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Cyclitol galactosides in embryos of buckwheat stem–leaf–seed explants fed D-*chiro*-inositol, *myo*-inositol or D-pinitol

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Abstract

Crop seeds accumulate soluble carbohydrates as part of their maturation process. In legume seeds, the major soluble carbohydrates are sucrose and its galactosides raffinose, stachyose and verbascose. In buckwheat (*Fagopyrum esculentum* Moench) seeds, the major soluble carbohydrates are sucrose and galactosides of D-*chiro*-inositol, named fagopyritols. This study was conducted to determine changes in soluble carbohydrate accumulation in embryos of buckwheat seeds after feeding solutions containing the free cyclitols D-*chiro*-inositol, *myo*-inositol and D-pinitol to stem–leaf–seed explants. Feeding D-*chiro*-inositol to explants resulted in a fourfold to fivefold increase in the accumulation of free D-*chiro*-inositol, fagopyritol A1 and fagopyritol B1 in embryos of mature seeds, but resulted in 30% less embryo dry weight compared to the control treatment without cyclitols. Feeding *myo*-inositol to buckwheat explants increased D-*chiro*-inositol in leaves and increased accumulation of fagopyritol A1 and fagopyritol B1 fivefold in embryos, fagopyritol A2 and fagopyritol B2 fourfold; fagopyritol A3 and fagopyritol B3 were also detected, with no reduction in accumulated embryo dry weight. Feeding D-pinitol to buckwheat explants resulted in accumulation of free D-pinitol in mature embryos, but not galactopinitols. D-Pinitol, galactopinitol A and galactopinitol B were not detected in embryos from explants fed solutions without D-pinitol. Feeding D-pinitol also resulted in reduced D-*chiro*-inositol accumulation by buckwheat seeds. The results indicate that *myo*-inositol may be the precursor to D-*chiro*-inositol synthesis, and fagopyritols accumulated in response to D-*chiro*-inositol availability in the embryo. We suggest that increasing *myo*-inositol in buckwheat maternal tissues may be an effective means to enhance the accumulation of D-*chiro*-inositol and fagopyritols in seeds, compounds that may be beneficial for the treatment of non-insulin-dependent diabetes mellitus.

Keywords: *Fagopyrum esculentum*, *Polygonaceae*, fagopyritol, D-*chiro*-inositol, buckwheat seed, cyclitol transport

Introduction

Crop seeds accumulate soluble carbohydrates as part of normal seed maturation (Horbowicz and Obendorf, 1994; Horbowicz *et al.*, 1998; Obendorf *et al.*, 1998). Embryos of buckwheat (*Fagopyrum esculentum* Moench, *Polygonaceae*) seeds accumulate six different fagopyritols (α-galactosides of D-*chiro*-inositol), in addition to sucrose and small amounts of D-*chiro*-inositol, *myo*-inositol, galactinol, raffinose and stachyose (Horbowicz *et al.*, 1998; Obendorf *et al.*, 2000; Steadman *et al.*, 2001b) (Fig. 1). Sucrose (42% of total) and fagopyritol B1 (40%) are the major soluble carbohydrates in buckwheat embryos (Horbowicz *et al.*, 1998).

In addition to raffinose family oligosaccharides (RFOs), some seeds also accumulate α-galactosides of cyclitols (Horbowicz and Obendorf, 1994; Obendorf, 1997). Two cyclitols found in buckwheat are D-*chiro*-inositol and *myo*-inositol (Horbowicz *et al.*, 1998; Obendorf *et al.*, 2000; Steadman *et al.*, 2001b). Unlike seeds of many legumes (Obendorf *et al.*, 1998; Peterbauer and Richter, 1998; Hoch *et al.*, 1999; Szczecinski *et al.*, 2000), buckwheat does not accumulate D-pinitol, D-ononitol or their galactosides (Fig. 1), and only small amounts of raffinose and stachyose accumulate in the axis of buckwheat embryos (Horbowicz *et al.*, 1998). *myo*-Inositol is common in seeds due to its importance in the metabolism of raffinose family galactosides, phytic

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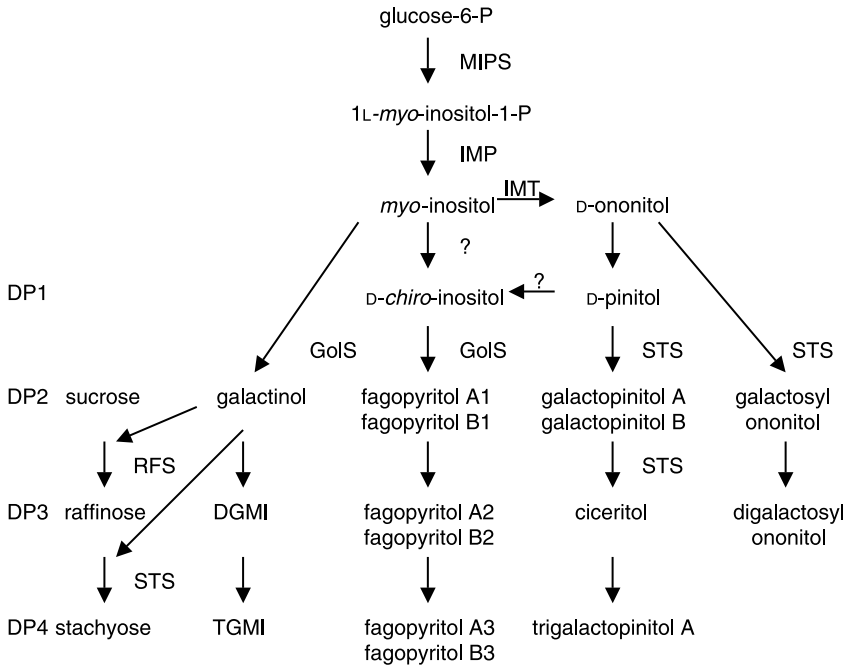

