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Feeding stem–leaf–pod explants of pea (*Pisum sativum* L.) with D-*chiro*-inositol or D-pinitol modifies composition of α-D-galactosides in developing seeds

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Abstract

Feeding stem–leaf–pod explants with D-*chiro*-inositol and D-pinitol was used as a method to modify α-D-galactosides in developing pea (*Pisum sativum*) seeds. Four genotypes differing in the composition of raffinose, stachyose and verbascose (raffinose family oligosaccharides or RFOs) in seeds – high RFOs (cv. Tiny), low RFOs (SZD175) and low verbascose (cv. Hubal and cv. Wt 506) – were studied. Although seeds of all examined pea lines were able to take up both D-*chiro*-inositol and D-pinitol, only D-*chiro*-inositol was effectively converted into its galactosides: mainly fagopyritol B1 (*O*-α-D-galactopyranosyl-(1 → 2)-D-*chiro*-inositol) and fagopyritol B2 (*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 2)-D-*chiro*-inositol). In seeds of pea lines naturally containing low levels of verbascose (cv. Hubal) and low RFOs (SZD175), the enhanced accumulation of fagopyritols depressed the RFO level by *c.* 64 and 20%, respectively. Moreover, in both genotypes, about 25 and 30% of total galactose bound in α-D-galactosides occurred in fagopyritols. D-Pinitol present in the pea seeds was converted into monogalactosides, but their accumulation was several-fold lower than that of fagopyritols and did not significantly influence the accumulation of RFOs. Pea seeds with the composition of soluble carbohydrates modified by feeding with either of the cyclitols were able to complete germination.

Keywords: galactosyl cyclitols, D-*chiro*-inositol, pea, D-pinitol, raffinose oligosaccharides, seeds

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Introduction

The raffinose family of oligosaccharides (RFOs), including raffinose, stachyose, verbascose and ajugose, are common constituents of legume seeds (Horbowicz and Obendorf, 1994). RFOs act as a storage material and are quickly degraded during the early stages of seed germination (Blöchl *et al.*, 2007; Lahuta and Goszczyńska, 2009). In maturing seeds, accumulation of RFOs coincides with the acquisition of seed desiccation tolerance (Obendorf, 1997). Legume seeds become germinable after attaining an appropriate level of RFOs during the maturation process. However, a high level of RFOs in legume seeds makes the seeds less desirable for human consumption. RFOs are considered an anti-nutritional factor because they are not digested by humans and monogastric animals, cause flatulence, and decrease the metabolizable energy of a diet (Coon *et al.*, 1990). Therefore, removal of RFOs from legume meal is an important target in plant breeding, which can be achieved by introduction of lines with a decreased content of stachyose or raffinose, as is done in soybean (Hitz *et al.*, 2002; Dierking and Bilyeu, 2008), or by genetic transformation. Suppression of the gene encoding galactinol synthase (GOLS: the key enzyme for the RFO biosynthetic pathway) in *Brassica napus*, using an antisense approach, substantially reduced the accumulation of galactinol (*O*-α-D-galactopyranosyl-(1 → 1)-L-*myo*-inositol) and stachyose in mature seeds (Bock *et al.*, 2009). Transgenic pea lines expressing the α-D-*GALACTOSIDASE* gene from coffee (*Coffea arabica*) showed a significant reduction (up to 40%) in the oligosaccharide content of seeds (Polowick *et al.*, 2009). An alternative strategy may be to transform plants with a gene encoding the enzyme that synthesizes D-ononitol (4-*O*-methyl-D-*myo*-inositol) (Chiera *et al.*, 2006). This method has resulted in overproduction of D-ononitol

and D-pinitol in somatic embryos, potentially at the expense of RFOs, although the effect on the level of RFOs in seeds has not been analysed. However, there is some indirect evidence suggesting that an elevated level of D-pinitol (3-*O*-methyl-D-*chiro*-inositol) or D-*chiro*-inositol in soybean, *Vicia hirsuta* [L.] S.F. Gray and *Vicia tetrasperma* L. Schreb., caused accumulation of appropriate galactosyl cyclitols and depressed the content of stachyose and/or verbascose in seeds (Gomes *et al.*, 2005; Lahuta *et al.*, 2005a, b). An increased supply of D-*chiro*-inositol and *myo*-inositol to developing seeds of soybean lines which contained relatively low stachyose or raffinose increased the level of fagopyritol B1 or stachyose, respectively, in seeds. However, feeding explants with D-pinitol did not alter the composition of RFOs in mature soybean seeds (Obendorf *et al.*, 2008).

