INHIBITION OF RAFFINOSE FAMILY OLIGOSACCHARIDES AND GALACTOSYL PINITOLS BREAKDOWN DELAYS GERMINATION OF WINTER VETCH (*VICIA VILLOSA* ROTH.) SEEDS

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ABSTRACT

Beside RFOs, which are commonly present in legume seeds, seeds of some species contain galactosyl pinitols (GPs). These carbohydrates, like RFOs, have been hypothesized to constitute an important energy and carbon skeletal source during germination. To test this hypothesis we have applied a specific α-galactosidase inhibitor (1-deoxygalactonojirimycin, DGJ) to germinating winter vetch (*Vicia villosa* Roth.) seeds, containing more galactosyl pinitols than RFOs. The breakdown of RFOs but not that of GPs was completely blocked in both embryonic axes and cotyledons tissues, during the first 18 h of imbibition in DGJ. The inhibitor only decreased the rate of GPs degradation. The inhibitory effect of DGJ on GPs degradation was partially alleviated by addition of sucrose or galactose to DGJ solutions. After three days of germination in water, RFOs and GPs disappeared in axial tissues of seeds imbibed in water, galactose or sucrose. Eighteen-hour imbibition of seeds in DGJ drastically reduced germination, by ca 50%, during the first three days. The inhibitory effect of DGJ decreased during the next seven days of germination. The presence of galactose or sucrose in imbibition solution initially stimulated seed germination, but later this effect was not statistically significant. Our study provides clear evidence that galactosyl pinitols play an important role in early winter vetch seeds germination. Additionally, we suggest that galactosyl pinitols can replace RFOs as reserve material necessary for early germination.

KEY WORDS: germination, galactosyl pinitols, raffinose family oligosaccharides, seed, winter vetch.

INTRODUCTION

The major soluble carbohydrates in legume seeds are sucrose and its α-D-galactosides: raffinose, stachyose and verbascose (raffinose family oligosaccharides, RFOs). RFOs are rapidly disappearing after the imbibition and breakdown of seeds, often completed before polymeric carbohydrates are mobilized (Bewley and Black 1994; Vidal-Valverde et al. 1998; Frias et al. 2000). The RFOs in axes are lost during the first two days of imbibition of soybean, pea and lupine seeds, whereas in cotyledons RFOs hydrolysis is prolonged for four-six days (Górecki and Obendorf 1997; Górecki et al. 1997). Verbascose, stachyose and raffinose are degraded progressively, while the level of monosaccharides increases gradually as germination progresses. Therefore, RFOs may be an essential source of rapidly metabolizable carbon for early germination events, as previously suggested (Downie and Bewley 2000). The mutation *mips* (*myo*-inositol phosphate synthase) in soybean seeds reduced stachyose level and field emergence of seeds (Meis et al. 2003). The latest study by Blöchl et al. (2007) on germinating pea seeds treated with a specific inhibitor of acidic and alkaline α-galactosidases, 1-deoxygalactonojirimycin (DGJ) (Asano et al. 2000) clearly indicated that inhibition of RFOs breakdown delays early pea seed germination. However, there is very little information on the role of galactosyl cyclitols present in seeds of some legumes (*Cicer*, *Glycine*, *Lentil*, *Lupinus*) during early seed germination. In soybean seedlings, galactinol, raffinose, galactosyl pinitols and fagopyritols (galactosides of D-*chiro*-inositol) disappeared in growing hypocotyls during the first 24 h of germination, whereas in cotyledons degradation of these carbohydrates (and stachyose) was prolonged up to 48-72 h (Górecki and Obendorf 1997). In yellow lu-