Figure 1. Proposed pathways for synthesis of cyclitols, cyclitol galactosides and raffinose family oligosaccharides (RFOs). Arrows without identification indicate that an enzyme catalysing the reaction has not been identified. Some reactions may be reversible. DP, degree of polymerization; DGMI, digalactosyl *myo*-inositol; TGMI, trigalactosyl *myo*-inositol; GolS, galactinol synthase (EC 2.4.1.123); IMP, *myo*-inositol-phosphate monophosphatase (EC 3.1.3.25); IMT, *myo*-inositol 4-methyltransferase (EC 2.1.1.129); MIPS, *myo*-inositol-phosphate synthase (EC 5.5.1.4); RFS, raffinose synthase (EC 2.4.1.82); STS, stachyose synthase (EC 2.4.1.67); ?, pathway not yet identified in higher plants (see review by Obendorf, 1997). For chemical structures, see Schweizer and Horman (1981), Quemener and Brillouet (1983), Horbowicz and Obendorf (1994), Obendorf (1997), Richter *et al.* (1997), Obendorf *et al.* (2000), Steadman *et al.* (2001b), Szczecinski *et al.* (2000) and Peterbauer *et al.* (2003).

acid and cell walls (Loewus and Murthy, 2000; Peterbauer and Richter, 2001). Buckwheat is an excellent dietary source of D-*chiro*-inositol in the form of its α-galactosides, fagopyritols, that accumulate in embryo tissues of seeds (Horbowicz *et al.*, 1998; Steadman *et al.*, 2000).

Fagopyritol B1 is the major α-galactoside accumulated in buckwheat embryos. The novel fagopyritol A series compounds, with the galactosyl α-(1 → 3) linkage to D-*chiro*-inositol, have been found only in buckwheat seeds (Obendorf *et al.*, 2000). Unique galactinol synthase homologues in buckwheat seeds form fagopyritol A1 and fagopyritol B1 (Ueda *et al.*, 2005). Soybean (*Glycine max* [L.] Merrill) galactinol synthase also forms fagopyritol B1 (Obendorf *et al.*, 2004). Fagopyritol A1 (Obendorf *et al.*, 2000) is isosteric with a putative insulin mediator, a galactosamine D-*chiro*-inositol (Larner *et al.*, 1988, 2003; Berlin *et al.*, 1990). Abnormal D-*chiro*-inositol metabolism and reduced insulin mediator have been found in subjects exhibiting non-insulin dependent diabetes mellitus (Larner and Huang, 1999; Larner, 2001). Consumption of buckwheat products has hypoglycaemic effects in diabetic patients (Lu *et al.*, 1992; Wang *et al.*, 1992). Feeding a buckwheat extract containing D-*chiro*-inositol and fagopyritols reduced serum glucose concentrations by 30–40% in streptozotocin-diabetic rats (Kawa *et al.*, 2003). Because of the potential health benefits, there is considerable interest in increasing the accumulation of fagopyritols in buckwheat seeds.

myo-Inositol phosphate synthase (MIPS, EC 5.5.1.4) is expressed both in maternal tissues and in embryo tissues (Johnson and Wang, 1996; Keller *et al.*, 1998; Hitz *et al.*, 2002), but the pathway for D-*chiro*-inositol formation has not been identified in higher plants (Fig. 1; reviewed by Obendorf, 1997). D-Pinitol and D-*chiro*-inositol are not formed in soybean embryos (Obendorf *et al.*, 1998, 2004; Odorcic and Obendorf, 2003), but are formed in maternal tissues (Dittrich and Brandl, 1987) and transported to seeds (Gomes *et al.*, 2005). Feeding D-*chiro*-inositol to soybean explants increased fagopyritol B1 and fagopyritol B2 concentrations in embryos of mature seeds, but did not increase raffinose and stachyose (Gomes *et al.*, 2005). We hypothesized that upon feeding free cyclitols to cut stems of buckwheat stem–leaf–seed explants, these carbohydrates would be taken up by maternal tissues

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and transported to seeds, resulting in large increases in accumulation of their respective galactosides in maturing embryos. Our objectives were to increase fagopyritols in buckwheat embryos for treatment of diabetes and to determine if accumulation of fagopyritols, galactinol or galactopinitols in embryos of buckwheat seeds could be enhanced by exogenously feeding the free cyclitols D-*chiro*-inositol, *myo*-inositol or D-pinitol to cut stems of buckwheat explants. Some of this work was part of an undergraduate research honours thesis (Ma, 2003).