The incorporation of cyclitols into the biosynthetic pathway of α-D-galactosides is closely associated with the substrate specificity of the enzymes or the presence of different isozymes. The first committed step in the biosynthetic pathway of RFOs is initiated by GOLS, which catalyses the synthesis of galactinol (*O*-α-D-galactopyranosyl-(1 → 1)-L-*myo*-inositol) from UDP-galactose and *myo*-inositol. GOLS can also use D-*chiro*-inositol (but not D-pinitol) as a galactosyl acceptor, producing fagopyritol B1 (Obendorf *et al.*, 2004) or fagopyritol B1 and fagopyritol A1 in a 4:1 mole ratio (Ueda *et al.*, 2005). Galactinol serves as a major galactosyl donor for the synthesis of raffinose and its higher homologues – stachyose and verbascose. Each step includes transfer of a galactose moiety from galactinol to an appropriate substrate: sucrose, raffinose and stachyose, respectively (Obendorf, 1997). A raffinose synthase (RS) channels sucrose into the RFOs pathway. RS, which was extracted from pea (*Pisum sativum*) seeds and partially purified, can also use D-ononitol and D-pinitol as galactosyl acceptors and synthesize their mono-galactosides (Peterbauer *et al.*, 2002a). Contrary to RS, stachyose synthase (STS) from pea seeds demonstrates higher affinity for D-ononitol and D-sequoitol (5-*O*-methyl-*myo*-inositol) as galactosyl acceptors, and can use ononitol galactoside as a galactose donor for stachyose synthesis (Peterbauer *et al.*, 2002b). STS purified from lentil (*Lens culinaris*) seeds can catalyse a range of various galactosyl transfer reactions. STS uses galactinol as a galactose donor and can catalyse the synthesis of galactosyl pinitol A (GPA, *O*-α-D-galactopyranosyl-(1 → 3)-4-*O*-methyl-D-*chiro*-inositol) from D-pinitol or ciceritol (*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 3)-4-*O*-methyl-D-*chiro*-inositol) from GPA (Hoch *et al.*, 1999). STS is a multifunctional enzyme that can also be responsible for the synthesis of verbascose (Peterbauer *et al.*, 2003). Less is known about the biosynthesis of tri-galactosides of cyclitols in seeds of some legumes (Yasui and Ohashi, 1990). In maturing seeds of winter vetch (*Vicia villosa*) both verbascose (tri-galactoside of sucrose) and tri-galactosyl pinitol A can be synthesized by the same enzyme, but presumably it is not STS (Lahuta, 2006).

The inhibitory effect of exogenously supplied D-*chiro*-inositol on the accumulation of RFOs in seeds of *V. hirsuta* (Lahuta *et al.*, 2005a) and *V. tetrasperma* (Lahuta *et al.*, 2005b) can be explained by competition between the GOLSs that catalysed synthesis of galactinol (galactoside of *myo*-inositol) and fagopyritol B1 (galactoside of D-*chiro*-inositol). A decreased level of galactinol can, in turn, inhibit the synthesis of verbascose or stachyose. Additionally, an increased level of fagopyritol B1 redirects the synthesis of α-D-galactosides into di- and tri-galactosyl D-*chiro*-inositols (fagopyritols A2, B2 and B3). Feeding D-pinitol to vetch explants (*V. hirsuta* and *V. tetrasperma*) increased its level in seeds and enhanced the content of galactosyl pinitols, simultaneously decreasing the accumulation of RFOs (Lahuta *et al.*, 2005a, b). Thus, it may be expected that in pea seeds, naturally containing *myo*-inositol as the sole cyclitol, introduction of D-pinitol, or D-*chiro*-inositol, could decrease the synthesis of RFOs via promotion of the synthesis of galactosyl cyclitols. This hypothesis has been verified in the present experiment, in which explants of four pea genotypes differing in the content and composition of RFOs in seeds were fed with D-pinitol and D-*chiro*-inositol. The results indicate that feeding stem–leaf–pod explants with cyclitols can be a useful strategy to monitor the potential of different genotypes, including cultivars and breeding lines, for modification of the composition of α-D-galactosides in seeds, without plant genetic transformation. The effect of the modification on seed germinability has also been tested.

Materials and methods

Plant material

Seeds of pea (*Pisum sativum*) were obtained from the Polish *Pisum* Genebank in Wiatrowo, which included cv. Tiny (catalogue no. Wt 2201), a vegetable-type pea with wrinkled seeds; cv. Hubal (Wt 7537), a fodder type; SZD175, a breeding line with smooth seeds; and Wt 506, a genebank accession belonging to *P. sativum* ssp. *asiaticum*. Our preliminary study on seed RFOs using 18 pea cultivars and 190 lines had shown that the total RFO levels in seeds ranged from 37.73 up to 110.00 mg g^{-1} of dry weight (DW), with the mean content (± SD) being 64.03 ± 3.42 mg (g DW)$^{-1}$. Seeds of different genotypes varied also in RFO composition: among RFOs, the dominant ones were verbascose (49% of genotypes), stachyose (36%) or both oligosaccharides at *c*. equal amounts (15% of genotypes).

