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Alpha - D-GALACTOSIDASE ACTIVITY IN STORED YELLOW LUPIN (Lupinus luteus L.) SEEDS

Lesław B. Lahuta, Ryszard J. Górecki, Dariusz Michalczyk, Agnieszka I. Piotrowicz-Cieślak

Department of Plant Physiology and Biotechnology, Warmia and Masuria University, Olsztyn, Poland

ABSTRACT

The activity of alpha-D-galactosidase and contents of soluble saccharides were studied in 'Juno' yellow lupin (*Lupinus luteus* L.) seeds stored. Seeds harvested at three stages of maturity (15, 25, 35 days after flowering - DAF) were stored at –21°C for two years (frozen immediately after harvest) or were dried after harvest to 8% of water content and stored at +20°C for 5 and 6 years. A high alpha-D-galactosidase activity in maturing and mature seeds could have been one of the causes of decreasing viability and vigour of the seeds stored. The hydrolysis of raffinose oligosaccharides decreased the ratio of these saccharides to sucrose. The decrease in the content of raffinose oligosaccharides was accompanied by an increase in galactosyl cyclitol contents.

Key words: alpha-D-galactosidase, yellow lupin, seed storage, raffinose, cyclitols

INTRODUCTION

Raffinose oligosaccharides and galactosyl cyclitols are the main sugar storage form in legume seeds which accumulate a low amount of starch, like soybean [2] and lupin [4]. The level of these sugars in embryonic tissues of lupin exceeds 20% of dry weight [20,22]. During seed germination, oligosaccharides are the primary source of energy and substrates for the synthesis of other compounds [5,6,14,17].

The hydrolysis of alpha-1→6 glycoside bonds between galactoside moieties of raffinose oligosaccharides, cell wall polysaccharides and storage glycoproteins is catalysed by alpha-D-galactosidase (EC 3.2.1.22). Mature seeds usually contain a few forms of alpha-D-galactosidase which differ in their molecular mass and activity.
In developing seeds the activity of alpha-D-galactosidase increases in the period of intensive synthesis of raffinose oligosaccharides and reaches the highest level at full ripeness. This increase may result from structural transformation of isoenzymes, which leads to changes in their specific activities [19]. In the maturing embryo, the processes of oligosaccharide synthesis and alpha-D-galactosidase reaction probably occur in different cellular compartments. In the cells of pea cotyledons alpha-D-galactosidase was restricted to vacuoles which are storing lectin precursors [10]. In the cells of soybean cotyledons alpha-D-galactosidase occurs in cisterns of the Golgi apparatus and may be deposited in protein bodies [11]. A similar observation was made in field bean [7]. Plant [18], studying the cotyledons of germinating narrow-leafed lupin, established a direct role of alpha-D-galactosidase and alpha-D-mannosidase in hydrolysis of glycoproteins and storage galactosides. In yellow lupin seeds the activity of alpha-D-galactosidase increases only at the beginning of germination [14] when the accumulated raffinose oligosaccharides and galactosyl cyclitols decompose completely [9].

While the role of alpha-D-galactosidase in the hydrolysis of saccharides and glycoproteins in germinating seeds is well recognised, the activity of this enzyme in non-germinating (stored) seeds remains to be elucidated. The content of oligosaccharides in seeds of various species was observed to decrease with an increase in the storage period [1,12,21,23,]. There was a positive correlation between the drop in raffinose oligosaccharides and slump in the seed longevity.

The present study was undertaken to compare the activity of alpha-D-galactosidase with quantitative and qualitative composition of oligosaccharides and galactosyl cyclitols in the yellow lupin seeds stored.