Materials and methods

Common buckwheat (Mancan cultivar) seeds were sown in greenhouse potting medium in 4-litre pots in the greenhouse at 27°C days (14 h) and 22°C nights (10 h) with natural light, supplemented by 640 μmol m^{-2} s^{-1} artificial light from Sylvania 1000 W metal halide lamps (Horbowicz and Obendorf, 1992). Buckwheat plants were grown in four sets of 40 pots, with four plants per pot. Self-incompatibility was useful to synchronize seed development (Obendorf *et al.*, 1993) by cross-pollination of a group of plants on a single day (Horbowicz and Obendorf, 2005). After opening of the first flowers, plants with pin flowers and plants with thrum flowers were separated, thinned to one plant per pot, and placed in separate growth chambers constantly at 18°C and 300 μmol m^{-2} s^{-1} fluorescent light. After 1 week at 18°C, corresponding to the temperature and plant age for optimum seed set (Slawinska and Obendorf, 2001; Taylor and Obendorf, 2001), plants from each growth chamber were removed, and flowers were hand-pollinated (pin × thrum; thrum × pin) to transfer pollen for legitimate cross-pollination (Morris, 1952). Plants were returned to their respective chambers. One week after pollination, and before rapid growth of the embryos in developing seeds, stem–leaf–seed explants were excised, placed in water and brought to the laboratory for feeding experiments.

Stem–leaf–seed explants, with 8–12 seeds per explant, were excised at the base of the second internode below the terminal cluster of racemes on the main stem of each buckwheat plant. Typical of the upper stem on buckwheat plants, each explant had two small, bract-like leaves. The triangular fruit (achene) forms a single seed. The dicotyledonous embryo is rich in fagopyritols (Horbowicz *et al.*, 1998), lipids (Horbowicz and Obendorf, 1992) and high-quality proteins (Elpidina *et al.*, 1990), and is embedded in a starchy endosperm (Steadman *et al.*, 2001a). In a preliminary experiment, explants were fed solutions containing 100 mM *myo*-inositol, 100 mM D-pinitol or 100 mM D-*chiro*-inositol to load explants with free cyclitols, or a control without cyclitols. Each solution contained 30 mM sucrose, 10 mM asparagine and 10 μM kinetin. To assess the time sequence of cyclitol movement, seeds were collected at 1 or 5 days of feeding, and also at 5 days of feeding plus 2, 4 or 7 days of slow drying of explants to simulate precocious maturation of seeds. Embryos were isolated by cutting the proximal end of the seed and gently removing the embryo, free of endosperm, through the basal cut. Embryos were excised from freshly harvested seeds, frozen in liquid nitrogen and, without additional drying, were analysed for soluble carbohydrates as described below. Due to the limited amount of material, we could not determine dry weight. Results were expressed on a per embryo basis for one or two seeds from each of two explants at each harvest time. Leaf discs (10 mg fresh weight) were collected at 1, 24 or 72 h of feeding, frozen in liquid nitrogen, and analysed for soluble carbohydrates.

In the preliminary experiment (see Table 1), considerable variability was observed among seeds at different feeding and drying times. Under these conditions, embryos increased in fresh weight 8–16 d after pollination, and increased in dry weight 12–20 d after pollination. In subsequent experiments (see Tables 2 and 3), the number of explants was increased, cyclitol concentrations were reduced, and the duration of feeding was increased to 2 weeks, when all embryos had reached maximum mass. Embryos were analysed for soluble carbohydrates after removal from mature seeds, and the results were expressed on a dry weight basis, using estimated water concentrations. The cut basal end of the internode (stem) of each explant was placed in a solution containing 50 mM *myo*-inositol, 50 mM D-pinitol or 50 mM D-*chiro*-inositol. A fourth solution without cyclitols served as control. All solutions contained 30 mM sucrose, 10 mM asparagine and 10 μM kinetin. Solutions were fed through the cut stems of the explants for 2 weeks at 25°C and 300 μmol m^{-2} s^{-1} fluorescent light (continuous), and explants were allowed to dry at 25 °C.

All treatments given to the buckwheat explants were assigned in a randomized complete block design. Each experiment was replicated over time a total of four times, using 2–4 explants per treatment from each of the four different sets of buckwheat plants. Explants that became contaminated or failed to transport cyclitols were excluded. One to five seeds per explant were harvested for analysis. Embryo fragments were collected by hand from gently crushed, mature, dry seeds. Separations were aided by use of a magnification lens (10 ×).