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Four genotypes were selected for feeding experiments, based on the composition of RFOs in seeds (Table 1): cv. Tiny, high RFOs [105.77 ± 5.38 mg (g DW)$^{-1}$, stachyose content was higher than verbascose], SZD175, low RFOs [42.38 ± 1.18 mg (g DW)$^{-1}$, verbascose content was higher than stachyose], cv. Hubal and Wt 506, moderate RFOs [58.02 ± 0.80 and 59.56 ± 2.43 mg (g DW)$^{-1}$, respectively] and low verbascose [8.31 ± 0.19 and 3.16 ± 0.04 mg (g DW)$^{-1}$, respectively]. It should be noted that the concentration of verbascose in seeds below 10 mg (g DW)$^{-1}$ was found in only four genotypes among 208 genotypes from the genebank.

Pea plants were grown in 10 dm^3 pots (20 cm diameter, 32 cm height, five plants per pot), containing soil (SUBSTRAL Osmocote, Scotts, Poland), under greenhouse conditions: 20–22°C in the day (14 h) and 14–16°C at night (10 h, February to April). Natural light was supplemented with fluorescent lamps (light intensity, *c*. 200 μmol m^{-2} s^{-1}). The plants were watered daily. All four genotypes exhibited distinct phenotypes. Tiny, Wt 506 and SZD175 developed white flowers (no anthocyanin synthesis excluding Wt 506), while cv. Hubal had pink-red flowers (anthocyanin synthesis). Seeds of Wt 506 and cv. Hubal had a coloured seed coat. Embryos of all the accessions had yellow cotyledons. The main stems of the tallest plants, Tiny and Wt 506 (180–200 cm) branched into 2–3 stems. The plants of Hubal and SZD175 were shorter (90 and 100 cm) and lacked stem branches. The SZD175 leaves were transformed into tendrils (the gene *afila*), while the other three pea lines developed leaves of a normal shape. The first flowers fully opened on 55–60 (cv. Tiny and WT 506), 65 (cv. Hubal) or 85 (SZD175) days after sowing. Fully opened flowers on the first node were tagged and pods were taken for analyses on days 20–22 after pollination (DAP) for determination of soluble carbohydrates in seeds before explant feeding experiments. Some of the plants were cultivated to full seed maturity for analysis of carbohydrate composition in seeds matured on the plant under the experimental conditions. Dry weight (DW) and water content of whole seeds were determined by drying seeds at 105°C for 24 h (10 seeds in each replicate). The results were shown as means of three replicates ± standard error (SE).

Explant feeding experiments

Explants of stem–leaf–pod of pea were used for feeding with D-pinitol and D-*chiro*-inositol. The explants were excised at 8–10 cm below and 1 cm above the node with the first pod [with seeds at 20–22 DAP, dry weight (DW) content 20–21% (fresh weight basis)]. Each explant included a section of the stem with one node, one leaf and one pod. Immediately after excising the bottom part of the stem, an explant was placed in a 10-ml glass tube containing 1 ml of 50 mM D-pinitol or 50 mM D-*chiro*-inositol solution. Experiments were performed in triplicate with ten plants per experiment. The temperature and light conditions were identical to those established for the plants grown in the greenhouse. After the first day of incubation, all the culture media had been absorbed by the explants, which were then subjected to a slow drying process for 3 weeks (at 20–22°C in the day and 14–16°C at night, 14/10 h day/night period, *c*. 40–50% air relative humidity). Soluble carbohydrates were determined in whole seeds before and after the feeding experiments.

Analysis of raffinose family oligosaccharides (RFOs) and galactosyl cyclitols

Before extraction of soluble carbohydrates, seeds and parts of seedlings, i.e. the epicotyl, root and cotyledons, were dried at 80°C for 18 h. Dry tissues were crushed in a mixed mill (MM 200, Retsch, Verder Group, Haan, Germany) set at the vibrational frequency of 22 Hz for 2 min. Carbohydrates were

Table 1. Contents of soluble carbohydrates [mg (g DW)$^{-1}$] in mature, dehydrated seeds of pea of genotypes varying in natural level of RFOs. Seed were derived from the Polish *Pisum* Genebank. Values are means ± SE ($n = 3$)

