

RESEARCH PAPER

Lipid and protein accumulation in developing seeds of three lupine species: *Lupinus luteus* L., *Lupinus albus* L., and *Lupinus mutabilis* Sweet

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Abstract

A comparative study was carried out on the dynamics of lipid accumulation in developing seeds of three lupine species. Lupine seeds differ in lipid content; yellow lupine (*Lupinus luteus* L.) seeds contain about 6%, white lupine (*Lupinus albus* L.) 7–14%, and Andean lupine (*Lupinus mutabilis* Sweet) about 20% of lipids by dry mass. Cotyledons from developing seeds were isolated and cultured *in vitro* for 96 h on Heller medium with 60 mM sucrose (+S) or without sucrose (–S). Each medium was additionally enriched with 35 mM asparagine or 35 mM NaNO₃. Asparagine caused an increase in protein accumulation and simultaneously decreased the lipid content, but nitrate increased accumulation of both protein and lipid. Experiments with [1-¹⁴C]acetate and [2-¹⁴C]acetate showed that the decrease in lipid accumulation in developing lupine seeds resulted from exhaustion of lipid precursors rather than from degradation or modification of the enzymatic apparatus. The carbon atom from the C-1 position of acetate was liberated mainly as CO₂, whereas the carbon atom from the C-2 position was preferentially used in anabolic pathways. The dominant phospholipid in the investigated lupine seed storage organs was phosphatidylcholine. The main fatty acid in yellow lupine cotyledons was linoleic acid, in white lupine it was oleic acid, and in Andean lupine it was both linoleic and oleic acids. The relationship between stimulation of lipid and protein accumulation by nitrate in developing lupine cotyledons and enhanced carbon flux through glycolysis caused by the inorganic nitrogen form is discussed.

Key words: Acetate, asparagine, carbon partitioning, fatty acids, nitrate, phospholipids, seed development, storage lipids, storage proteins, sucrose.

Introduction

Metabolic pathways of storage proteins, oil, and starch have been well described; however, the mechanisms determining the differential partitioning of seed reserves into the major storage components remain largely unknown. Most pathways are branched, and there are alternative routes, futile cycles, and product turnover. A major metabolic activity in developing seeds is the conversion of sucrose via glycolysis to oil and lipids (Baud *et al.*, 2008; Wei *et al.*, 2008). Sucrose is imported from the photosynthetic tissues of the mother plant and converted via

glycolysis into the primary substrate for plastidic fatty acid synthesis, i.e. acetyl-CoA (Rawsthorne, 2002; Hills, 2004). However, the synthesis of oil in developing seeds is dependent on the supply of many precursors, such as sucrose, acetate (Vigeolas *et al.*, 2003; Vigeolas and Geigenberger, 2004), glucose 6-phosphate, phosphoenol pyruvate, pyruvate, and malate (Rawsthorne, 2002) or glycerol 3-phosphate (Vigeolas *et al.*, 2007). In *Brassica napus*, enhancement of sugar metabolism and/or sink strength may provide many more precursors for seed lipid synthesis,

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and thus increase the oil content (Li *et al.*, 2006). However, sucrose has a dual function as a transport and nutrient sugar and as a signal molecule triggering storage-associated processes. Sucrose acts at the transcriptional and post-transcriptional levels, thereby affecting carbon fluxes. It is a key player within the regulatory network controlling seed differentiation (Weber *et al.*, 2005; Rolland *et al.*, 2006). In *B. napus* and sunflower, exogenous amino acids are used as a nitrogen and carbon source for protein synthesis, but not as a carbon source for plastidic fatty acid synthesis (Schwender and Ohlrogge, 2002; Schwender *et al.*, 2006; Alonso *et al.*, 2007). However, in soybean, there is a significant flux of carbon from amino acids into lipids. Amino acid carbon, via malic enzyme and pyruvate, is imported into the plastid and accounts for about 10% of lipid carbon (Allen *et al.*, 2009).

There are many data confirming that the biosyntheses of storage protein and oil in developing oil-storing seeds are independent processes. A decrease in the amount of protein or oil in seeds does not generally lead to a compensating increase in other major storage compounds. This has been shown, for example, in *Arabidopsis* *abi* and *aba* mutants where storage protein content is strongly reduced but oil content does not change significantly (Finkelstein and Somerville, 1990), and in the *wr1* mutant where oil is reduced by 80% but storage protein content is similar to that of wild-type seeds (Focks and Benning, 1998). In two lines of *B. napus* with a 10% difference in oil content there is only a 1% difference in protein content (Li *et al.*, 2006). In contrast to oil-storing seeds, in protein-storing seeds there is a negative correlation between protein and oil accumulation. It has been well documented in soybean (Shannon *et al.*, 1972; Brim and Burton, 1979; Sebern and Lambert, 1984; Wilcox and Cavins, 1995; Wilcox, 1998; Cober and Voldeng, 2000; Wilcox and Shibles, 2001; Chung *et al.*, 2003; Lardizabal *et al.*, 2008). In soybean there is also a negative relationship between accumulation of seed protein and carbohydrates (Wilcox and Shibles, 2001). Moreover, in soybean, protein deposition starts in the peripheral (abaxial) region of seeds (Perez-Grau and Goldberg, 1989), but the oil level is the highest in the inner (abaxial) part of cotyledons and lowest in the embryo axis and abaxial region of cotyledons. The inner region of cotyledons shows an ~5-fold higher lipid content compared with peripheral regions (Borisjuk *et al.*, 2005).

Lupine plants in field conditions thrive in symbiosis with dinitrogen-fixating *Rhizobium* sp., which supply the host plant with nitrogen in the form of asparagine. This is a dominant amino acid in legume seeds. For example, in lupine germinating seeds, asparagine can constitute up to 30% of dry matter (Lehmann and Ratajczak, 2008) and from 33% to 49% of the free amino acid pool in developing soybean seeds (Hernández-Sebastià *et al.*, 2005). In lupine seeds, the major storage proteins are globulins, called conglutins (Ratajczak *et al.*, 1988, 1996, 1999). It has been well documented that asparagine stimulates synthesis of polypeptides related to both

conglutinins α and β in yellow lupine seeds (Ratajczak *et al.*, 1988, 1996). A positive correlation between free asparagine and seed protein content at maturity was confirmed in soybean. The asparagine content in developing cotyledons may be a determinant of storage protein biosynthesis (Hernández-Sebastià *et al.*, 2005). In contrast to asparagine, nitrate is an unfavourable source of nitrogen, which not only does not stimulate, but sometimes even depresses both synthesis and accumulation of total globulins in developing yellow lupine seeds (Ratajczak *et al.*, 1988, 1996). Since nitrate limits the synthesis of storage proteins the question can be raised of how nitrate stimulates the synthesis of storage oil in developing lupine seeds.

Here a comparative study is presented on the dynamics of lipid accumulation in developing seeds of three lupine species with different lipid content: yellow lupine (*Lupinus luteus* L.; lipid content about 6%), white lupine (*Lupinus albus* L.; lipid content 7–14%), and Andean lupine (*Lupinus mutabilis* Sweet; lipid content about 20%). For comparison, oil accumulation in dry seeds of soybean might range from 12% to 26% (Zhou *et al.*, 2006). Whereas yellow and white lupine are crops of the Old World (Winch, 2006), Andean lupine has been commonly grown in South America, especially in the Andean highlands (Putnam, 1993; Cady, 2004; Winch, 2006). However, Andean lupine has also already been grown experimentally in Europe, South Africa, Australia (Davis, 1981), and Russia (Kurlovich, 2002; Cady, 2004). It is important to find breakpoints in the network of lipid metabolism in lupine tissues that determine the efficiency of synthesis of lipids. The level of storage lipids varies depending on species, but also depending on trophic conditions. Studies conducted on *in vitro* cultivated immature cotyledons isolated from developing lupine seeds will allow the relationship between the intensity of lipid accumulation and trophic conditions to be specified. The trophic factors (i.e. sucrose, asparagine, and nitrate) were supplied in compound media. ^{14}C -Labelled acetic acid was used as a precursor for lipid synthesis. Total lipid content together with particular lipid fractions was analysed. The differences in ultrastructure of the tissues of organs that accumulate lipids in various trophic conditions were identified. A relationship between storage lipid and protein accumulation in developing lupine seeds was determined. The presented data give the biochemical and anatomical characteristics of differences in the synthesis of storage lipids in yellow, white, and Andean lupine. In *in vitro* grown developing lupine cotyledons, the effect of availability of carbon and nitrogen sources on lipid synthesis was determined.