Abbreviations:
RFOs – raffinose family of oligosaccharides; GPs – α-D-galactosides of D-pinitol; GPA – galactosyl pinitol A; GPB – galactosyl pinitol B; DGPA – di-galactosyl pinitol A (ciceritol); TGPA – tri-galactosyl pinitol A; DGJ – 1-deoxygalactonojirimycin; DGMI – di-galactosyl-*myo*-inositol

pine seeds, loss of RFOs and galactosyl cyclitols in axial tissues preceded visible germination (Górecki et al. 1997). However, all the above species contain only small amounts of galactosyl cyclitols (mainly α-D-galactosides of D-pinitol and less D-*chiro*-inositol). In seeds of buckwheat (*Fagopyrum esculentum* Moench. Polygonaceae), containing predominantly galactosides of D-*chiro*-inositol instead of RFOs, disappearance of these compounds in axes and cotyledons is completed after 18-20 hours of dehulled achenes germination. The loss of fagopyritol B1 (mainly galactoside) in the axes was closely associated with the onset of rapid germination (Horbowicz et al. 1998). In the present study we have tested the effect of α-galactosidases inhibitor (DGJ) on the degradation of RFOs and α-D-galactosides of D-pinitol (galactosyl pinitols) in relation to early germination of winter vetch (*Vicia villosa* Roth.) seeds. This species has been chosen because of its higher concentration of galactosyl pinitols than RFOs in seeds (Lahuta 2006). Therefore, inhibition of α-galactosidase by DGJ can be an appropriate method for understanding the involvement of both types of α-D-galactosides (RFOs and galactosyl pinitols) in seed germination.

MATERIALS AND METHODS

Winter vetch seeds (*Vicia villosa* Roth, cv. Minikowska, from Rolnas, Poland) were surface-sterilized and imbibed in sterile water (controls) or water solutions of 250 μM DGJ (Sigma-Aldrich, Vienna, Austria), 50 mM galactose, 25 mM sucrose and combination of these for 18 h. Imbibed seeds were then short washed three times to remove DGJ and sugars, transferred to Petri dishes (wetted filter paper with water) and kept at 24°C in the dark for germination for 10 days. A seed was considered to be germinated when the radicle pierced the seed coat. Soluble carbohydrates were extracted and assayed in axes and cotyledons of dry, imbibed (18 h) and germinated seeds (after three days of germination), as described previously (Lahuta 2006).

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

RESULTS AND DISCUSSION

Dry winter vetch embryos contained sucrose, RFOs, D--pinitol and galactosyl pinitols (GPs) as main soluble carbohydrates (Table 1). In the RFOs fraction verbascose dominated, whereas the main GP was ciceritol. The concentration of both types of α-D-galactosides in the axes was ca 2-fold higher than that in cotyledons. Higher concentration of α-D-galactosides in the axis than in cotyledons is characteristic for legumes (Obendorf 1997; Peterbauer and Richter 2001) and can be a result of faster maturation of axial tissues than cotyledons. Imbibition of winter vetch seed initiated fast degradation of GPs and RFOs in the embryo (Fig. 1), which initially involved oligosaccharides of a higher degree of polymerization: verbascose (among RFOs, Fig. 1A), ciceritol and TGPA among GPs (Fig. 1B). In seeds of other legumes (lupine, soybean, pea) degradation of RFOs or RFOs and galactosyl cyclitols starts during seed imbibition (Bewley and Black 1994) and, similarly to winter vetch, galactosides of a higher degree of polymerization are degraded earlier. Quemener and Brillouet (1983) reported that the in vitro hydrolysis rate of ciceritol by α--D-galactosidase is much lower than for raffinose, stachyo-

TABLE 1. The concentration of soluble carbohydrates in embryonic axes and cotyledons of dry winter vetch (*Vicia villosa* Roth. cv Minikowska) seeds. Data represent means ± SE ($n = 3$).

		Axis	Cotyledon
		mg g^{-1} dry weight	
Sucrose		28.61±0.56	12.33±0.44
myo-Inositol		0.86±0.03	0.30±0.02
Galactinol		1.01±0.03	0.37±0.00
DGMI		2.13±0.08	0.85±0.03
Total RFOs		33.08±1.74	14.30±0.42
	Raffinose	2.54±0.04	0.99±0.03
	Stachyose	8.90±0.60	4.08±0.16
	Verbascose	21.64±1.09	9.23±0.24
D-Pinitol		4.76±0.03	2.09±0.12
Total GPs		46.88±0.97	28.24±0.75
	GPA	4.22±0.17	1.92±0.06
	GPB	1.53±0.04	0.69±0.02
	Ciceritol	26.31±0.31	15.75±0.38
	TGPA	14.81±0.83	9.87±0.29
Total		117.35±2.13	58.49±1.75
RFOs/GPs ratio		0.70±0.02	0.51±0.00