MATERIALS AND METHODS

The experiments investigated maturing seeds of 'Juno' yellow lupin (*Lupinus luteus* L.) grown on plots of the Agricultural Experiment Station at Tomaszkowo in the vicinity of Olsztyn over the 1991, 1992 and 1994 seasons. The seeds were collected at 5-day intervals, beginning from the 15th day after flowering from 3 lower pods. Having air-dried under laboratory conditions (the temperature of 18-20°C, 60-70% of RH) to 6-8% of water content, the seeds were placed for storage in tightly closed containers. In 1994, right after harvest the maturing seeds were frozen in liquid nitrogen and stored at –21°C. In 1997 the study defined the alpha-D-galactosidase activity as well as quantitative and qualitative composition of soluble sugars (with gas chromatography, GC).

Determination of seed vigour and viability. The seed viability was assessed with the germination method (10 days in wet paper towels at 20°C), while vigour with the sprout growth analysis (after 5 days of germination at 20°C) and the measurement of seed leachate electroconductivity [8].

Determination of alpha-D-galactosidase activity. The activity of alpha-D-galactosidase was determined following Lehle et al. [15] and McCleary et al. [16]. 50 mM HEPES - NaOH, pH 7.4, constituted the extraction buffer. Following centrifugation (10 000 x g, 20 min, 4°C), the supernatant was fractionated with ammonium sulphate added to obtain 40% and 80% saturation. The salted-out fractions were dialysed for 24 hours with the 0.005 M phosphate buffer (pH 5.0 at the temperature of 4°C). Following centrifugation (10 000 x g, 10 min, 4°C), 0.1 ml of supernatant was withdrawn to a glass test tube and 0.2 ml of 0.1M phosphate buffer (pH 4.5) and 0.1 ml of alpha-D-p-O-nitrophenylgalactopiranoside (0.01% substrate in 0.1M phosphate buffer, pH 4.5) were added. The reaction (15 min. at 37°C) was completed adding 2.6 ml (cold) of 0.1 M solution of Na_2CO_3 in 0.1M NaOH. The optical density was measured at the wavelength of 420 nm. A standard curve was plotted for pure p-nitrophenol (Sigma) at the concentration range of 5-50 micro g/3ml (for Ex1000, y=35.05x + 14.02, where y - absorbance, x - p-nitrophenol concentration).

Determination of sugars with gas chromatography. The soluble dry and frozen seed sugars were extracted with a 50% ethanol (30 min, 90°C) with the internal standard – phenyl alpha-D-glucoside. The extracts were centrifuged (10 000 x g, 10 min), filtered through micro-spin filters (10 000 MWCO, Lida) and dried with a stream of nitrogen. Sugars were derived with TMSI-pyridine solution, while trimethylsilyl-derivates were separated on capillary DB1 column (J & W Scientific, 15 m in length, 0.25 micro m thick film) with programmed temperature column heating [9].

RESULTS

Seed viability and vigour. Seeds harvested on 15 DAF were not capable of germination. An increase in viability was observed at the phase of the cotyledon cell enlargement and dry weight accumulation. Mature seeds showed the highest vigour and viability. A decrease in vigour, manifested by shortened sprouts, and several-fold increased seed leachate electroconductivity was recorded in seeds that had been stored for 6 years (collected in 1991). The seed leachate electroconductivity was highest in unripe seeds (15 and 25 DAF) stored for 5 and 6 years (after 6 years much higher than after 5 years). Increased permeability of cell membranes did not visibly affect the high (100%) germination capacity of mature yellow lupin seeds (Table 1).

Table 1. Viability and vigour of 'Juno' yellow lupin seeds

Year of harvest	DAF	Viability (%)		Length of seedling (mm)		Electroconductivity (microS·cm^{-1}·seed^{-1}	
		1*	2*	1	2	1	2
1992	15	0	0	0	0	6.4 ± 0.6	10.3 ± 0.2
	25	5.0 ± 2.0	50 ± 5.8	14.0 ± 2.0	20.9 ± 6.5	6.3 ± 0.2	11.1 ± 0.9
	35	78.0 ± 6.2	100	68.0 ± 4.3	65.0 ± 7.5	3.1 ± 0.5	3.2 ± 0.2
1991	15	0	0	0	0	1.3 ± 0.1	71.3 ± 0.7
	25	2.0 ± 12	6.7 ± 3.3	0	0.96 ± 0.9	1.1 ± 0.01	18.1 ± 0.6
	35	94.0 ± 2.1	100	95.0 ± 11	53.6 ± 2.2	0.8 ± 0.01	5.5 ± 1.2