To demonstrate accumulation of D-*chiro*-inositol in leaf tissues after feeding *myo*-inositol to buckwheat leaf–stem–seed explants, these explants were excised

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Table 1. Comparison of soluble carbohydrates (μg embryo^{-1}) extracted from embryos of developing seeds (12 d after pollination) after feeding buckwheat explants for 5 d with solutions containing 100 mM D-*chiro*-inositol, 100 mM *myo*-inositol or 100 mM D-pinitol, or a control without cyclitols, all in 30 mM sucrose, 10 mM asparagine and 10 μM kinetin

Soluble carbohydrate	D-*chiro*-Inositol ($N = 3$)	*myo*-Inositol ($N = 2$)	D-Pinitol ($N = 3$)	Control ($N = 2$)
D-*chiro*-Inositol	101.7 ± 12.0*	70.8 ± 45.4	13.0 ± 8.0	12.4 ± 6.3
Fagopyritol A1	71.2 ± 35.5	14.9 ± 3.3	14.8 ± 7.4	14.2 ± 11.0
Fagopyritol B1	326.4 ± 170.1	46.6 ± 27.5	55.6 ± 27.7	76.3 ± 61.6
Fagopyritol A2	4.0 ± 3.1	0.9 ± 0.9	0[a]	2.7 ± 2.7
Fagopyritol B2	2.4 ± 1.6	0.8 ± 0.7	0	3.4 ± 2.7
myo-Inositol	3.1 ± 0.8	4.8 ± 1.1	3.1 ± 0.9	9.7 ± 6.7
Galactinol	3.3 ± 0.3	11.4 ± 7.7	2.2 ± 1.1	10.3 ± 7.2
DGMI	0	0.4 ± 0.4	0	1.5 ± 1.2
D-Pinitol	0	0	265.1 ± 35.9*	0
Galactopinitol A[b]	0	0	4.0 ± 2.0	0
Galactopinitol B[b]	0	0	1.5 ± 0.2*	0
Sucrose	186.8 ± 52.0	150.1 ± 61.7	108.0 ± 20.0	368.1 ± 127.5

DGMI, digalactosyl *myo*-inositol.
Values are means ± SE.
*Means are significantly different ($P < 0.05$; *t*-test) from the control.
[a]0 = not detected.
[b]Identification not confirmed.

at the base of the third internode below the terminal cluster of racemes, to include one large leaf in addition to the two small, bract-like leaves. Five replicate explants were fed solutions containing 50 mM *myo*-inositol, 30 mM sucrose, 10 mM asparagine and 10 μM kinetin. Another five replicate explants were fed control solutions without cyclitols (30 mM sucrose, 10 mM asparagine and 10 μM kinetin). Three 1-cm^2 leaf discs were harvested from the leaf blade of each explant at 0 (1 h), 1, 2, 3, 4 and 5 d after feeding commenced, frozen in liquid nitrogen, and stored at −80°C.

Table 2. Comparison of soluble carbohydrates [mg (g dry weight)$^{-1}$] extracted from embryos of mature, dry seeds after feeding buckwheat explants with solutions containing 50 mM D-*chiro*-inositol, 50 mM *myo*-inositol or 50 mM D-pinitol, or a control without cyclitols, all in 30 mM sucrose, 10 mM asparagine and 10 μM kinetin for 14 d

Soluble carbohydrate	D-*chiro*-Inositol ($N = 24$)	*myo*-Inositol ($N = 22$)	D-Pinitol ($N = 18$)	Control ($N = 15$)
D-*chiro*-Inositol	1.6 ± 0.4*	0.8 ± 0.2*	0.1 ± 0.0	0.3 ± 0.2
Fagopyritol A1	2.9 ± 0.5*	3.8 ± 0.7*	0.6 ± 0.1	0.7 ± 0.1
Fagopyritol B1	17.9 ± 3.3*	23.6 ± 4.1*	2.8 ± 0.4	3.9 ± 0.4
Fagopyritol A2	0.9 ± 0.3	1.9 ± 0.4*	0.5 ± 0.1	0.5 ± 0.1
Fagopyritol B2	2.0 ± 0.6	3.0 ± 0.7*	0.5 ± 0.2	0.6 ± 0.2
Fagopyritol A3	0[a]	2.2 ± 0.6*	1.3 ± 1.0	0.6 ± 0.2
Fagopyritol B3	0	0.4 ± 0.2*	0	0
myo-Inositol	0.5 ± 0.2	0.6 ± 0.1*	0.1 ± 0.0	0.2 ± 0.1
Galactinol	2.7 ± 1.3	1.3 ± 0.4*	0.2 ± 0.0	0.2 ± 0.0
DGMI	0.7 ± 0.4	0.8 ± 0.2*	0.0 ± 0.0	0.1 ± 0.1
D-Pinitol	0	0	3.1 ± 0.6*	0
Galactopinitol A[b]	0	0	0.6 ± 0.1*	0
Galactopinitol B[b]	0	0	0.3 ± 0.0*	0
Sucrose	24.4 ± 8.7	29.4 ± 7.2*	4.4 ± 0.9	4.8 ± 0.6
Raffinose	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Stachyose	0.4 ± 0.2	0.7 ± 0.2	0.2 ± 0.1	0.4 ± 0.2
Dry weight of embryos (mg ± SE)	2.8 ± 0.3*	4.8 ± 0.5	4.3 ± 0.5	4.9 ± 0.6
Percent (%) of control weight	57	99	89	100

DGMI, digalactosyl *myo*-inositol.
Values are means ± SE.
*Means are significantly different ($P < 0.05$; *t*-test) from the control.
[a]0 = not detected.
[b]Identification not confirmed.