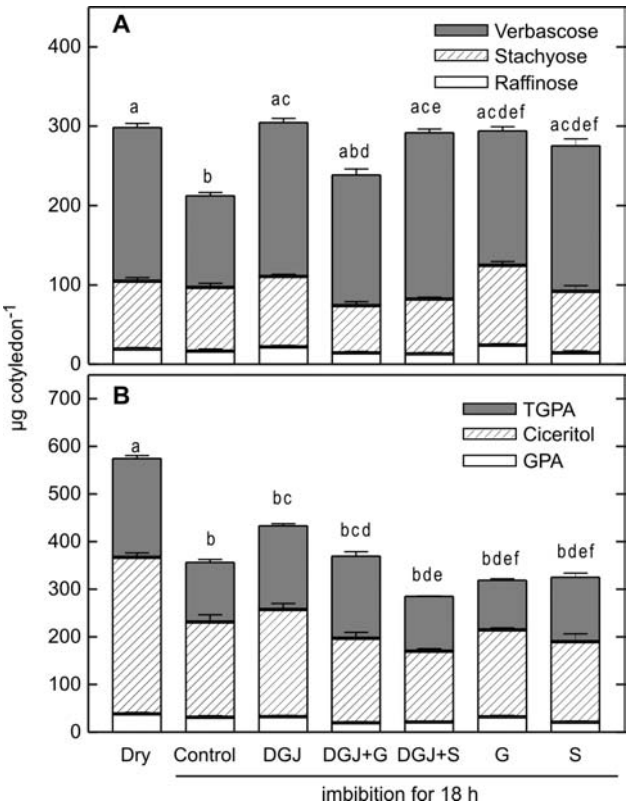

Fig. 1. The content of RFOs (A) and GPs (B) in winter vetch cotyledon before (Dry) and after 18 hours of seeds imbibition in water (Control), DGJ, sugars (S – sucrose, G – galactose), mixture of DGJ plus galactose (DGJ+G), and DGJ plus sucrose (DGJ+S). Data represent means ± SE ($n = 3$). For statistical analysis were subjected total RFOs and total GPs. Bars with the same letters are not significantly different ($P<0.05$) after a Tukey correction for multiple comparisons.

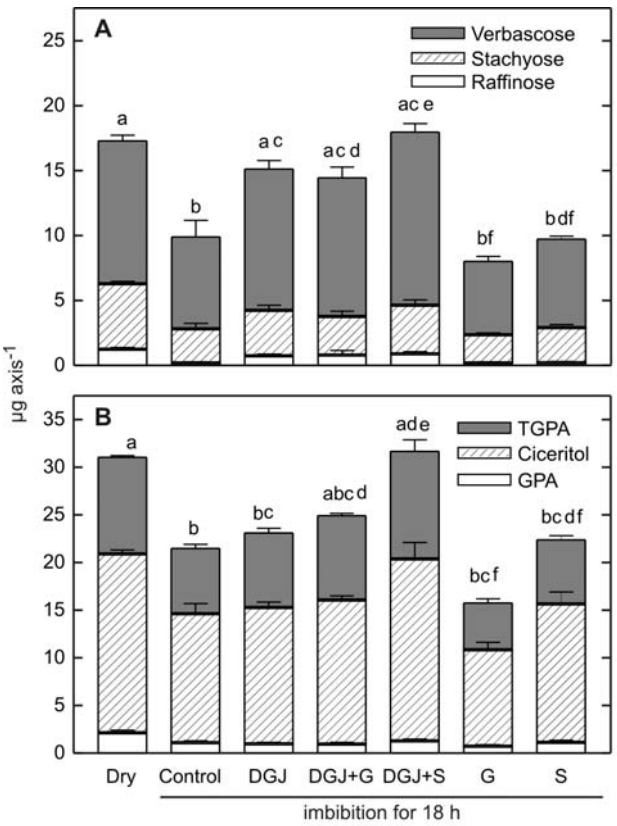

Fig. 2. The content of RFOs (A) and GPs (B) in winter vetch embryonic axes before (Dry) and after 18 hours of seeds imbibition in water (Control), DGJ, sugars (S – sucrose, G – galactose), mixture of DGJ plus galactose (DGJ+G), and DGJ plus sucrose (DGJ+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs and total GPs. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