1* - freshly harvested seeds
2* - stored seeds, 1997 analysis

Alpha-D-galactosidase. In developing seeds of yellow lupin, the activity of alpha-D-galactosidase (per seed) increased since 15 DAF till full seed maturity, irrespective of the year of harvest (Fig. 1). In mature seeds, kept in dry storage (collected in 1991 and 1992), activity of alpha-D-galactosidase was higher than in seeds stored at -21°C. In the older seeds (from 1991 and 1992), the activity of alpha-D-galactosidase was lower than in the seeds harvested in 1994 (Table 2).

Table 2. Activity of alpha-D-galactosidase in extracts of mature (35 DAF) 'Juno' yellow lupin seeds after fractionation with ammonium sulphate (in ng of p-nitrophenol x min^{-1}x mg^{-1} protein)

Year of harvest (Time of storage, years)	Crude extract Crude extract	Supernatant fractionated with ammonium sulphate	
		to 40% of saturation	to 80% of saturation
1994 (3)	118.99	282.42	632.07
1992 (5)	128.95	239.27	466.06
1991 (6)	125.99	236.40	528.38

Raffinose oligosaccharides (RFOs) and cyclitols. The highest contents of raffinose oligosaccharides and cyclitols were recorded in mature seeds (35 DAF, Fig. 2). In the seeds collected 25 DAF the level of oligosaccharides and cyclitols was several-fold lower, while in the seeds harvested 15 DAF those saccharides were not detected. In mature seeds stored since 1991, the contents of cyclitols (myo-inositol, D-pinitol) and galactosyl cyclitols (galactinol, fagopyritol B1, B2, ciceritol) were higher than in seeds stored since 1992, while the contents of stachyose and verbascose were lower. Unripe seeds from 1991 (15 and 25 DAF) contained more myo-inositol and sucrose than the seeds from 1992, while the contents of other saccharides did not differ significantly. The contents of saccharides in seeds from dry storage, irrespective of the seed maturity level, were higher than in seeds frozen immediately after harvest (in 1994) and stored at -21°C. Dry storage of seeds could have resulted in a gradual decrease in raffinose oligosaccharides to sucrose ratio (Table 3).

Fig. 1. Activity of α-D-galactosidase in 'Juno' yellow lupin seeds U = μ g of *p*-nitrophenol x min^{-1} x $seed^{-1}$)

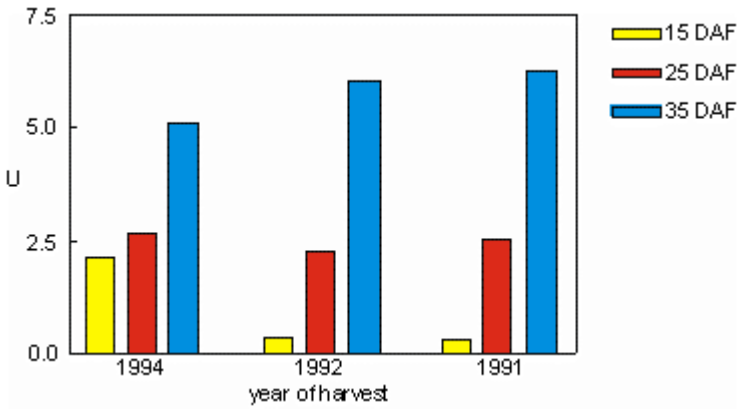

Fig. 2. Content of ethanol soluble carbohydrates in the 'Juno' yellow lupin seeds stored

