydration. Consequently, the galactopinitol content was about twice as high as galactinol at the end of drying. However, the induced galactopinitol content in immature seeds was about half that of mature seeds.

Discussion

We describe a novel pinitol galactoside which has been identified as *O*-α-D-galactopyranosyl-(1 → 1)-3-*O*-methyl-*chiro*-inositol. The structure of this galactopinitol was verified by HPLC, GC-MS and NMR. A galactopinitol has previously been detected in a wide range of legume seeds, and was identified as *O*-α-D-galactopyranosyl-(1 → 2)-4-*O*-methyl-*chiro*-inositol for the major component of pinitol galactosides (Beveridge et al. 1977). Schweizer and Horman (1981) have also shown another minor isomer, *O*-α-D-galactopyranosyl-(1 → 2)-3-*O*-methyl-*chiro*-inositol, in soybean. Thus *O*-α-D-galactopyranosyl-(1 → 1)-3-*O*-methyl-*chiro*-inositol is the third isomer of galactopinitol and appears to be the major one in leucaena seeds. Our result seems to be in conflict with that of Beveridge et al. (1977) who claimed that *O*-α-D-galactopyranosyl-(1 → 2)-4-*O*-methyl-*chiro*-inositol was the major isomer in leucaena seeds and other pasture legume seeds. Since they did not detail the method, the reason for the difference is not clear.

Galactopinitol accumulated during seed maturation and rapidly decreased in the final stages of development (Fig. 3). Ganter et al. (1991) reported the presence of *O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 2)-3-*O*-methyl-D-*chiro*-inositol in seeds of *Mimosa scabrella*. We also detected the similar trigalactosyl cyclitol and a higher order of galactosyl cyclitols in mature leucaena seeds (data not shown). The rapid decrease of galactopinitol is probably associated with the biosynthesis of higher galactosyl oligomers.

Pinitol was detected throughout the seed development by HPLC, but high amounts of pinitol were found only at the beginning of galactopinitol accumulation and low amounts were present in mature seeds (data not shown). Since the *myo*-inositol is a substrate of galactinol synthase for the synthesis of galactinol (Dey 1990), pinitol probably is a precursor of galactopinitol.

Cyclitols such as pinitol and *myo*-inositol might act as membranes protectants (Crowe et al. 1988, Smirnoff and Cumbes 1989, Sommer et al. 1990), chemicals related to drought tolerance (Ford 1984, Nguyen and Lamant 1988, Keller and Ludlow 1993) and salt tolerance (Gorham et al. 1981). Galactosyl cyclitols such as galactinol and galacto-*chiro*-inositol in maturing seeds have been recently proposed as playing a role in stabilizing the structures of macromolecules and membranes during desiccation (Horbowicz and Obendorf 1994). The physiological role of galactopinitol, another species of galactosyl cyclitols, to desiccation tolerance of seeds needs further study.

Germination percentage of the immature seeds at 70 DPA increased during the process of artificial drying (Fig. 4) as reported in other orthodox seeds (Leprince et al. 1990). Germination decreased to 37% after 8 d drying (moisture content down to 24%) even though the mole ratio of oligosaccharides to sucrose is well above 1.0. The oligosaccharides/sucrose mole ratio is likely only a part of the total picture of desiccation tolerance, which also involves maturation proteins (Blackman et al. 1991) and membrane phase stability (Crowe and Crowe 1986). This dehydration damage probably is because of underdevelopment of seed tissue. Decreased germination percentage was also observed at the maturation stage (Fig. 3A). This decrease in germination is related to the formation of seed coat dormancy because scarification applied to the seed coat resulted in 98% germination.

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