Materials and methods

Plant material

Plants of yellow lupine (*L. luteus* L.) cv. Juno, white lupine (*L. albus* L.) cv. Butan, and Andean lupine (*L. mutabilis*

Sweet) were grown on experimental fields of the Plant Breeding Station Smolice Division in Przebędowo (Poland) in 2006 and 2007. Pods containing seeds at the five successive developmental stages (I–V, according to the morphological and cytological classification; Woźny *et al.*, 1984; Ratajczak, 1986) were collected. Seeds from each developmental stage were isolated from the pods, frozen in liquid nitrogen, kept at -70°C , and used for determination of the total lipid level and soluble protein. Pods containing seeds in developmental stage III were surface-sterilized by 30 s immersion in 96% (v/v) ethanol and 20 min in 0.2% HgCl_2 , and were finally washed five times with sterile, distilled water. Seeds were isolated from the pods and classified with respect to their uniform size. Cotyledons were separated from embryo axes and divided into three parts. One part was frozen in liquid nitrogen, stored at -70°C , and used for determination of the total lipid level, phospholipids, free fatty acids, and soluble protein. The second part was used for ultrastructural observation. The third part was used for preparation of *in vitro* culture. Isolated cotyledons were placed in 10 cm Petri dishes containing 11 ml of Heller medium (Heller, 1954) with 60 mM sucrose (+S) or without sucrose (–S). Each medium was additionally enriched with 35 mM asparagine or 35 mM NaNO_3 . The amino acid solutions were sterilized by passing them through 0.22 μm Millipore filters before adding to autoclaved media. The cotyledon *in vitro* cultures were maintained at a stable temperature of 25°C and a constant illumination of $75\ \mu\text{M}$ light quantum $\text{m}^{-2}\ \text{s}^{-1}$. After 96 h of culture, cotyledons were washed in water and used for experiments. One part was frozen in liquid nitrogen, kept at -70°C , and used for determination of the total lipid level, phospholipids, fatty acids, and soluble protein. The second part was used for ultrastructural observation. The third part was used for radiolabelling experiments with $[1-^{14}\text{C}]\text{acetate}$ and $[2-^{14}\text{C}]\text{acetate}$.

Metabolism of $[1-^{14}\text{C}]\text{acetate}$ and $[2-^{14}\text{C}]\text{acetate}$ in developing cotyledons

Cotyledons which were previously grown for 96 h in *in vitro* conditions were incubated in solutions of $[1-^{14}\text{C}]\text{acetate}$ and $[2-^{14}\text{C}]\text{acetate}$ as described previously (Borek *et al.*, 2003). Each culture medium (8 ml) was enriched with 925 kBq of specifically labelled acetate. Cotyledons

were incubated with constant rotary shaking and constant illumination of $75\ \mu\text{M}$ light quantum $\text{m}^{-2}\ \text{s}^{-1}$. Pieces of filter paper soaked with 200 μl of 20% KOH were placed above the surface of the incubation medium. After 120 min of incubation, the pieces were removed and their radioactivity was measured using a Packard Tri-Carb 2100 scintillation counter. Cotyledons were washed in water, frozen in liquid nitrogen, and kept at -70°C for determination of the radioactivity of the chloroform:methanol fraction (used for determination of total lipid; see below).

Determination of total lipid, phospholipids, and total fatty acids

Total lipids were extracted from seeds using chloroform:methanol (2:1, v/v) containing 0.05% butylated hydroxytoluene (BHT), according to Allen *et al.* (1966), as described by Pukacka (1991). The amounts of lipids were determined gravimetrically. Phospholipids were determined in aliquots of the lipid extracts separated by one-dimensional TLC in chloroform:methanol:acetic acid:water (85:15:10:3.5, v/v; Nichols *et al.*, 1965), using original phospholipid standards. Spots containing phospholipids and detected with iodine vapour were scraped off for analysis of phosphorus. The phosphorus content was estimated according to Ames (1966). Total fatty acids (as methyl esters, after transmethylation of the extracted lipids with 0.5 M KOH in methanol for 15 min at 70°C and extraction in hexane) were determined by using an Agilent 6890 gas chromatograph, column 30 m DB25, temperature 200°C , and a flame ionization detector (FID).

Preparation of tissues for transmission electron microscopy

Fragments of isolated cotyledons from 96 h *in vitro* culture were fixed in Carnoy's half-strength fixative (Karnowsky, 1965), i.e. in a mixture of 4% glutaraldehyde and 4% paraformaldehyde (1:1, v/v). Post-fixation was conducted in 1% OsO_4 . The samples were stained in a 2% aqueous solution of uranyl acetate. Dehydration was performed in a series of acetone solutions. The objects were embedded in epoxy resin of low viscosity (Spurr, 1969). Ultrathin sections were prepared by using an Ultratome III (LKB). They were stained in 5% uranyl acetate and 0.5% lead

Table 1. Fresh weight (FW; mg) and dry matter (DM; % of fresh weight) content in seeds from five developmental stages
Mean weight of single seed \pm SD.

Developmental stage	Yellow lupine		White lupine		Andean lupine	
	FW	DM	FW	DM	FW	DM
I	55.5 \pm 4.6	11.6 \pm 0.7	112.1 \pm 10.0	16.5 \pm 0.2	69.7 \pm 9.1	15.2 \pm 0.9
II	110.3 \pm 8.1	13.4 \pm 1.4	265.1 \pm 20.6	17.3 \pm 0.8	130.0 \pm 11.0	16.2 \pm 1.3
III	240.3 \pm 10.6	15.1 \pm 1.3	608.7 \pm 46.3	18.5 \pm 0.2	249.9 \pm 17.0	19.7 \pm 1.0
IV	418.4 \pm 23.8	34.8 \pm 0.1	769.6 \pm 64.8	35.1 \pm 2.1	279.6 \pm 15.2	32.2 \pm 5.8
V	234.7 \pm 34.4	43.7 \pm 1.6	668.8 \pm 62.2	43.1 \pm 4.4	210.5 \pm 13.0	42.5 \pm 4.2

citrate and observed under a transmission electron microscope (TEM-1200 Ex JEOL).

Protein determination

Whole seeds or cotyledons were homogenized in 0.1 M TRIS-HCl buffer, pH 7.8 (4 ml for 1 g of fresh weight). The homogenate was centrifuged for 20 min at 22 000 *g*. Protein concentration in the supernatant was determined according to Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

The experiments were carried out in 2006 and repeated in 2007, with three replications in each year. The results are the mean \pm SD of the data obtained in the two years, with the exception of the data from experiments with [14 C] acetate (Figs 9, 10) that represent only one breeding season. Significance of differences between mean values was determined with the Student's *t*-test. The statistical analysis was used only in assessing the significance of the differences caused by asparagine and NaNO₃ added to the +S and -S medium variants of *in vitro* culture.