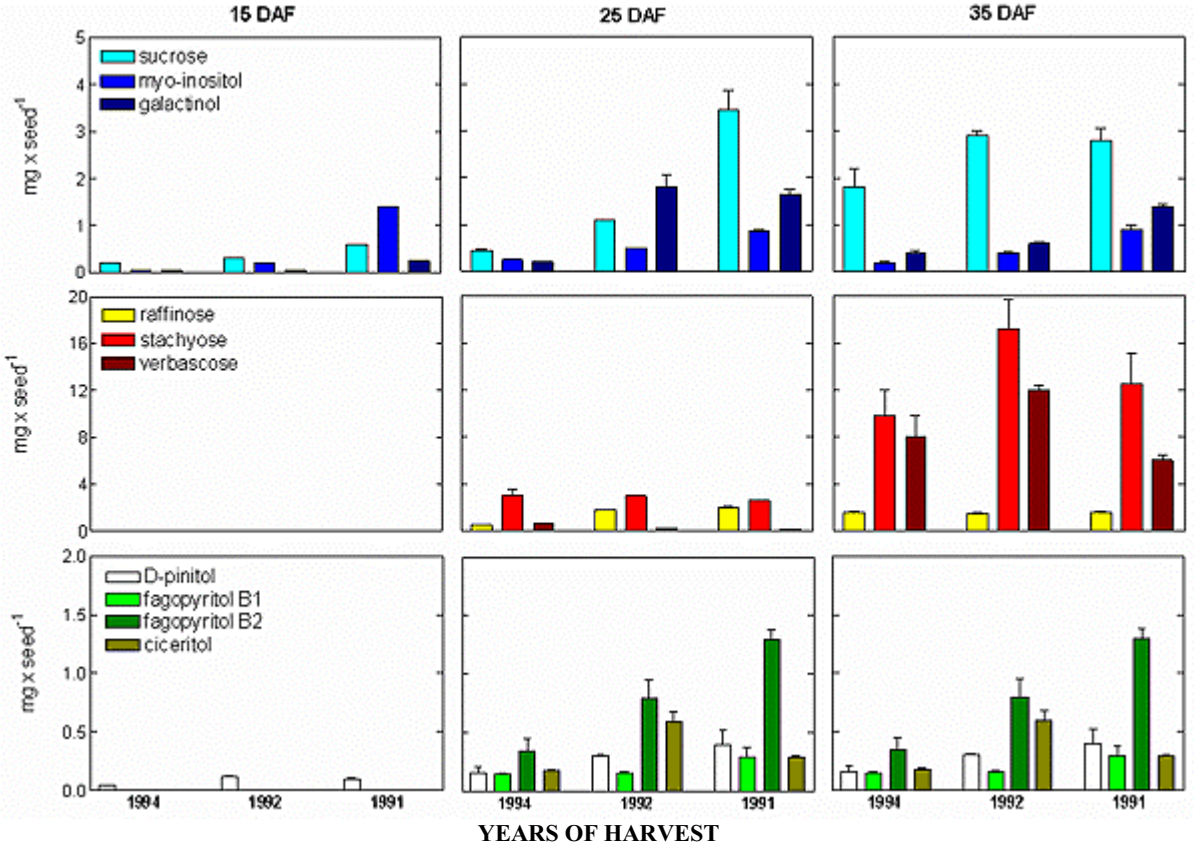

Table 3. Raffinose oligosaccharides: sucrose ratio in 'Juno' yellow lupin seeds stored

Year of harvest (Time of storage, years)	Seed harvested at	
	25 DAF	35 DAF
1994* (3)	10.0	10.7
1992 (5)	3.9	9.7
1991 (6)	1.2	6.7