se and verbascose. Porter et al. (1990) indicated that hydrolysis of stachyose in soybean seeds is a rate-limiting step and soybean α-galactosidase simultaneously hydrolyzes two substrates: stachyose and raffinose. By analogy, it can be expected that hydrolysis of verbascose and trigalactosyl pinitol A in winter vetch seeds can be limited by the rate of hydrolysis of products: stachyose and ciceritol, respectively. Additionally, degradation of ciceritol and next GPA produces D-pinitol, which can by a factor that reduces the sensitivity of α-galactoside to hydrolysis by α-galactosidase (Quemener and Brillouet 1983).

During the first 18 hours of imbibition, degradation of RFOs in winter vetch embryos was completely inhibited by DGJ at 250 µM concentration (Figs 1A and 2A). However, DGJ only partially blocked degradation of GPs (Figs 1B and 2B). In pea seed treatment with 50 µM DGJ, RFOs breakdown was blocked completely during 42 hours after imbibition (Blöchl et al. 2007). Addition of galactose to DGJ solution slightly decreased the inhibitory effect of DGJ on degradation of both types of galactosides in cotyledons, but not in embryonic axes of winter vetch seeds. Addition of sucrose to DGJ did not relieve the inhibitory effect of DGJ on RFOs degradation, but stimulated hydrolysis of GPs in cotyledons (Fig. 1), and completely blocked degradation of GPs in embryonic axes (Fig. 2). Galactose and sucrose used instead of DGJ also inhibited degradation of RFOs, but not that of GPs in cotyledons (Fig. 1). Both sugars did not change degradation of galactosides in embryonic axes (Fig. 2). Although the levels of sucrose and D--pinitol increased according to hydrolysis of galactosides, as expected, no noticeable increase in the content of free galactose occurred (data not shown).

The inhibition of GPs and RFOs degradation by DGJ delayed winter vetch seed germination by approximately 50% during the first three days after imbibition (Fig. 3). In pea imbibed in 50 µM DGJ the germination rates decreased by approximately 70% (Blöchl et al. 2007). Exogenous supply of galactose only, but not that of sucrose, was capable of relieving most of the inhibitory effect of DGJ on pea germination (Blöchl et al. 2007). In our study, winter vetch seeds imbibed in galactose and sucrose solutions initially germinated faster than control seeds, indicating high embryo demand on easy metabolizable carbohydrates even in the initializing seed germination. Initially, differences in percentage of germinated seeds were observed up to 3rd day of germination. Although later the germination of seeds imbibed in galactose and DGJ/sugars solution gradually increased, the effect of DGJ was still evident.

In the axes of seeds germinated for three days (previously imbibed in water, galactose and sucrose), RFOs were completely degraded (Fig. 4A) and the content of GPs declined to a very low level (Fig. 4C). Following the hydroly-

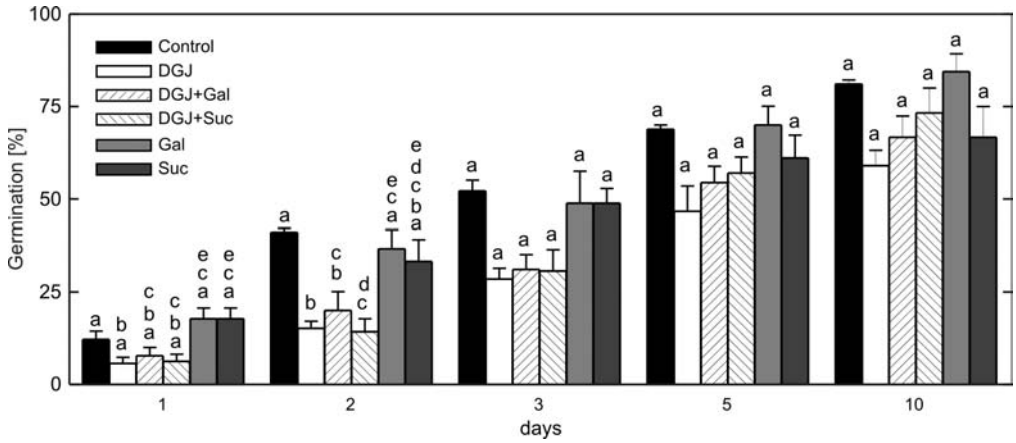

Fig. 3. Germination of winter vetch seeds imbibed for 18 hours in water (Control) or DGJ, galactose (Gal), sucrose (Suc) and mixture of them (DGJ+Gal, DGJ+Suc), and then kept on wet (in water) germination paper for 10 days. Data represent means ± SE (n = 3). Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