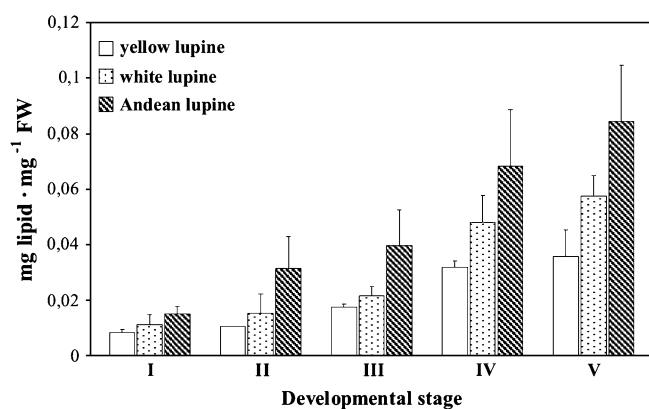


Fig. 1. Total lipid level in whole seeds from five developmental stages.

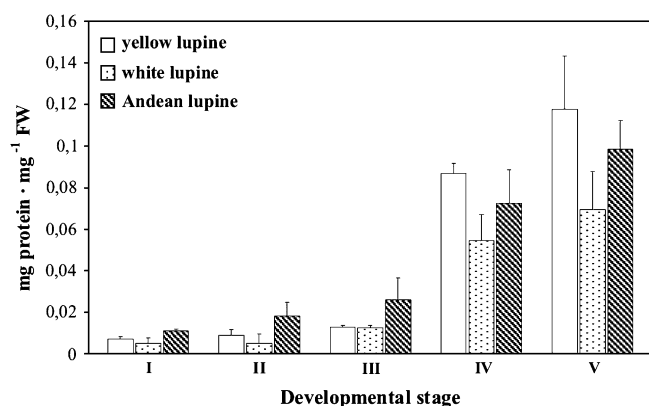


Fig. 2. Soluble protein content in whole seeds from five developmental stages.

Results

Five stages (denoted I–V) can be distinguished in the lupine seed developmental period, based on morphological and anatomical traits. This classification of yellow lupine seeds was described in detail by Woźny *et al.* (1984) and Ratajczak (1986). In white and Andean lupine seed, the same stages of developmental were distinguished. Briefly, in the first two stages, endosperm was still present in liquid form (stage I) or in the form of a gelatinous lump (stage II). In stage III, seeds were light green. Endosperm disappeared and a dark green embryo filled the entire interior of the seed. In stage IV, seeds were at their biggest, the embryo was a little less green than in the preceding stage, and the embryo axis began turning yellow. Little pronounced brown

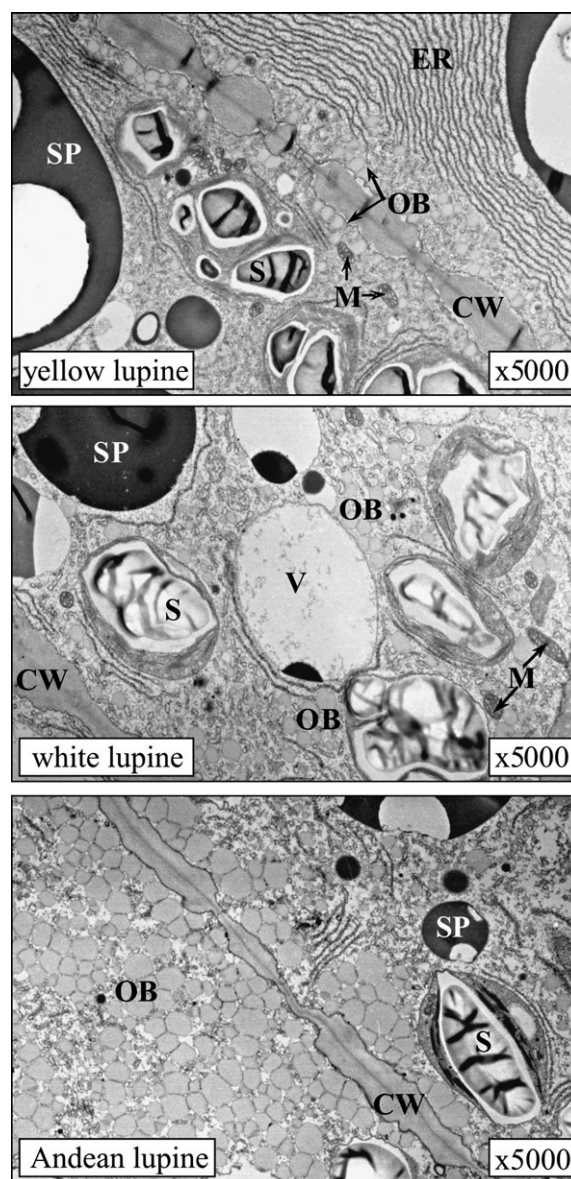


Fig. 3. Electron micrographs of cotyledons isolated from seeds from developmental stage III (used for preparation of *in vitro* culture). CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; OB, oil body; S, starch; SP, storage protein; V, vacuole.

Table 2. Fresh weight (FW; mg) and dry matter (DM; % of fresh weight) content in cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h

Mean weight of a single cotyledon \pm SD. Control, cotyledons isolated from seeds used for preparation of *in vitro* culture; +S, medium with 60 mM sucrose; –S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃, medium with 35 mM NaNO₃. Statistical significance at ***P* < 0.01.

	Yellow lupine		White lupine		Andean lupine	
	FW	DM	FW	DM	FW	DM
Control	67.1 \pm 14.2	12.2 \pm 1.8	179.7 \pm 20.0	20.8 \pm 2.5	74.7 \pm 6.9	17.5 \pm 1.0
+S	94.6 \pm 3.8	13.6 \pm 1.6	193.6 \pm 11.7	20.7 \pm 3.1	99.3 \pm 6.2	20.1 \pm 3.3
+S+Asn	102.4 \pm 10.0	12.2 \pm 1.8	197.9 \pm 6.4	20.4 \pm 3.7	108.1 \pm 12.7	21.7 \pm 3.7
+S+NO ₃	90.3 \pm 10.4	13.8 \pm 2.5	190.1 \pm 18.6	21.4 \pm 3.6	96.1 \pm 7.3	22.0 \pm 2.1
–S	74.1 \pm 5.5	9.0 \pm 2.0	196.9 \pm 16.5	17.5 \pm 3.1	90.4 \pm 4.3	19.5 \pm 2.8
–S+Asn	96.7 \pm 10.5**	8.9 \pm 2.1	199.2 \pm 12.3	15.4 \pm 3.2	96.5 \pm 10.2	20.9 \pm 2.0
–S+NO ₃	77.0 \pm 8.0	9.1 \pm 2.2	199.0 \pm 9.6	17.7 \pm 3.1	95.2 \pm 10.0	19.7 \pm 2.5

spots appeared on the seed coat (except for white lupine seeds). In the last developmental stage (V), seeds were desiccated, the embryo axis was yellow, and cotyledons were light green/yellow. Brown spots (mottling) were clearly visible on the yellow and Andean lupine seed coat (data not presented). The seed fresh weight increased in consecutive developmental stages up to stage IV, but the highest content of dry matter was found in seeds from stage V (Table 1).

The total lipid level in the five developmental stages was determined. In the three lupine species the lipid level increased almost linearly during the seed developmental period and reached the maximal level in stage V. In that stage, the highest content of lipid was in Andean lupine seeds, whereas the lowest was noted in the seeds of yellow lupine (Fig. 1). For the following experiments, cotyledons from developmental stage III were used, because in that stage the lipid level was 45–50% of the maximum content. In consecutive stages the soluble protein content was also increased, but the highest protein accumulation took place in stages IV and V (Fig. 2). In these developmental stages, in contrast to the lipid level, the highest amount of protein was observed in the seeds of yellow lupine.