	Genotype			
Carbohydrate	cv. Tiny High RFOs	SZD175 Low RFOs	cv. Hubal Low verbascose	Wt 506 Low verbascose
Sucrose	41.92 ± 1.45	14.08 ± 0.59	24.71 ± 0.66	23.99 ± 0.77
myo-Inosiol	2.10 ± 0.16	0.88 ± 0.04	1.70 ± 0.09	1.19 ± 0.00
Galactinol	1.42 ± 0.08	0.83 ± 0.02	1.45 ± 0.08	1.61 ± 0.01
Total RFOs	105.77 ± 5.38	42.38 ± 1.18	58.02 ± 0.80	59.56 ± 2.43
Raffinose	20.35 ± 1.81	4.94 ± 0.16	14.56 ± 0.24	11.10 ± 0.55
Stachyose	44.45 ± 2.91	13.65 ± 0.38	35.16 ± 0.41	45.29 ± 1.84
Verbascose	40.96 ± 0.65	23.79 ± 0.97	8.31 ± 0.19	3.16 ± 0.04
Total	151.66 ± 4.24	58.41 ± 1.23	86.28 ± 1.56	86.85 ± 1.62

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extracted from 40–45 mg of meal (in 1.5-ml tubes) with 800 μl of 50% (v/v) ethanol containing 100 μg of xylitol as an internal standard. The homogenate was shaken on Genie 2 vortex (Scientific Industries, New York, USA) for 1 min, heated at 90°C for 30 min and centrifuged at 20,000 *g* for 20 min at 4°C. Aliquots (400 μl) of clear supernatant were transferred to 1.5-ml tubes containing 300 μl of a 50% (v/v) slurry of ion-exchange resins (Dowex 50W × 8, H^+, and Dowex 2W × 8, formate, Sigma-Aldrich, St. Louis, Missouri, USA). The samples were shaken at 1300 rpm for 45 min and were centrifuged at 20,000 *g*. Aliquots of the supernatants (200 μl) were evaporated to dryness in a rotary evaporator centrifuge (at 40°C). Samples were stored over silica gel in a desiccator to remove traces of water residues. Carbohydrates were derivatized with 200 μl of a mixture of trimethylsilyl imidazole:pyridine (1:1, v/v) at 80°C for 45 min. Trimethylsilyl (TMS)-derivatives of soluble carbohydrates were analysed by capillary gas chromatography as described previously (Lahuta, 2006). The gas chromatograph (GC 2010, Shimadzu, Kyoto, Japan) was equipped with an auto-injector (AOC-20i), an auto-sampler (AOC-20s) and a flame-ionization detector. The TMS-derivatives of carbohydrates were separated on a Zebron ZB-1 capillary column (15 m length, 0.25 mm diameter, 0.1 μm film; Phenomenex, Torrance, California, USA). Helium was used as a carrier gas. The column was operated with an initial temperature of 150°C adjusted to 335°C at 20°C min^{-1} and the final temperature was held for 0.75 min (total time 10 min). The injector port was operated in the split mode (10:1) at 335°C, and the detector was maintained at 350°C.

Soluble carbohydrates were quantified from the standard curves; the ratios of the area of signals for each known compound to the area of the signal for xylitol, the internal standard, were plotted against known amounts (over the range 10–250 μg) of each compound. The results of analyses are means of three independent replicates ± SE.

Standards of D-pinitol and D-*chiro*-inositol were obtained from Industrial Research Ltd (Lower Hutt, New Zealand), galactinol was obtained from Wako Pure Chemicals Industries Ltd (Neuss, Germany) and verbascose originated from Megazyme (Wicklow, Ireland). The other carbohydrates were purchased from Sigma-Aldrich and commercially unavailable standards of galactosyl pinitols were isolated and purified from seeds of winter vetch (*Vicia villosa*) as described earlier (Szczeciński *et al.*, 2000). Galactosides of D-*chiro*-inositol (fagopyritol B1, B2) were isolated and purified from seeds of common buckwheat (*Fagopyrum esculentum*) according to the method described by Horbowicz *et al.* (1998). The identity of fagopyritol B1, B2, A1 and galactosyl pinitol A and B synthesized in pea seeds fed with D-*chiro*-inositol or D-pinitol was confirmed by a gas chromatography–mass spectrometry (GC/MS) method. TMS-derivatives of soluble carbohydrates were separated on a Zebron ZB-5MSi capillary column (30 m length, 0.25 mm diameter, 0.25 μm film; Phenomenex) using a gas chromatograph coupled with the mass spectrometer (GCMS-QP2010, Shimadzu). The column initial temperature was 150°C, programmed to 325°C at 20°C min^{-1} and held at 325°C for 15 min. The injection port was operated at 325°C at split mode (20:1), and helium (1.0 ml min^{-1}) was used as the carrier gas. The ion source (EI) voltage was 70 V, ion source temperature 260°C, interface temperature 325°C. Mass spectra were collected with scan speed 1250 in a scan range of 100–650 m/z (mass to charge ratio). The fagopyritols and galactosyl pinitols were identified by the comparison of the retention time and mass spectrum of each compound with the retention time and mass spectrum of a known standard.