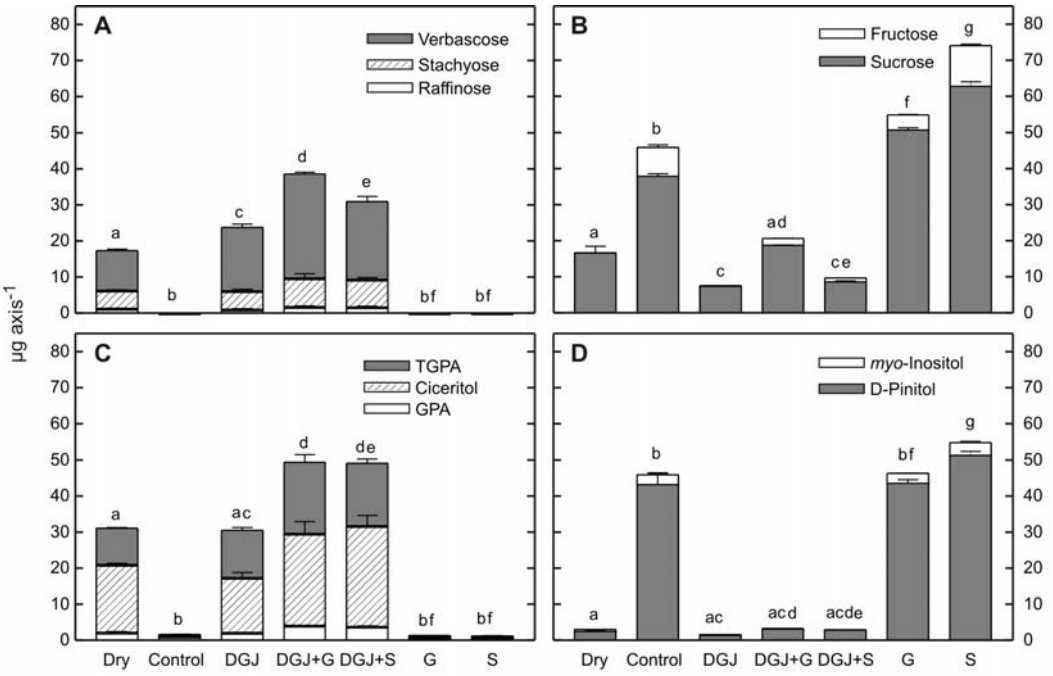

Fig. 4. The content of RFOs (A), sucrose and fructose (B), GPs (C), cyclitols (D) in embryonic axes of winter vetch seed before (Dry) and after three days of germination of seed imbibed for 18 hours in water (Control), DGJ, sugars (S – sucrose, G – galactose), and mixture of DGJ/sugars (DGJ+G, DGJ+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs, total GPs, sucrose + fructose and *myo*-inositol + D-pinitol. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