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Table 3. Comparison of soluble carbohydrates [mg (g DW)$^{-1}$] extracted from embryos of mature buckwheat seeds after feeding buckwheat explant solutions containing 50 mM D-*chiro*-inositol, 50 mM D-pinitol and 50 mM D-*chiro*-inositol or 50 mM D-pinitol, or a control without cyclitols, all in 30 mM sucrose, 10 mM asparagine and 10 μM kinetin for 14 d

Soluble carbohydrate	D-*chiro*-Inositol ($N = 18$)	D-*chiro*-Inositol and D-pinitol ($N = 12$)	D-Pinitol ($N = 23$)	Control ($N = 9$)
D-*chiro*-inositol	33.9 ± 4.4*	10.9 ± 2.5*	2.5 ± 0.6	2.1 ± 0.4
Fagopyritol A1	7.4 ± 1.1*	6.6 ± 1.4	3.8 ± 0.5	4.1 ± 0.5
Fagopyritol B1	69.2 ± 6.4*	51.3 ± 8.2*	17.3 ± 1.7*	24.9 ± 2.6
Fagopyritol A2	1.4 ± 0.5*	1.4 ± 0.3*	0.7 ± 0.2*	4.1 ± 0.4
Fagopyritol B2	1.4 ± 0.2*	1.8 ± 0.4*	1.5 ± 0.3*	6.2 ± 0.7
Fagopyritol A3	0.1 ± 0.1*	0.2 ± 0.1*	0.5 ± 0.3*	3.1 ± 0.4
Fagopyritol B3	0[a]	0	0	0.5 ± 0.5
myo-Inositol	0.3 ± 0.1	0.2 ± 0.0	0.5 ± 0.1*	0.1 ± 0.0
Galactinol	0.5 ± 0.1*	0.3 ± 0.1*	0.8 ± 0.3	1.7 ± 0.4
DGMI	0*	0*	0.1 ± 0.0*	1.5 ± 0.3
D-Pinitol	0	31.7 ± 3.4*	195.6 ± 27.9*	0
Galactopinitol A[b]	0	1.4 ± 0.2*	2.1 ± 0.4*	0
Galactopinitol B[b]	0	0.7 ± 0.1*	0.7 ± 0.1*	0
Sucrose	18.1 ± 3.4*	26.5 ± 9.0	27.9 ± 4.3	44.3 ± 8.0
Raffinose	0	0	0	0
Stachyose	0.1 ± 0.1*	0.1 ± 0.1*	0 ± 0*	0.9 ± 0.2

DGMI, digalactosyl *myo*-inositol.
Values are means ± SE.
*Means are significantly different ($P < 0.05$; *t*-test) from the control.
[a]0 = not detected.
[b]Identification not confirmed.

Soluble carbohydrates were extracted from embryos and leaf discs and analysed. Embryo tissues were isolated, weighed and homogenized in a ground-glass homogenizer with 2.2 ml of ethanol: water (1:1, v/v), containing 300 μg of phenyl α-D-glucoside as an internal standard, and centrifuged at 27,000 × *g* for 20 min. Leaf discs frozen in liquid nitrogen were pulverized to a fine powder in a cold mortar. The frozen powder was homogenized in 600 μl ethanol:water (1:1, v/v) containing 100 μg of phenyl α-D-glucoside as an internal standard, and centrifuged at 15,000 × *g* for 15 min. Clear supernatants were passed through a 10,000 MW cutoff filter and evaporated to dryness with nitrogen gas. Residues were stored overnight in a desiccator above P_2O_5 to remove traces of water, and the dry residue was derivatized with trimethylsilylimidazole:pyridine (1:1, v/v) for 45 min at 80 °C. Analysis of derivatized soluble carbohydrates was performed as described by Horbowicz and Obendorf (1994), using a Hewlett Packard 6890 Series gas chromatograph. Soluble carbohydrate compositions of embryos are reported as mean ± SE of the mean on a dry weight basis for each mature buckwheat seed analysed. Soluble carbohydrates in leaf discs were expressed on a leaf area basis. A value of zero (0) means the compound was not detected. Statistical differences ($P < 0.05$) between means were verified by a *t*-test.

In a substrate competition experiment (see Table 3), explants were fed solutions containing 50 mM D-pinitol, 50 mM D-*chiro*-inositol, or 50 mM D-pinitol and 50 mM D-*chiro*-inositol in combination, or a control solution without cyclitols; all solutions contained 30 mM sucrose, 10 mM asparagine and 10 μM kinetin for 2 weeks as above. Since ambient relative humidities were variable at harvest, seeds were transferred to desiccators and equilibrated to 6% moisture during a period of 7 d over a saturated solution of LiCl (12% relative humidity). Embryos were excised from mature, dry seeds and analysed for soluble carbohydrates as described above.