Germinability

Seeds matured on the plant under greenhouse conditions and seeds matured on the explants fed with cyclitols were germinated on wet germination paper towels in the dark at 20°C for 7 days (20 seeds in each of three replicates). Number of seeds with a radicle protruded from the seed coat and reaching 5 mm length was counted. Soluble carbohydrates were determined in the embryo axis (epicotyl plus root) and cotyledons.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) and Tukey's post-hoc test (if overall $P < 0.05$) for multiple comparisons.

Results

In developing pea seeds at 20–22 DAP, sucrose was the major soluble carbohydrate (Table 2). Seeds also contained small amounts of glucose, fructose, *myo*-inositol, galactinol, raffinose and stachyose. At this stage of development, seeds did not contain verbascose. Incubation of stem–leaf–pod explants only with water (control) followed by slow drying decreased the concentration of sucrose and stimulated accumulation of RFOs in seeds, similarly to processes in seeds naturally maturing on the mother plant (Fig. 1). In seeds of the lines containing high (Tiny) or low (SZD175) concentrations of RFOs, stachyose or verbascose were the dominant carbohydrates, respectively (Fig. 1A and B). In seeds of pea genotypes with low verbascose content (Hubal and Wt 506), stachyose was the major oligosaccharide (Fig. 1C and D).

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Table 2. The fresh weight (FW), dry weight (DW) and the concentration of soluble carbohydrates [mg (g DW)$^{-1}$] in developing pea seeds (at 20–22 DAP) before feeding experiments. Values are means ± SE ($n = 3$)

	Genotype			
	cv. Tiny High RFOs	SZD175 Low RFOs	cv. Hubal Low verbascose	Wt 506 Low verbascose
FW, mg seed^{-1}	123.84 ± 2.47	256.88 ± 2.88	274.22 ± 5.90	103.55 ± 2.36
DW, as % FW	20.07 ± 0.09	24.59 ± 0.25	21.49 ± 0.08	21.09 ± 0.07
Carbohydrate				
Sucrose	254.57 ± 11.73	115.92 ± 0.60	185.40 ± 2.01	197.62 ± 7.03
Glucose	1.94 ± 0.05	0.75 ± 0.04	1.42 ± 0.15	2.84 ± 0.73
Fructose	1.82 ± 0.09	0.94 ± 0.09	2.23 ± 0.16	3.89 ± 0.84
myo-Inosiol	9.66 ± 0.80	2.61 ± 0.01	9.18 ± 0.05	5.88 ± 0.18
Galactinol	1.51 ± 0.07	2.58 ± 0.01	0.51 ± 0.02	0.62 ± 0.02
Total RFOs	2.76 ± 0.04	4.32 ± 0.08	0.64 ± 0.01	1.74 ± 0.09
Raffinose	1.68 ± 0.07	2.39 ± 0.07	0.64 ± 0.01	1.59 ± 0.13
Stachyose	0.85 ± 0.05	1.93 ± 0.02	–	0.15 ± 0.04
Verbascose	–	–	–	–
Total	272.27 ± 12.24	127.08 ± 0.66	199.38 ± 2.05	212.58 ± 8.79

Seeds of all investigated genotypes were able to take up exogenously applied cyclitols, which do not naturally occur in pea plants. The efficiency of the absorption of cyclitols by seeds as both free form and galactoside-bound form ranged from 33 to 68% for D-*chiro*-inositol and from 14 to 37% for D-pinitol (calculated as % of cyclitols found in seeds to the total amount of cyclitols absorbed by explants). The highest efficiency was found in Hubal seeds.