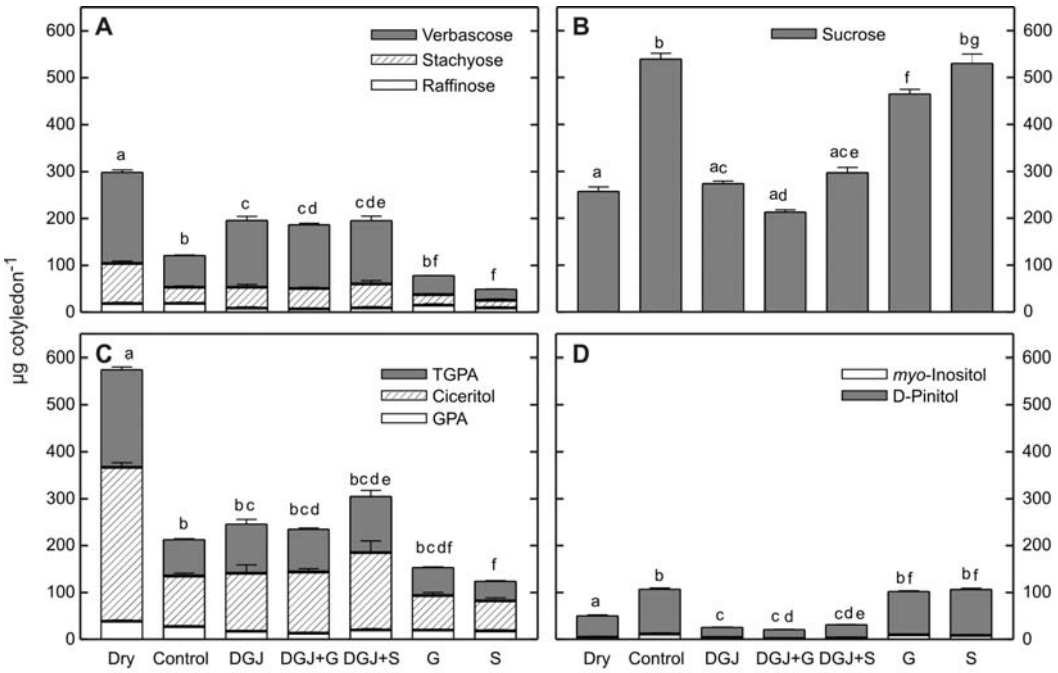

Fig. 5. The content of RFOs (A), sucrose and fructose (B), GPs (C), cyclitols (D) in cotyledon of winter vetch seed before (Dry) and after three days of germination of seed imbibed for 18 hours in water (Control), DGJ, sugars (S – sucrose, G – galactose), and mixture of DGJ/sugars (DGJ+G, DGJ+S). Data represent means ± SE (n = 3). For statistical analysis were subjected total RFOs, total GPs, sucrose + fructose and *myo*-inositol + D-pinitol. Bars with the same letters are not significantly different (P<0.05) after a Tukey correction for multiple comparisons.

sis of oligosaccharides and galactosyl pinitols, the levels of sucrose, fructose (Fig. 4B), D-pinitol and *myo*-inositol (Fig. 4D) increased. In control cotyledons, the content of RFOs and GPs decreased two- and three-fold, respectively, as compared to dry seeds (Figs 5A and C). It should be noted that in cotyledons of seeds imbibed in galactose and sucrose the content of RFOs and GPs was the lowest among tested seeds. Analogously to the axes, cotyledons were observed to contain higher levels of sucrose and D-pinitol (Figs 5B and D). The concentration of free galactose was very low and comparable to the level of glucose (below 0.15 mg g^{-1} DW, data not shown). Previous findings indicated that the rate of degradation of ciceritol, presented in lentil, chickpea and white lupine seeds, by α-galactosidase is lower than in the case of raffinose or stachyose (Quemener and Brillouet 1983; Frias et al. 2000), presumably as a re-

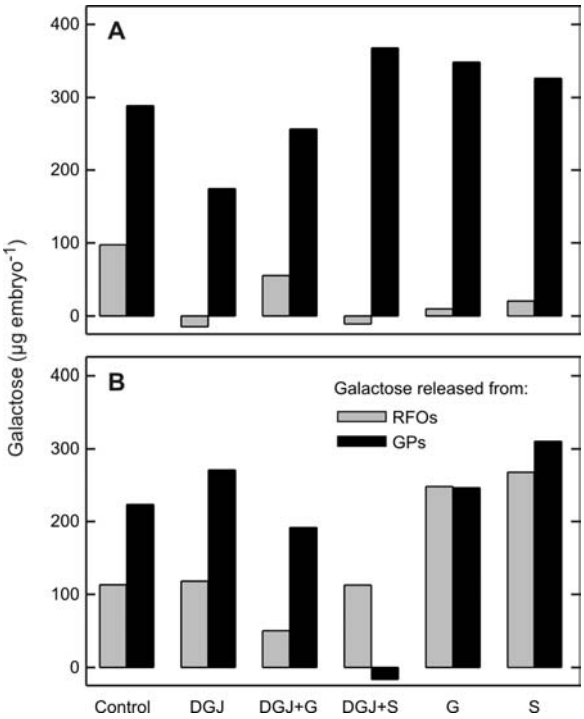

Fig. 6. (A) The average amount of galactose released from RFOs and GPs during 18 hours of seeds imbibition in water (control) and DGJ, DGJ/sugars and sugars (G, S – galactose, sucrose) solutions (differences between dry and imbibed seed) and (B) during following germination for three days (differences between the content of galactose at 18th hour of imbibition and third day of germination). The mean content of released galactose in whole embryo was calculated on the molecular formula of each oligosaccharide (among RFOs) and galactosyl pinitol (among GPs) (therefore SE is absent).