DISCUSSION

In various species seed drying shows a stimulatory effect on the synthesis of raffinose oligosaccharides [3,9,12,13]. In the present study the stimulatory effect of seed drying on oligosaccharides (and possibly galactosyl cyclitols) biosynthesis could have been manifested by: (i) the accumulation of galactinol (at 15 and 25 DAF), which is the basic donor of galactose moiety for the synthesis of RFOs [15] and (ii) by an increased saccharide content in seeds from dry storage, as compared to those stored at -21°C. The sucrose content was similar in all seed samples from dry storage. Seeds harvested in 1991, compared to those from 1992, contained more myo-inositol, galactinol and raffinose, yet less verbascose and stachyose. The breakdown of oligosaccharides was occurred both in the mature seeds (35 DAF) and in those collected 25 DAF. This result could be explained by a higher alpha-D-galactosidase activity in the seeds from 1991.

Taufel et al. [21] established a direct effect of an increased temperature (30-32°C) and higher air RH (90-98%) on the breakdown of raffinose oligosaccharides in the stored seeds of bean, soybean and pea. Under similar conditions (35°C, 100% RH), raffinose and sucrose declined in maize kernels [1]. With increased seed water content, the hydrolysis of raffinose could be catalysed by alpha-D-galactosidase and the breakdown of sucrose by invertase. In the present study the water content in the seeds stored (dried after harvest in 1991 and 1992) was 6% and 8% in seeds harvested 35 and 15 DAF, respectively. It is not clear if alpha-D-galactosidase can be active under such a low seed hydration. Probably some differences in the level of intracellular protein hydration and a direct contact of enzyme with oligosaccharides may enable the hydrolysis reaction in tissues of the seeds stored. A decreased level of raffinose saccharides in frozen seeds may result from a high activity of alpha-D-galactosidase in mature seeds, as compared with dry seeds, and, on the other hand, from oligosaccharide synthesis occurring over seed drying.

The pattern of quantitative and qualitative changes in galactosyl cyclitols composition differed from that of raffinose oligosaccharides. In the lupin seeds stored, galactose released from oligosaccharides was probably used as a substrate for the synthesis of galactosyl cyclitols (fagopyritol B2, mainly), whose content gradually increased, which may be one of the ways to decrease reducing sugars known to have an adverse effect on the viability of the seeds stored [12].

The decrease in the ratio of raffinose oligosaccharides to sucrose was noticed in ageing seeds of several species, which is positively correlated with a decrease in seed vigour and accelerated ageing of the embryo [12]. In the present study, the vigour of the seeds stored was lower, as compared with freshly harvested seeds. The biggest slump in vigour (manifested by a decreased seedling length and increased membrane permeability) was noticed in the seeds stored over the longest period and harvested at the lowest level of maturity.

The results of the present study demonstrate a relationship between an increase in the activity of alpha-D-galactosidase and decrease in the content of raffinose oligosaccharides (and their ratio to sucrose) in the yellow lupin seeds stored. The concurrent drop in the seed vigour may confirm a direct relationship between the longevity of yellow lupin seeds and the content of raffinose oligosaccharides in seeds.

CONCLUSIONS

1. The present study demonstrates crucial effects of alpha-D-galactosidase on the RFO breakdown in the yellow lupin seeds stored.
2. An increase in the storage time decreased the ratio of RFO to sucrose in seeds of different maturity, which could have decreased the cell membrane integrity and seed vigour; due to these changes, the seeds lost their germination ability.
3. Alpha-D-galactosidase plays a crucial role in metabolic changes of soluble carbohydrates in the seeds stored. However, the catalytic properties and the ability of alpha-D-galactosidase to hydrolyse RFO at a low tissue hydration level are still unknown and need further investigations.

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Submited:

Lesław B. Lahuta, Ryszard J. Górecki, Dariusz Michalczyk, Agnieszka I. Piotrowicz-Cieślak
Department of Plant Physiology and Biotechnology
Warmia and Masuria University
Plac Łódzki 3, 10-718 Olsztyn, Poland