Different lipid levels in seeds of the three lupine species were clearly visible in the ultrastructure of cotyledon parenchyma cells. Cells from the central part of cotyledons from stage III were investigated. In yellow lupine cotyledons, oil bodies were small and sparse. They were sited mostly near the cell wall and vacuole, and rarely were dispersed in the cytoplasm (Fig. 3). In white lupine cotyledons, oil bodies were more numerous than in yellow lupine cotyledons but they were distributed evenly in the cytoplasm. The highest number of oil bodies was in Andean lupine cotyledons. They were visible mostly near the cell wall and in different parts of the cytoplasm, where they were aggregated in clusters (Fig. 3). Thus, clear superiority of Andean lupine in accumulating oil was noticed at the ultrastructural level in developmental stage III. Moreover, large starch granules were visible in cotyledons from developmental stage III of each investigated lupine species (Fig. 3).

Cotyledons from seeds from developmental stage III were isolated and cultured *in vitro* on Heller medium (Heller, 1954) supplemented with (+S) or deprived of 60 mM sucrose (–S). The media were additionally enriched with 35 mM asparagine or 35 mM NaNO₃. Sucrose, asparagine, and NaNO₃ influenced fresh weight and dry matter content in cotyledons. However, almost all differences were not statistically significant (Table 2). Asparagine stimulated the fresh weight of cotyledons of the three species grown on both +S and –S media, and in yellow and white lupine cotyledons it caused a decrease in dry matter content. NaNO₃ stimulated the fresh weight to a lesser extent than asparagine in cotyledons grown on –S medium, and caused a decrease in cotyledons grown on +S medium. The highest dry matter content was noticed in cotyledons grown on medium supplemented with NaNO₃ (with the exception of Andean lupine cotyledons grown on –S medium).

The total lipid level in *in vitro* grown cotyledons of each investigated species was higher in organs fed with sucrose (compare +S and –S; Fig. 4). Yellow and white lupine cotyledons grown *in vitro* on medium without sucrose (–S) contained less lipids than cotyledons used for preparation of *in vitro* culture (control in Fig. 4). In Andean lupine cotyledons the lipid level was higher (with and without sucrose) than in cotyledons used for preparation of *in vitro* culture (Fig. 4). Addition of asparagine to the medium (+S and –S) caused a decrease, whereas addition of NaNO₃ caused an increase in total lipid level. The inhibitory effect of asparagine was the clearest and was statistically significant in yellow and Andean lupine cotyledons cultured on medium without sucrose (decrease of about 20.6% and 22.3%, respectively). In yellow, white, and Andean lupine cotyledons fed with sucrose and NaNO₃, the lipid level was the highest among all the variants studied (increase of about 7.8, 8.6, and 10.2%, respectively; Fig. 4). The stimulatory effect of NaNO₃ on lipid accumulation was clearly visible in the ultrastructure of cotyledon parenchyma cells (Figs 5–7). It was estimated that the highest number of oil bodies accumulating storage lipids in cotyledons of three lupine species was in organs cultured on medium with

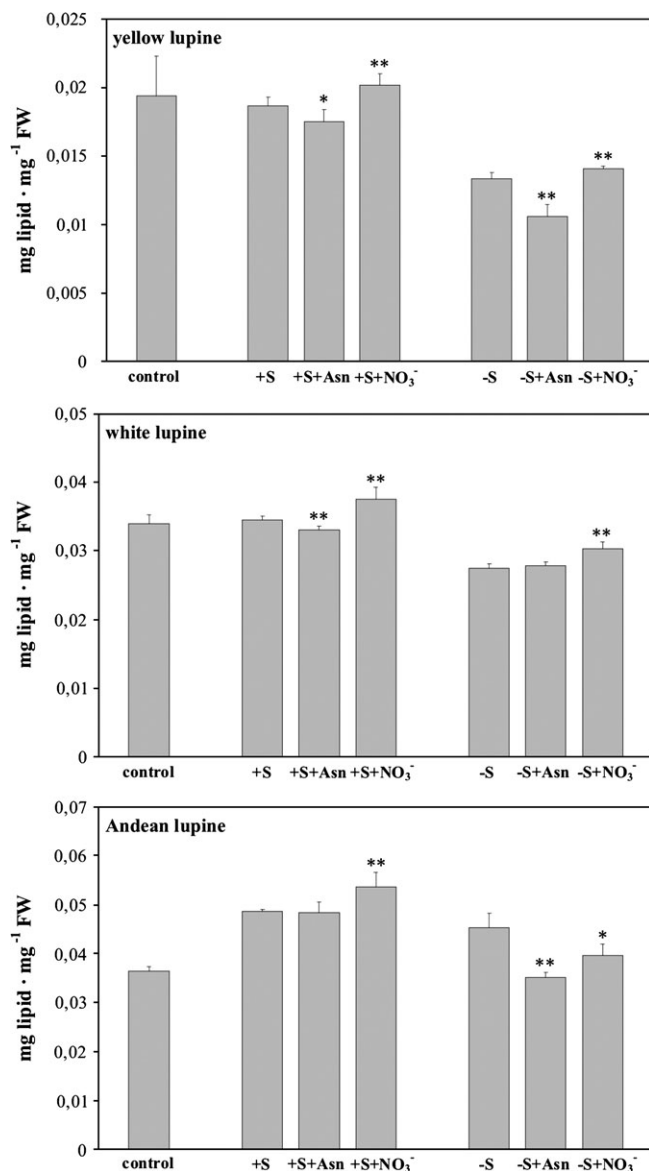


Fig. 4. Total lipid level in cotyledons isolated from seeds from developmental stage III grown *in vitro* for 96 h. Control, cotyledons isolated from seeds, used for preparation of *in vitro* culture; +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Statistical significance at **P* < 0.05; ***P* < 0.01.

sucrose and with NaNO₃. In cotyledons cultured on medium supplemented with asparagine, the number of lipid bodies was the lowest.

Soluble protein content in cotyledons of the three lupine species was clearly higher in organs cultured *in vitro* (in each culture variant) than in cotyledons isolated from developing seeds (Fig. 8). Sucrose caused an increase in protein content, and addition of asparagine to the medium (both +S and -S) resulted in an even greater increase in protein content. In yellow, white, and Andean lupine cotyledons, asparagine caused an increase of 28.1, 32.0, and 30.5% for +S medium and of 16.3, 25.1, and 22.5% for -S medium, respectively. A similar effect was caused by NaNO₃, but it

was weaker than in the case of asparagine (increase of 16.1, 9.3, and 11.4% for +S medium and of 6.0, 9.4, and 2.9% for -S medium, respectively; Fig. 8).

To determine the pathways of acetate in developing cotyledons, the liberation of ¹⁴CO₂ and incorporation of carbon atoms from specifically labelled acetate were measured. The highest amount of radioactive CO₂ was produced by yellow lupine cotyledons and the lowest by white lupine cotyledons (Fig. 9). The radioactivity of liberated CO₂ was markedly higher with carbon atoms coming from position C-1 than position C-2 of acetate. In an extreme case, the radioactivity of CO₂ with C-1 was >100-fold higher than the radioactivity of CO₂ with C-2 (white lupine, -S+NO₃⁻). In yellow and white lupine, more radioactive CO₂ was produced by cotyledons fed with sucrose, compared with cotyledons grown on medium without sucrose. There were no differences between +S and -S variants in Andean lupine. Asparagine and NaNO₃ caused a decrease in liberation of ¹⁴CO₂ (with C-1 and C-2 of acetate) but the inhibitory effect of asparagine was greater than that caused by NaNO₃. The exception was ¹⁴CO₂ with C-2 of acetate liberated by white lupine cotyledons when asparagine stimulated production of CO₂ with C-2 of acetate (Fig. 9). The highest radioactivity of the lipid fraction was in yellow and Andean lupine cotyledons (Fig. 10). In yellow lupine cotyledons from each medium variant, more radioactivity came from C-1 of acetate. In white lupine cotyledons, such a relationship was observed only in organs fed with sucrose (+S), whereas in Andean lupine it was seen in cotyledons grown on medium without the sugar (-S). NaNO₃ caused a decrease in the radioactivity of the lipid fraction in yellow and white lupine cotyledons fed with sucrose. In Andean lupine cotyledons, the same effect occurred in organs grown on medium both with and without the sugar. Asparagine caused no effect in yellow lupine cotyledons. In white lupine, the radioactivity of the lipid fraction was reduced only in cotyledons fed with sucrose, and in cotyledons of Andean lupine cultured on medium without sucrose (Fig. 10).