sult of specific activity of α-D-galactosidase (Petek et al. 1969; Dey et al. 1983).

In winter vetch seeds treated with DGJ and DGJ/sugars, the content of RFOs and GPs in axial tissues increased in comparison to axes of dry seeds (Figs 4A and C), presumably because of the transport of galactosides from cotyledons to growing roots and epicotyl. However, it is also possible that enzymes for RFOs synthesis can remain functional at early germination stages, as has been shown in germinating tomato seeds (Downie et al. 2003). In pea seeds, addition of galactose to the inhibitor solution significantly increased the RFOs content (Blöchl et al. 2007). In our study, galactose and sucrose added to DGJ solution also increased the content of both types of galactosides in the axes, but not in cotyledons.

For comparison of the amounts of galactose released from oligosaccharides and galactosyl pinitols, first the amounts of galactose bound in each oligosaccharide or galactosyl pinitol were computed (based on the molecular formula of each galactoside) in a whole embryo before imbibition, after 18 hours of imbibition and after three days of germination. Then, the differences between the total amounts of galactose in RFOs and GPs before and after imbibition (Fig. 6A) or between 18th hour and third day of germination (Fig. 6B) were calculated. Thus, an average amount of released galactose clearly indicates that during the first 18 hours of germination most of galactose was released from GPs. In seeds imbibed in DGJ, degradation of RFOs was completely stopped, whereas that of GPs was decreased by ca 45% (Fig. 6A). Additionally, the content of galactose bound in RFOs slightly increased, presumably because the enzymes for RFOs biosynthesis remain functional in germinating seeds. A similar effect was found in the case of seeds imbibed in a mixture of DGJ and sucrose. Addition of galactose to DGJ solution partially relieved the inhibitory effect of DGJ on degradation of RFOs and GPs. Galactose and sucrose stimulated faster release of galactose from GPs and partially inhibited breakdown of RFOs (Fig. 6A), which coincided with a higher germination rate (Fig. 3). During the germination, control embryos utilized similar amounts of galactose released from RFOs as during the first 18 hours of imbibition (ca 100 µg embro^{-1}). Comparable amounts of galactose derived from RFOs were found in embryos initially imbibed in DGJ and DGJ/sucrose (Fig. 6B). However, the amount of galactose released from GPs was still two-fold higher than that from RFOs.

The same type of chemical linkage [α-(1→6)-*O*-D-glycoside bound] between galactosyl residues is present in both types of galactosides. Thus, it can be expected that inhibition of degradation of galactosides by DGJ should stop degradation of both types of galactosides. Our results indicate that each treatment leading to inhibition of RFOs degradation simultaneously stimulated degradation of GPs (Figs 1B and 6A). The reason for this is not clear. In our study seeds were imbibed in DGJ solutions for only 18 hours and later were transferred to Petri dishes containing only water. Therefore, the effect of DGJ was associated with its uptake by imbibed embryos. On the other hand, the stimulatory effect of galactose and, to a lesser extent, sucrose on the GPs degradation can suggest that galactose induces expression of new form of α-galactosidase, preferentially degrading galactosides of D-pinitol. It is also possible that faster degradation of GPs than RFOs in winter vetch seeds is associated with substrate specificity of α-galactosidase present in winter vetch seeds. Both hydrolytic and galactosyltransferase activity of α-galactosidase was found in seeds of *V. sativa* (Petek et al. 1969). However, seeds of this species accumulate only RFOs (Lahuta et al. 2005). The data obtained during our study indicate that investigations on the characteristic of α-galactosidase from winter vetch seeds during germination may be necessary to discover the causes of preferential degradation of GPs rather than RFOs.

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