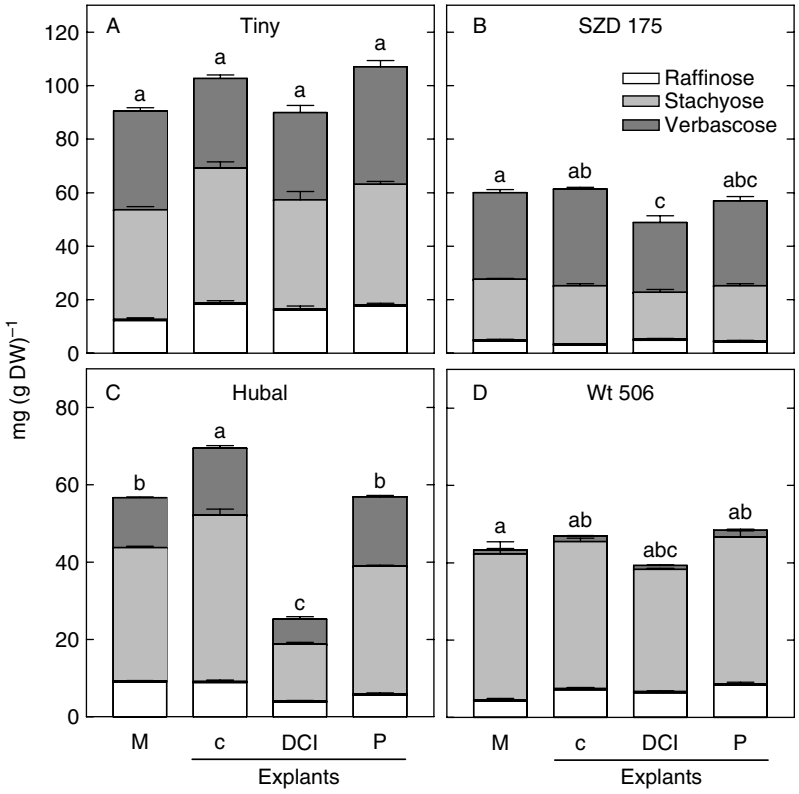

Figure 1. Contents of raffinose family oligosaccharides in seeds of pea (*Pisum sativum* L.) of four genotypes – high RFOs, cv. Tiny (A); low RFOs, SZD 175 (B); low verbascose, cv. Hubal (C) and WT 506 (D) – matured on the mother plant (M) or on the stem–leaf–pod explant fed with water (c), D-*chiro*-inositol (DCI), or D-pinitol (P). Means ± SE ($n = 3$). Bars with the same letters are not significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons for total RFO concentrations.

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Seeds of Tiny (high RFOs content) and Wt 506 (low verbascose) from explants fed with D-*chiro*-inositol accumulated similar amounts of total RFOs to those found accumulated in seeds matured on the explants fed with water or matured on the mother plant (Fig. 1A and D). Feeding D-*chiro*-inositol caused a statistically significant ($P < 0.05$) decrease in the accumulation of RFOs in seeds of Hubal and SZD175, as compared to seeds from explants fed with water and D-pinitol or matured on the plants (Fig. 1B and C). In contrast to D-*chiro*-inositol, D-pinitol had no significant effects on the composition and concentration of RFOs in seeds of any of the investigated pea lines. Both cyclitols were accumulated in pea seeds in a free and bound form as galactosyl cyclitols (Fig. 2). Seeds of the low verbascose genotypes (Hubal, Wt 506) accumulated significantly less free D-*chiro*-inositol (Fig. 2A) and more free D-pinitol (Fig. 2C) than seeds of the high RFOs (Tiny) and low RFOs genotype (SZD175). Enhanced levels of both D-*chiro*-inositol and D-pinitol in seeds did not affect the concentration of endogenous *myo*-inositol (data not shown).

In seeds containing D-*chiro*-inositol, its mono-galactosides fagopyritol B1 and B2 accumulated. Fagopyritol A1 and A2 were present in trace amounts [$< 0.16\,\text{mg}\,(\text{g DW})^{-1}$, data not shown]. The highest amounts of fagopyritol B1 [$17\,\text{mg}\,(\text{g DW})^{-1}$] were synthesized in seeds of the low verbascose genotype (Wt 506, Fig. 2B). Pea seeds containing D-pinitol synthesized its two mono-galactosides: galactosyl pinitol A (GPA) and galactosyl pinitol B (GPB) and traces [$< 0.02\,\text{mg}\,(\text{g DW})^{-1}$] of di-galactosyl pinitol A, named ciceritol. The mean total concentration of GPA + GPB for all investigated genotypes [$2.3\,\text{mg}\,(\text{g DW})^{-1}$] was approximately sixfold lower than that of fagopyritols [$15\,\text{mg}\,(\text{g DW})^{-1}$]. The concentration of galactosyl pinitols in seeds of low RFO and low verbascose genotypes was significantly higher than that in high RFO seeds (Fig. 2D). In seeds of all pea genotypes fed with D-pinitol, D-*chiro*-inositol was present only at a very low level [$0.2–0.3\,\text{mg}\,(\text{g DW})^{-1}$]. In seeds of Wt 506 from explants fed with D-pinitol, fagopyritol B1 was also formed [$0.06\,\text{mg}\,(\text{g DW})^{-1}$].