The trophic conditions of *in vitro* culture had a significant influence on the phospholipid content in developing cotyledons (Fig. 11), and changes in the phospholipid level were similar to changes in the level of total lipid (Fig. 4). The dominant phospholipid in cotyledons of the three lupine species was phosphatidylcholine. In yellow and Andean lupine cotyledons grown on medium without sucrose (-S), asparagine caused a decrease in all phospholipids. In cotyledons fed with sucrose (+S) this relationship was restricted only to the dominant phospholipid, i.e. phosphatidylcholine (Fig. 11). In white lupine cotyledons, sugar deficiency clearly reduced phosphatidylcholine and increased the phosphatidic acid level. Asparagine and NaNO₃ in white lupine cotyledons fed with sucrose did not cause significant changes in phospholipid content; however, in cotyledons cultured on medium without sucrose, a higher level of almost all phospholipids was observed (Fig. 11). The dominant fatty acid was linoleic acid in yellow lupine, oleic acid in white lupine, and oleic and linoleic acid in

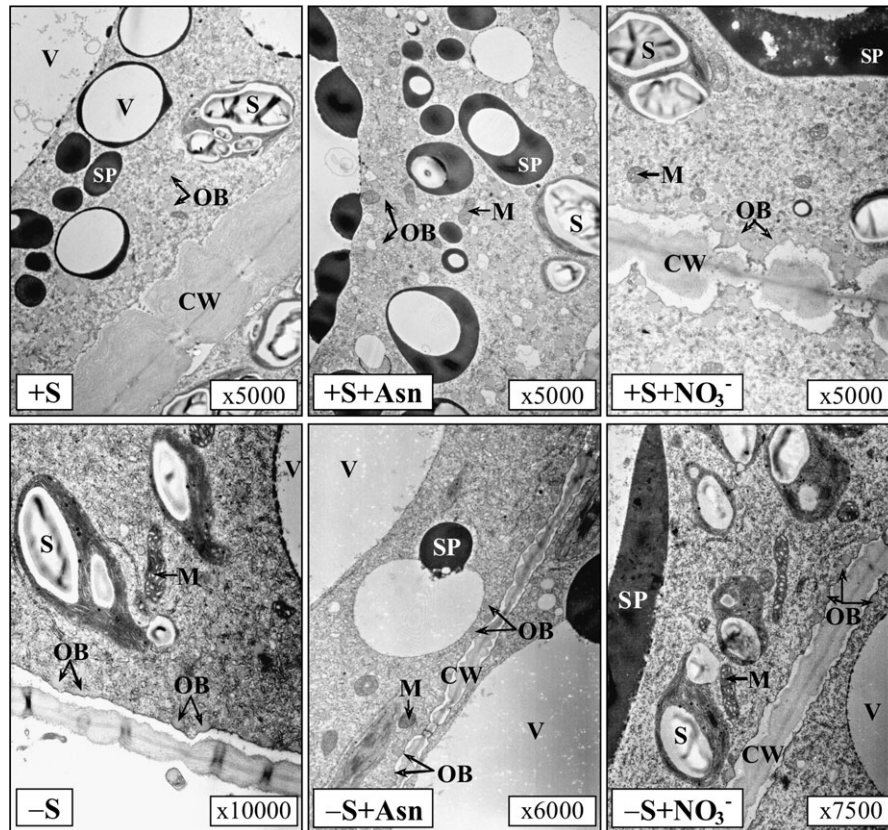


Fig. 5. Electron micrographs of yellow lupine cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. CW, cell wall; M, mitochondrion; OB, oil body; S, starch; SP, storage protein; V, vacuole.

Andean lupine cotyledons (Fig. 12). The highest content of erucic acid was noticed in white lupine cotyledons. In yellow lupine cotyledons there were vestigial quantities of this fatty acid, whereas Andean lupine cotyledons contained no erucic acid (Fig. 12). In contrast to phospholipids, generally there were no significant changes in fatty acid composition caused by *in vitro* culture conditions, with the exception of oleic acid, whose level in yellow lupine cotyledons fed with sucrose was decreased by asparagine (Fig. 12).

Discussion

A study on the influence of sucrose, asparagine, and nitrate on accumulation of storage lipids and proteins in developing seeds of three types of lupine seed was conducted. Developing cotyledons grown *in vitro* for 96 h were used. Asparagine added to the medium caused an increase of protein accumulation in cotyledons of all three lupine species (Fig. 8). Lipid accumulation was not stimulated or was reduced by asparagine (Fig. 4). In comparison, nitrate increased protein accumulation to a lesser extent than asparagine, but significantly stimulated lipid accumulation. Stimulation of lipid synthesis by nitrate in developing cotyledons can be interpreted in relation to findings obtained by metabolic flux analysis of *B. napus* embryos (Junker *et al.*, 2007). In *B. napus*, the inorganic form of

nitrogen enhances glycolytic efficiency. The results of [¹⁴C]acetate incorporation experiments indirectly prove the stimulatory effect of nitrate on glycolytic efficiency in developing lupine seeds. Nitrate decreased incorporation of ¹⁴C of acetate into the lipid fraction especially in yellow lupine cotyledons grown on medium with sucrose (+S+NO₃⁻ in Fig. 10). This result can be interpreted in the following way: enhancement of glycolysis by nitrate resulted in increased synthesis of acetyl-CoA from unlabelled sucrose. This led to enhanced carbon flux into lipids, and consequently lowered the radioactivity of the lipid fraction by dilution (Fig. 10), with a simultaneous increase in total lipid level (Fig. 4).

The inorganic form of nitrogen also stimulated protein accumulation in developing lupine seeds, although the provision of additional carbon skeletons is essential for amino acid synthesis. The main source of carbon skeletons for amino acid synthesis in developing seeds is the anaplerotic pathway involving phosphoenol pyruvate carboxylase (PEPC). In developing *B. napus* embryos, nitrate causes enhanced PEPC activity, and also triggers enhanced carbon flux through PEPC. Glycolysis enhanced by the inorganic nitrogen form provides more carbon skeletons for increased amino acid synthesis and simultaneously increases carbon flux into lipids (Junker *et al.*, 2007). Asparagine significantly stimulated protein accumulation but not lipid accumulation in lupine cotyledons. Therefore, in lupine