Fructose, glucose, maltose, sucrose, raffinose, stachyose, *myo*-inositol, phenyl α-D-glucoside, trimethylsilylimidazole and pyridine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). D-Pinitol and D-*chiro*-inositol were purchased from Industrial Research Limited (Lower Hutt, New Zealand). Galactinol was purified from lemon balm (*Melissa officinalis* L.) leaves. Fagopyritols and digalactosyl *myo*-inositol [DGMI; α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 1)-1L-*myo*-inositol] were purified from buckwheat seeds (Obendorf *et al.*, 2000; Steadman *et al.*, 2001b). Galactopinitol standards were a gift. When needed, soluble carbohydrate standards were purified by carbon (Mallinckrodt Baker Inc., Phillipsburg, New Jersy, USA) –Celite (Supelco, Bellefonte, Pennsylvania, USA) column chromatography (Whistler and Durso, 1950).

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Results

Preliminary feeding–drying time experiment (Experiment 1)

Leaf discs from explants contained *myo*-inositol [0.40 ± 0.01 μg (mg fresh weight)$^{-1}$], D-*chiro*-inositol (0.48 ± 0.06 μg mg^{-1}), glucose (3.3 ± 1.3 μg mg^{-1}), fructose (4.0 ± 1.7 μg mg^{-1}), and sucrose (2.1 ± 0.9 μg mg^{-1}). Fagopyritols, galactopinitols, galactinol, raffinose, stachyose or D-pinitol were not detected. Concentrations of D-*chiro*-inositol in leaf discs were fivefold or tenfold higher than in the control treatment at 24 or 72 h, respectively, after feeding explants with D-*chiro*-inositol (data not shown). After feeding explants with D-pinitol for 1, 24 or 72 h, free D-pinitol concentrations in leaf disks were 0.2, 6.5 or 15.2 μg mg^{-1}fresh weight, respectively.

After the first day of feeding buckwheat explants, all embryos had accumulated sucrose (150–300 μg embryo^{-1}) and small amounts of free D-*chiro*-inositol (2–6 μg embryo^{-1}) and free *myo*-inositol (1–5 μg embryo^{-1}). Fagopyritols, galactinol, galactopinitols, raffinose and stachyose were not detected in embryos from all feeding treatments (data not shown). Only those explants fed D-pinitol accumulated free D-pinitol in the embryo (35 μg embryo^{-1}) after 1 d of feeding.

After feeding D-*chiro*-inositol to explants for 5 d, embryos had accumulated eightfold more free D-*chiro*-inositol than embryos in the control treatment (Table 1). Fagopyritol A3, fagopyritol B3, raffinose, stachyose, D-pinitol and galactopinitols were not detected. After feeding *myo*-inositol to explants for 5 d, the concentration of *myo*-inositol in embryos was not different from the control. Only embryos from explants fed D-pinitol for 5 d accumulated large amounts of free D-pinitol (Table 1). Very small peaks (similar to background) on the gas chromatograms, corresponding to the retention times of galactopinitol A and galactopinitol B, were insufficient for identification of these compounds. If present, the amounts of galactopinitol A and galactopinitol B were small in embryos from explants fed D-pinitol for 5 d (Table 1), and did not increase during slow drying for 2, 4 or 7 d (data not shown). Since data were variable for all treatments, the number of explants was increased in subsequent experiments, and feeding duration was extended to 2 weeks (21 d after pollination and after maximum embryo dry weight).

Main cyclitol feeding experiment (Experiment 2)

After feeding 50 mM D-*chiro*-inositol to buckwheat explants for 2 weeks, the free D-*chiro*-inositol concentration was fivefold higher in embryos of mature dry seeds, compared to embryos from the control treatment without cyclitols (Table 2). Feeding D-*chiro*-inositol also increased the concentrations of fagopyritol A1 and fagopyritol B1 in embryos fourfold. Concentrations of the higher fagopyritol oligomers, free *myo*-inositol, galactinol, DGMI, sucrose, raffinose, stachyose and reducing sugars in embryos were not significantly different from the control (data not shown). D-Pinitol and galactopinitols were not detected. Sucrose concentrations were variable. After feeding explants D-*chiro*-inositol, embryos from mature dry seeds had 40% less dry matter than control embryos (Table 2). In a separate experiment, leaf discs harvested from explants at 2–5 d during *myo*-inositol feeding accumulated tenfold more D-*chiro*-inositol (Fig. 2) and 70-fold more *myo*-inositol (data not shown) compared to the control. During feeding, sucrose and maltose increased fivefold and glucose and fructose increased 20-fold in leaves of explants, but the mean values were not significantly different ($P > 0.05$, *t*-test) between explants fed *myo*-inositol and those fed a control solution (data not shown).

Feeding 50 mM *myo*-inositol to buckwheat explants slightly increased free D-*chiro*-inositol in embryos from mature seeds, but the concentrations of fagopyritols in embryos were three- to fourfold higher than in embryos from the control without cyclitols (Table 2). Concentrations of free *myo*-inositol, galactinol and DGMI in embryos were significantly higher than in the control, but remained low. D-Pinitol