The explant feeding with cyclitols followed by slow drying did not stop the increase in seed dry weight, which was an approximately twofold increase. This increase must be a result of translocation of carbon sources remobilized from maternal tissues. Seeds fed with cyclitols were viable, but their germinability was lower (mean for water or cyclitol treatment approximately 60%) than that of seeds matured on the mother plants (mean for genotypes 80%). In the epicotyl, root and cotyledons of 7-day-old seedlings, galactosyl cyclitols were completely degraded and tissues contained free cyclitols: D-pinitol or D-*chiro*-inositol

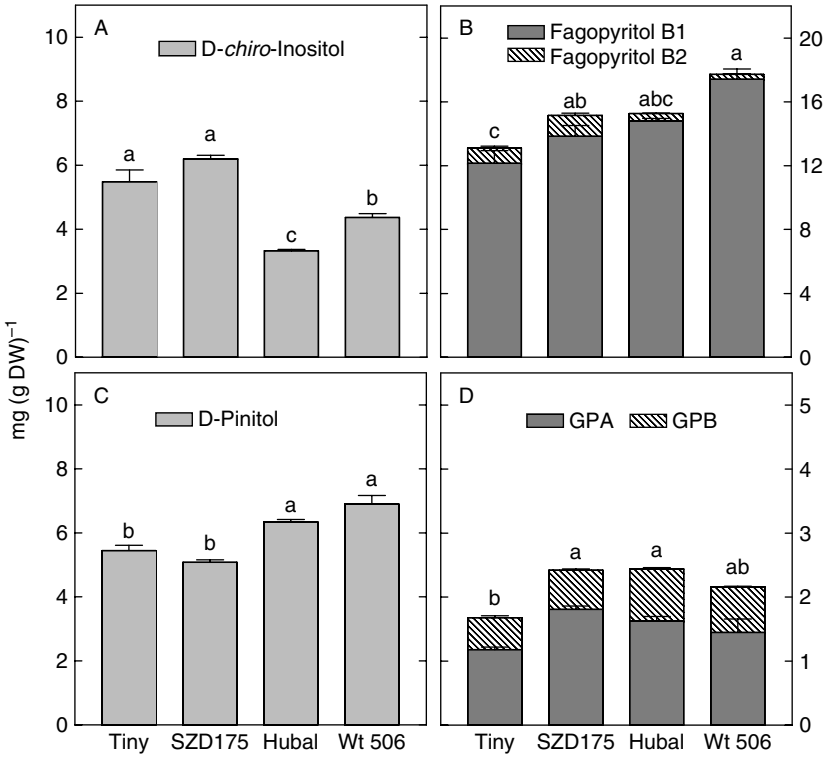

Figure 2. Contents of cyclitols (A, C) and galactosyl cyclitols (B, D) in seeds of pea matured on stem–leaf–pod explants fed with D-*chiro*-inositol (A, B) or D-pinitol (C, D). Means ± SE ($n = 3$). Bars with the same letters are not significantly different ($P < 0.05$) after a Tukey's correction for multiple comparisons.

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(data not shown). The concentration of released free D-*chiro*-inositol in seedling tissues reached levels of 3% DW (Tiny) to 4.6–4.8% DW (SZD175, Hubal and Wt 506). The concentration of D-pinitol was lower, ranging from 1.5% DW (Tiny) to 2.5–2.9% DW (SZD175, Hubal and Wt 506).

Discussion

A concentration of sucrose much greater than that of monosaccharides (fructose plus glucose, Table 2) is characteristic of the middle stage of the development of legume seeds (Weber *et al.*, 2005). Sucrose, the main carbon source for embryonic metabolism transported from the pod into developing seeds, can be hydrolysed in the seed coat by cell wall-bound invertases, and/or can flow directly into the apoplastic space surrounding the embryo. The uptake of both monosaccharides and sucrose by the embryo is possible only via appropriate sugar transporters (Weber *et al.*, 1997; Zhou *et al.*, 2009). In pea plants sucrose is the predominant sugar transported into developing seeds (Tegeder *et al.*, 1999). *Myo*-inositol can be synthesized in embryonic tissues (Chiera and Grabau, 2007) and/or transported from maternal tissues to the embryo (Karner *et al.*, 2004; Gomes *et al.*, 2005). Among several monosaccharide transporters recently identified in *Arabidopsis*, polyol/monosaccharide transporter AtPMT5 is a low specificity H^+-symporter that mediates the energy-dependent uptake of hexoses, pentoses, linear polyols of various chain lengths (3–6 carbons), and of inositol across the plasma membrane (Klepek *et al.*, 2005). Also, a highly specific transporter for *myo*-inositol and inositol derivatives (i.e. D-*chiro*-inositol) was identified in *Arabidopsis* as an energy-dependent, plasma membrane-localized H^+-inositol symporter (Schneider *et al.*, 2007). However, in *Arabidopsis*, sucrose and a small amount of raffinose, but not cyclitols, are transported through the phloem. The ability of developing seeds of soybean (Gomes *et al.*, 2005; Obendorf *et al.*, 2008) and vetch (Lahuta *et al.*, 2005a, b) to take up exogenously applied *myo*-inositol, D-*chiro*-inositol and D-pinitol suggests that a common cyclitol transporter(s) exists in legumes.