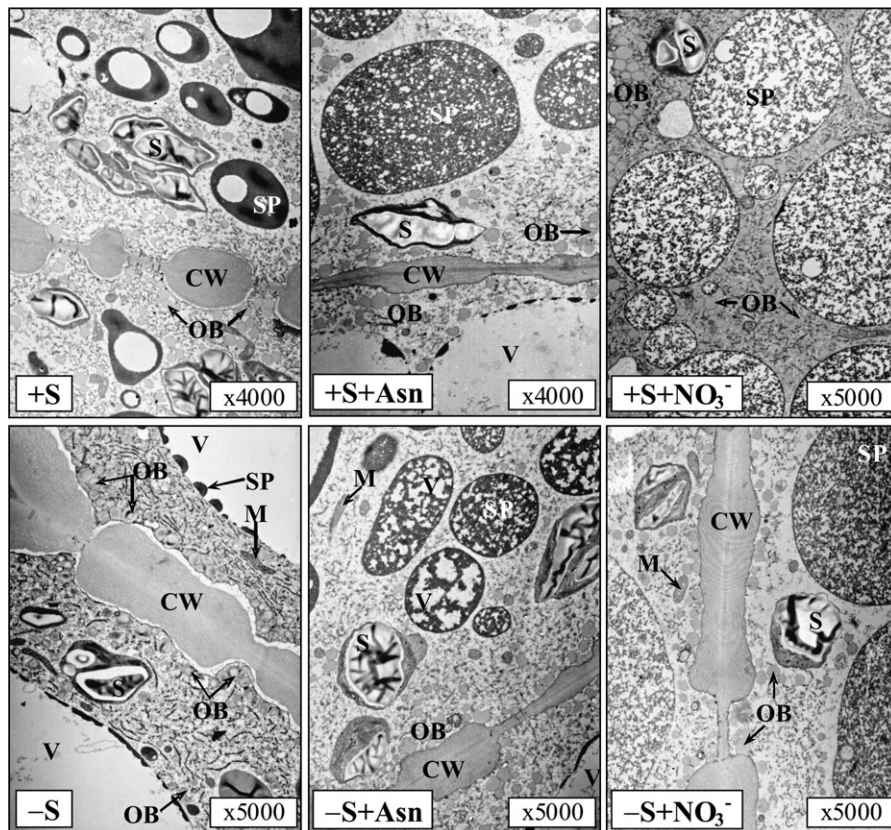


Fig. 6. Electron micrographs of white lupine cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. CW, cell wall; M, mitochondrion; OB, oil body; S, starch; SP, storage protein; V, vacuole.

seeds, carbon flux from amino acids to fatty acids was not confirmed by the results of this study as had been proved in developing soybean seeds (Allen *et al.*, 2009).

In *in vivo* conditions nitrogen from root nodules is supplied to the developing lupine seeds mainly in the form of asparagine (Atkins *et al.*, 1975; Lea and Milfin, 1980). The stimulatory effect of asparagine on protein accumulation in cotyledons grown *in vitro* is probably due to the fact that culture variants with added asparagine copy natural conditions to the greatest degree. In *in vitro* conditions, an important carbon source for protein synthesis (especially in -S variants) was probably starch. Numerous and large starch granules were observed in cotyledons isolated from seeds from developmental stage III of the three lupine species (Fig. 3). Starch granules were also visible in cotyledons grown for 96 h *in vitro* (Figs 5–7). This is a temporary starch, because mature air-dry lupine seeds do not contain starch (Młodzianowski and Wesołowska, 1975; Hoffmannowa and Zielińska, 1981; Borek *et al.*, 2006). The important feature of storage tissues or organs is accumulation of storage compounds in quantities considerably covering their energetic and structural needs. Therefore, in cotyledons grown *in vitro* on medium without sucrose (-S), autophagy caused by sugar starvation was not observed. Autophagy often occurs in cells, tissues, or organs directly dependent on an external source of assimilates, e.g. in cell culture of sycamore (Journet *et al.*, 1996), in cell suspen-

sions of rice and tobacco (Chen *et al.*, 1994; Moriyasu and Ohsumi, 1996), or in sugar-starved lupine embryo axes (Borek and Ratajczak, 2002; Morkunas *et al.*, 2003; Borek *et al.*, 2006).

Differences in carbon flux to proteins and lipids occurred among the three lupine species. Lack of sucrose in the medium (-S) caused limited lipid accumulation in yellow and white lupine cotyledons, compared with cotyledons used for preparation of *in vitro* culture (control in Fig. 4), but not protein accumulation (Fig. 8). In Andean lupine cotyledons, sucrose added to the medium (+S) stimulated accumulation of both lipid and protein (in comparison with the control) to a much greater extent than in other lupine species. However, in Andean lupine cotyledons, even the lack of sucrose in the medium did not restrict lipid accumulation (in comparison with control) in any of the three *in vitro* culture variants, i.e. -S, -S+Asn, and -S+NO₃⁻ (Fig. 4). Moreover, in conditions of sucrose deficiency, asparagine decreased the lipid content to the greatest extent (-S+Asn versus -S), and lipid levels were also decreased in cotyledons cultured on medium with nitrate (-S+NO₃⁻ versus -S in Fig. 4). A negative relationship between lipid and protein accumulation in the cotyledons of the three lupine species grown in the presence of asparagine (Figs 4, 8) is in agreement with literature data regarding such a relationship in soybean (Shannon, *et al.*, 1972; Brim and Burton, 1979; Sebern and Lambert, 1984;

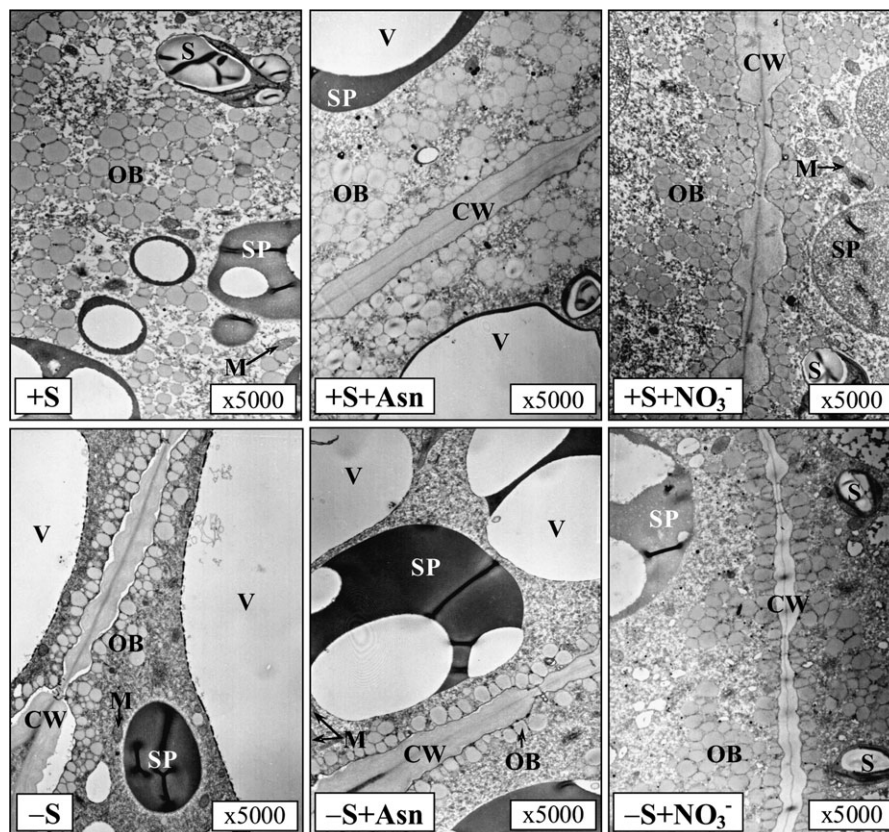


Fig. 7. Electron micrographs of Andean lupine cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. CW, cell wall; M, mitochondrion; OB, oil body; S, starch; SP, storage protein; V, vacuole.

Wilcox and Cavins, 1995; Wilcox, 1998; Cober and Voldeng, 2000; Wilcox and Shibles, 2001; Chung *et al.*, 2003; Lardizabal *et al.*, 2008). However, a negative lipid/protein relationship does not become obvious when nitrate serves as the nitrogen source. In yellow and white lupine cotyledons, nitrate caused an increase in content of both lipid and protein in cotyledons regardless of whether they were cultured on medium with or without sucrose. These data indicate that in *in vitro* conditions, carbon flux into lipid and protein varies in different lupine species and depends on the availability of organic carbon as well as the form of nitrogen, i.e. organic or inorganic.