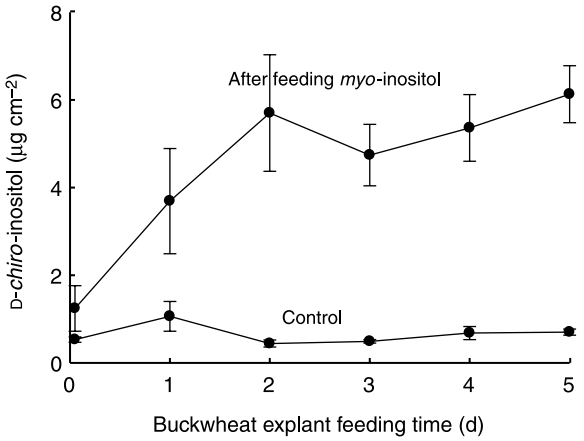

Figure 2. D-*chiro*-Inositol accumulation in leaf discs of buckwheat explants fed a solution of 50 mM *myo*-inositol, 30 mM sucrose, 10 mM asparagine and 10 μM kinetin or a control solution (30 mM sucrose, 10 mM asparagine and 10 μM kinetin) for 1 h to 5 d. Values are mean ± SE of the mean of extracts from three 1-cm^2 leaf discs for five replicate explants per treatment each day. Means for explants fed *myo*-inositol for 1 to 5 d are significantly different ($P < 0.05$, *t*-test) from the control.

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and galactopinitols were not detected. Sucrose concentration was fivefold higher than in the control treatment, while concentrations of raffinose, stachyose and reducing sugars (data not shown) were not significantly different from the control. Embryo dry weight was the same as that of the control (Table 2).

Feeding 50 mM D-pinitol to buckwheat explants increased free D-pinitol in embryos of mature, dry seeds, but had no significant effect on the concentrations of other soluble carbohydrates or the dry weight of embryos (Table 2). Galactopinitol A and galactopinitol B did not accumulate (their presence was not confirmed).

Substrate competition experiment (Experiment 3)

Feeding both D-*chiro*-inositol and D-pinitol to buckwheat explants resulted in a 68% lower free D-*chiro*-inositol concentration in embryos of mature dry seeds, compared to embryos from explants fed D-*chiro*-inositol, while the concentration of other soluble carbohydrates was similar (Table 3). The concentration of free D-pinitol in buckwheat embryos from explants fed both D-*chiro*-inositol and D-pinitol was 84% less than in embryos from explants fed D-pinitol. While very large concentrations of free D-pinitol accumulated in embryos from explants fed D-pinitol or both D-*chiro*-inositol and D-pinitol, its galactosides, galactopinitol A and galactopinitol B, did not accumulate, or, if present, were in very small concentrations. Concentrations of the remaining soluble carbohydrates were similar in embryos from explants fed D-pinitol and in embryos from explants fed a control solution without cyclitols (Table 3).

Discussion

Two free cyclitols were found naturally in buckwheat embryos: D-*chiro*-inositol and *myo*-inositol (Horbowicz *et al.*, 1998; Obendorf *et al.*, 2000; Steadman *et al.*, 2001b). Control explants fed a solution without cyclitols accumulated D-*chiro*-inositol and *myo*-inositol, primarily as fagopyritols and galactinol, respectively, in embryos. Sucrose, *myo*-inositol and D-*chiro*-inositol, plus small and variable amounts of reducing sugars, were the first soluble carbohydrates to accumulate in developing seeds, as previously described (Horbowicz *et al.*, 1998). Fagopyritol B1 and lesser amounts of fagopyritol A1 then accumulated, with a decline in free D-*chiro*-inositol, and served as precursors to the subsequent accumulation of the higher fagopyritol oligomers (Fig. 1). Galactinol accumulated in a transient pattern in embryos, while free *myo*-inositol remained relatively low, reflecting the role of *myo*-inositol in the synthesis of galactinol (Ueda *et al.*, 2005), phytin and cell walls (Loewus and Murthy, 2000). Reducing sugars declined to trace amounts, and only small amounts of raffinose and stachyose were detected in mature dry embryos.

Exogenously supplied D-*chiro*-inositol fed to buckwheat explants markedly increased the concentration of free D-*chiro*-inositol in embryos, and fagopyritol A1 and fagopyritol B1 concentrations increased dramatically in response to the supply of D-*chiro*-inositol to embryos. The digalactosyl oligomers, fagopyritol A2 and fagopyritol B2 (Fig. 1), increased in lower concentrations. Since fagopyritols were not detected in leaves or maternal tissues, fagopyritol A1 and fagopyritol B1 were formed in embryos in response to the supply of free D-*chiro*-inositol. Previous work demonstrated that a unique galactinol synthase formed fagopyritol A1 and fagopyritol B1 using D-*chiro*-inositol as the galactosyl acceptor and UDP-galactose as the galactosyl donor (Ueda *et al.*, 2005). Typical ratios for fagopyritol A1 to fagopyritol B1, fagopyritol A2 to fagopyritol B2, and fagopyritol A3 to fagopyritol B3, respectively, were 1:6.12, 1:1.28, and 1:0.25 in extracts from mature seed tissues (Steadman *et al.*, 2000, 2001b), indicating that the fagopyritol A series compounds were favoured for elongation. Fagopyritol A1 and fagopyritol B1 accumulations were enhanced by cool temperature, while accumulations of their higher galactosyl oligomers (fagopyritol A2, fagopyritol B2, fagopyritol A3 and fagopyritol B3) were enhanced by warm temperature (Horbowicz *et al.*, 1998; Horbowicz and Obendorf, 2005). Accumulation of higher oligomers in embryos of explants was limited, since high temperatures were avoided. In contrast to the synthesis of fagopyritol A1 and fagopyritol B1, the higher fagopyritol oligomers were not formed by buckwheat galactinol synthase (Ueda *et al.*, 2005). An enzyme to catalyse the elongation to their respective di- and tri-galactosides in buckwheat embryos has not been identified (Fig. 1). Legume seed stachyose synthase (STS, galactinol:raffinose galactosyltransferase, EC 2.4.1.67) exhibited elongase activities that formed higher α-galactosyl oligomers (Hoch *et al.*, 1999; Peterbauer *et al.*, 2002), and also catalysed the formation of α-galactosides of D-pinitol (Hoch *et al.*, 1999). Stachyose synthase activity has not been reported in buckwheat, and raffinose and stachyose accumulation was limited to very low concentrations, mostly in the axis of buckwheat embryos (Horbowicz *et al.*, 1998).