In developing pea seeds (20–22 DAP), the presence of galactinol and small amounts of raffinose and stachyose (Table 2) indicated that the RFO biosynthetic pathway was initiated. In seeds of garden pea isoline *RRRbRb* and SD1, both galactinol synthase and stachyose synthase were active at the early stage of development (Peterbauer *et al.*, 2001). Thus cyclitols absorbed by an explant and transported to seeds can be introduced into the RFO pathway. Indeed, formation of its galactosides was observed in seeds accumulating D-*chiro*-inositol (Fig. 2A and B). The multifunctional enzymes GOLS (Ueda *et al.*, 2005) and STS (Peterbauer *et al.*, 2002b) could be involved in this process. Surprisingly, the formation of the di-galactoside of D-*chiro*-inositol, i.e. fagopyritol B2, was very low, regardless of pea genotypes. This can mean that STS in pea seeds has a low affinity for fagopyritol B1 as a galactosyl acceptor. If STS, in fact, is responsible for the synthesis of galactosides of D-pinitol (Hoch *et al.*, 1999; Peterbauer and Richter, 2001), it can be expected that these compounds should accumulate to a considerable amount in pea seeds fed with D-pinitol. Unexpectedly, the accumulation of galactosyl pinitol A and B was very low (Fig. 2D), despite the concentration of D-pinitol being as high as that of D-*chiro*-inositol (Fig. 2A and C). Although the concentration of D-pinitol was approximately twofold lower (Fig. 2C) than that of raffinose (Fig. 1), the concentration of galactosyl pinitols (Fig. 2D) was several-fold lower than the concentrations of stachyose and verbascose (Fig. 1). Therefore, it can be assumed that pea STS has a very low affinity for D-pinitol as a galactosyl acceptor. Consequently, D-pinitol did not alter the accumulation of RFOs in pea seeds (Fig. 1). Similar results were recently obtained in a study on soybean lines (Obendorf *et al.*, 2008). However, we found that seeds of two vetch species fed D-pinitol significantly increased the content of mono-, di- and tri-galactosides of D-pinitol. The resulting concentrations of D-pinitol galactosides were much higher than the natural accumulation of galactosyl pinitols (Lahuta *et al.*, 2005a, b). Recently, we have demonstrated that seeds of garden vetch (*Vicia sativa*), which naturally accumulate only RFOs, supplied with D-pinitol were able to form mono-, di- and tri-galactosides of D-pinitol (Lahuta *et al.*, 2010). Thus, it can be suggested that STS in seeds of pea or soybean differs in catalytic properties from STS present in seeds of *Vicia* spp. In pea seeds supplied with D-*chiro*-inositol, the accumulation of verbascose (SZD175, Fig. 1B) or all RFOs (cv. Hubal, Fig. 1C) declined or remained unaffected (cv. Tiny and Wt 506, Fig. 1A and D). Interestingly, based on the molecular formula we calculated that the total amount of galactose bound into fagopyritols was equal in seeds, regardless of pea genotype.

On the other hand, it should be emphasized that feeding with cyclitols did not affect the genetically determined composition of RFOs in pea seeds. Stachyose dominated among RFOs in seeds of Tiny, verbascose in SZD175, and seeds of the two other genotypes, Hubal and Wt 506, contained the lowest amounts of verbascose, regardless of seed maturation on the plant or on an explant (Fig. 1), analogous to seeds derived from the germplasm collection (Table 1).

Generally, our results indicate low susceptibility of pea seeds to replacement of RFOs by galactosyl cyclitols. In two genotypes, in which supplying D-*chiro*-inositol caused a significant decrease in the

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concentration of total RFOs in seeds, one belongs to fodder cultivars (Hubal) and the second (SZD175) to peas used for human nutrition. Further investigations concerning the effects of different D-*chiro*-inositol concentrations, duration of explant feeding and seed developmental stages should more precisely determine the biological potential of both pea genotypes for modification of α-D-galactoside composition in seeds. In effect, they could be helpful in deciding whether genetic transformation of peas with genes encoding enzymes synthesizing D-*chiro*-inositol could be legitimate. This is important with respect to the fact that the enzymes directly synthesizing D-*chiro*-inositol have not yet been discovered. Moreover, our results eliminate the transformation of peas with genes encoding enzymes synthesizing D-pinitol (*myo*-inositol methyltransferase and D-ononitol epimerase) as a strategy for decreasing the RFOs in pea seeds.

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