Differences in liberation of ¹⁴CO₂ and incorporation of carbon of acetate into the lipid fraction in cotyledons of the three lupine species can be partially caused by different uptake of acetate. However, some results provide information about [¹⁴C]acetate metabolism in all three lupine species. Results from radiolabelling experiments performed on *in vitro* grown developing cotyledons provided additional data about the regulatory mechanism in lipid and protein accumulation. Previous studies conducted on the organs of germinating seeds of yellow lupine showed that the C-1 atom of acetate is liberated mostly as CO₂ in the tricarboxylic acid (TCA) cycle, whereas the C-2 atom of acetate is localized mostly in sugars and amino acids (Borek *et al.*, 2003). The results of this work also confirm intensified use of the C-1 atom of acetate in respiration

(Fig. 9) and the C-2 atom in anabolic processes (Fig. 10) in developing lupine embryos. The lowered lipid level in cotyledons grown *in vitro* in different trophic conditions (Fig. 4) can be explained by the data presented in Fig. 10. A decreased lipid level in the absence of sucrose and additional changes caused by asparagine and nitrate are due to a deficit of substrates for lipid synthesis, not to changes in the structure of the biosynthetic apparatus, because incorporation of radiolabelled carbon atoms of acetate was only slightly lower in cotyledons grown on medium without sucrose. In cotyledons of yellow lupine it was even more intensive and was not modified by asparagine and nitrate (Fig. 10). A similar mechanism occurs in the sucrose-starved embryo axes of germinating pea seeds. Respiration of embryos is very sensitive to sucrose starvation, but the decline in respiration activity observed in intact starved embryos results from the exhaustion of respiratory substrates in tissues of the embryos and not from degradation of mitochondria (Morkunas *et al.*, 2000).

In developing cotyledons of the three lupine species, liberation of ¹⁴CO₂ was significantly lower than incorporation of carbons of acetate into the lipid fraction. In an extreme case, the radioactivity of CO₂ with C-1 of acetate was >100-fold lower than the radioactivity of the lipid fraction with C-1, and the radioactivity of CO₂ with C-2 of acetate was >11 500-fold lower than the radioactivity of the lipid fraction with C-2 (white lupine, -S+NO₃⁻, Figs 9, 10).

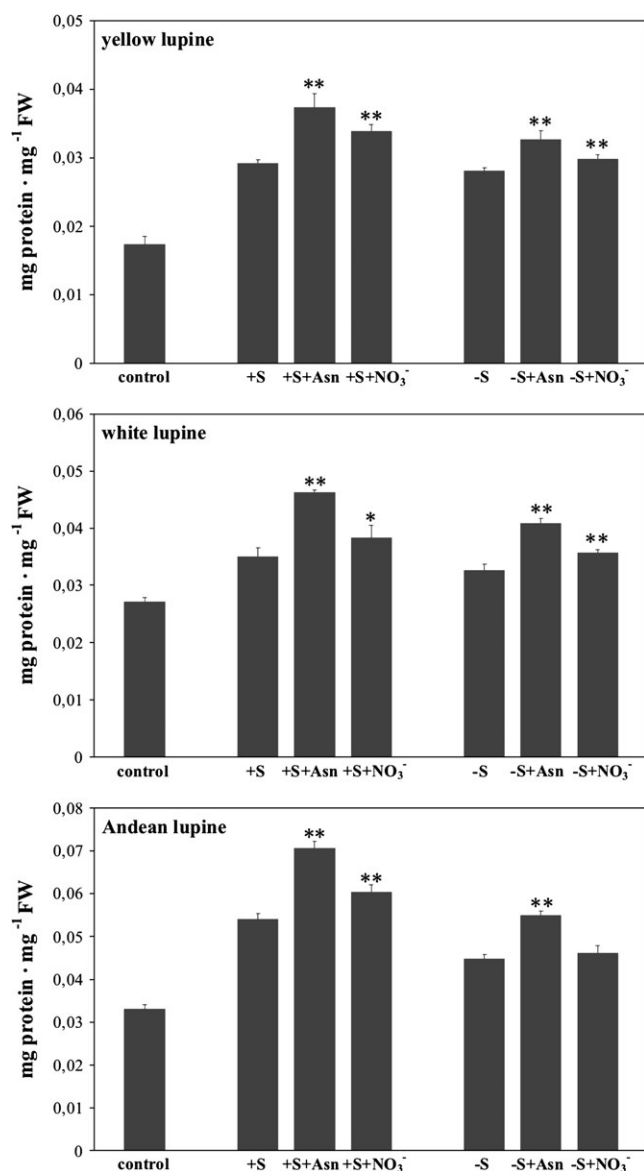


Fig. 8. Soluble protein content in cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. Control, cotyledons isolated from seeds used for preparation of *in vitro* culture; +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Statistical significance at **P* < 0.05; ***P* < 0.01.

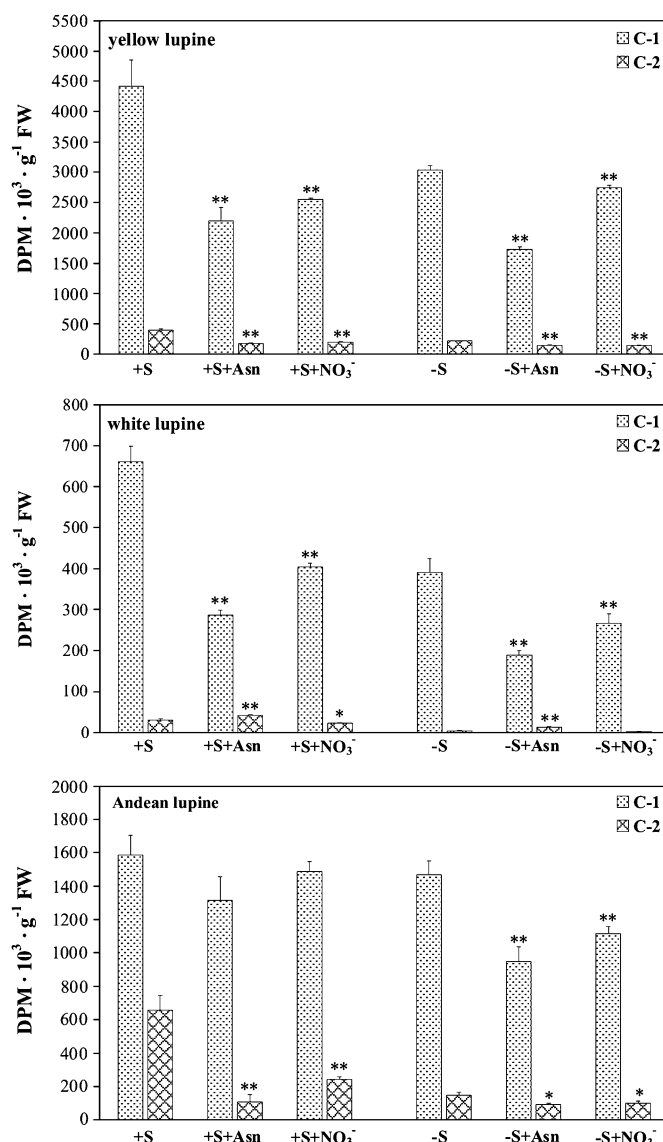


Fig. 9. Radioactivity of ¹⁴CO₂ liberated by cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h and incubated for 120 min in solutions of [1-¹⁴C]acetate (C-1) and [2-¹⁴C]acetate (C-2). Radioactivity of added acetate: 925 kBq. +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Data are from one (representative) breeding season. Statistical significance at **P* < 0.05; ***P* < 0.01.