Feeding D-*chiro*-inositol to buckwheat explants resulted in decreased embryo dry weight. Gomes *et al.* (2005) also reported a shrivelling of soybean seeds and reduced cotyledon dry weight after feeding free D-*chiro*-inositol to soybean explants. Feeding *myo*-inositol to buckwheat explants dramatically increased the concentrations of free D-*chiro*-inositol in maternal tissues and fagopyritol B1 in embryos without a reduction in embryo dry weight.

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Buckwheat embryos accumulated D-pinitol only after treatments with D-pinitol supplied exogenously, since buckwheat does not naturally synthesize D-pinitol (Horbowicz and Obendorf, 1994; Horbowicz *et al.*, 1998; Steadman *et al.*, 2000). Feeding D-*chiro*-inositol and D-pinitol together to isolated soybean embryos reduced D-*chiro*-inositol uptake into embryos by 50%, compared to feeding D-*chiro*-inositol alone; D-*chiro*-inositol did not inhibit the uptake of D-pinitol (Odorcic and Obendorf, 2003; Obendorf *et al.*, 2004). Similarly, a 68% reduction in accumulation of D-*chiro*-inositol in the presence of D-pinitol was also observed in buckwheat embryos, presumably through reduced transport and/or uptake of D-*chiro*-inositol into embryo tissues.

It was previously hypothesized that D-*chiro*-inositol synthesis may be through D-pinitol as an intermediate (Horbowicz and Obendorf, 1994; Obendorf, 1997); however, the present data do not support this hypothesis for buckwheat. Feeding D-pinitol to buckwheat explants did not increase D-*chiro*-inositol or fagopyritol accumulation in embryos. Since D-pinitol was not detected in buckwheat, it is unlikely that D-pinitol could serve as an intermediate for D-*chiro*-inositol synthesis (Fig. 1). While exogenously fed D-pinitol accumulated as free D-pinitol in buckwheat embryos, galactopinitols did not accumulate in response to supply of free D-pinitol. This is consistent with the evidence that buckwheat galactinol synthase can form fagopyritols (Ueda *et al.*, 2005), but neither buckwheat nor soybean galactinol synthase use D-pinitol as a substrate (Obendorf *et al.*, 2004; Ueda *et al.*, 2005).

Buckwheat embryos accumulate only very low amounts of stachyose, mostly in the axis (Horbowicz *et al.*, 1998). Since stachyose synthase catalyses the synthesis of D-pinitol galactosides in soybean (T.P. Lin and R.L. Obendorf, unpublished, 1998) and some other seeds (Hoch *et al.*, 1999; Peterbauer *et al.*, 2002), the lack of galactopinitol A and galactopinitol B accumulation in embryos of buckwheat explants fed D-pinitol, despite high amounts of the accumulated substrate D-pinitol, is consistent with the minimal accumulation of stachyose in buckwheat embryos.

While we cannot rule out the possibility of D-*chiro*-inositol synthesis in buckwheat embryos, our data are consistent with the hypothesis that D-*chiro*-inositol in maternal tissues was transported to seeds (embryos), as has been demonstrated in soybean (Odorcic and Obendorf, 2003; Obendorf *et al.*, 2004; Gomes *et al.*, 2005). A 20-fold increase in fagopyritol B1 and a tenfold increase in fagopyritol B2 were found in embryos after feeding free D-*chiro*-inositol to soybean explants (Gomes *et al.*, 2005). If D-*chiro*-inositol were synthesized naturally in the embryo, feeding this exogenous source should not be expected to produce such a noticeable increase in fagopyritols in either buckwheat or soybean embryos. The synthesis of D-*chiro*-inositol thus appears to be directly from *myo*-inositol (Fig. 1), since D-pinitol, an intermediate in the alternative pathway, is not naturally present in buckwheat, whereas D-*chiro*-inositol increased after feeding *myo*-inositol.

Collectively, these results lead to the suggestion that an increase in the supply of D-*chiro*-inositol, possibly by increasing *myo*-inositol in maternal tissues, may result in enhanced fagopyritol accumulation in embryos of maturing buckwheat seeds, compounds with potential hypoglycaemic effects in patients with non-insulin dependent diabetes mellitus.

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