It is possible that low liberation of ¹⁴CO₂ is caused by low efficiency of the TCA cycle in carbon flux to storage compounds in lupine cotyledons, as has been documented in developing embryos of *B. napus* (Schwender et al., 2006) and soybean (Allen et al., 2009). Additionally, liberation of ¹⁴CO₂ is low because of its reassimilation mainly by PEPC (Junker et al., 2007) and RuBisCO (Schwender et al., 2004; Sriram et al., 2004; Allen et al., 2009). Probably, in developing lupine cotyledons grown *in vitro*, carbon flux through the TCA cycle is low, as in embryos of *B. napus* (Schwender et al., 2006) and soybean (Allen et al., 2009), and sucrose delivered by the mother plant, similarly to the

in *in vitro* conditions, is mainly metabolized through glycolysis to acetyl-CoA used for lipid synthesis.

In this report, data concerning photosynthetic activity were not presented; however, the intensive green colour of cotyledons from developmental stage III can indicate that in developing lupine seeds photosynthesis is active, as in developing soybean seeds (Borisjuk et al., 2005; Rolletschek et al., 2005; Allen et al., 2009). Acetyl-CoA (delivered by pyruvate kinase) and ATP and NADPH (generated during the light-dependent phase of photosynthesis) are essential for fatty acid synthesis. Moreover, an additional source of NADPH can be the oxidative pentose phosphate pathway,

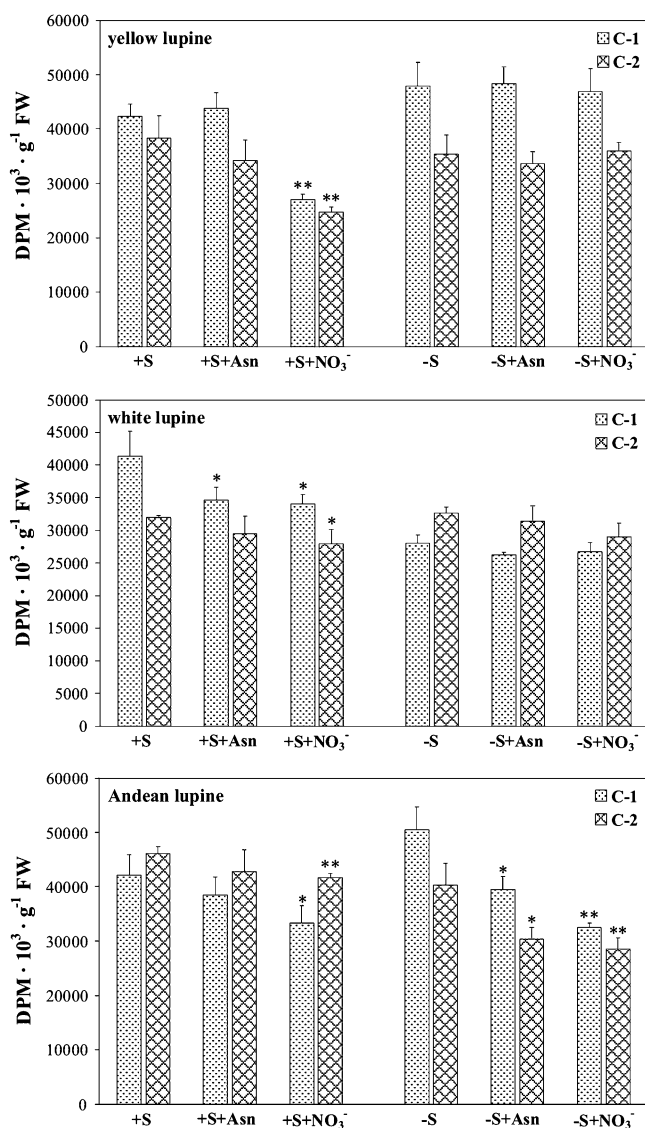


Fig. 10. Radioactivity of the lipid fraction (2 ml of chloroform:methanol) of cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h and incubated for 120 min in solutions of [1-¹⁴C]acetate (C-1) and [2-¹⁴C]acetate (C-2). Radioactivity of added acetate: 925 kBq. +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Data are from one (representative) breeding season. Statistical significance at **P* < 0.05; ***P* < 0.01.

as has been proved in soybean embryos (Sriram *et al.*, 2004).

Lupine oil could also be a valuable source of phosphatidylcholine in the human diet. The content of fatty acids in lupine oil is similar to that of oils from other oilseed crops. Yellow lupine oil, like soybean and sunflower oil, contains a high amount of linoleic acid, whereas white lupine oil, similarly to Canola oil, contains a high amount of oleic acid (Dyer *et al.*, 2008). Nevertheless, due to the relatively low lipid level in mature seeds of yellow and white lupine, industrial production of oil is unprofitable. More promising are seeds of Andean lupine, which contain

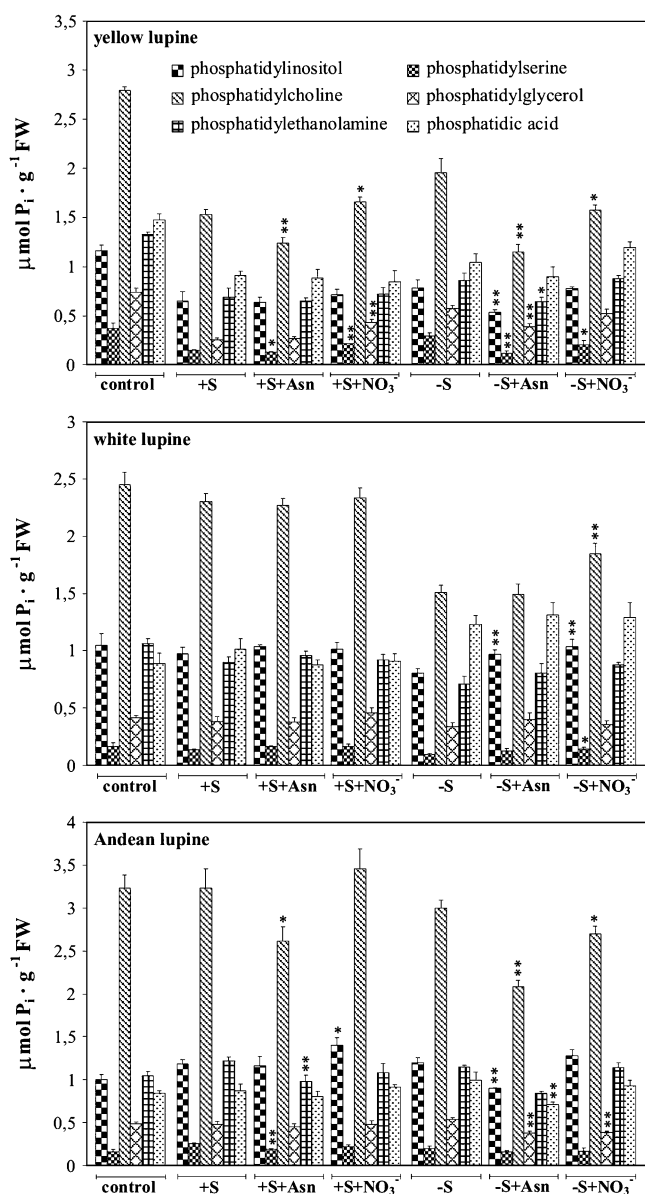


Fig. 11. Phospholipid content in cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. Control, cotyledons isolated from seeds used for preparation of *in vitro* culture; +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Statistical significance at **P* < 0.05; ***P* < 0.01.

about 20% of lipids in dry mass. Andean lupine oil contains a high amount of oleic and linoleic acids, combining features of soybean, Canola, and sunflower oils (Dyer *et al.*, 2008). Andean lupine, however, will require intensive selection and agronomical research in order to obtain varieties that grow and yield well in Polish climatic conditions. This type of work has already been done in Germany, and Andean lupine is proposed as a crop suitable for European climatic conditions (Roemer and Jahn-Deesbach, 1988). Moreover, the percentage content of fatty acids in the three lupine species was remarkably stable under the influence of the trophic conditions

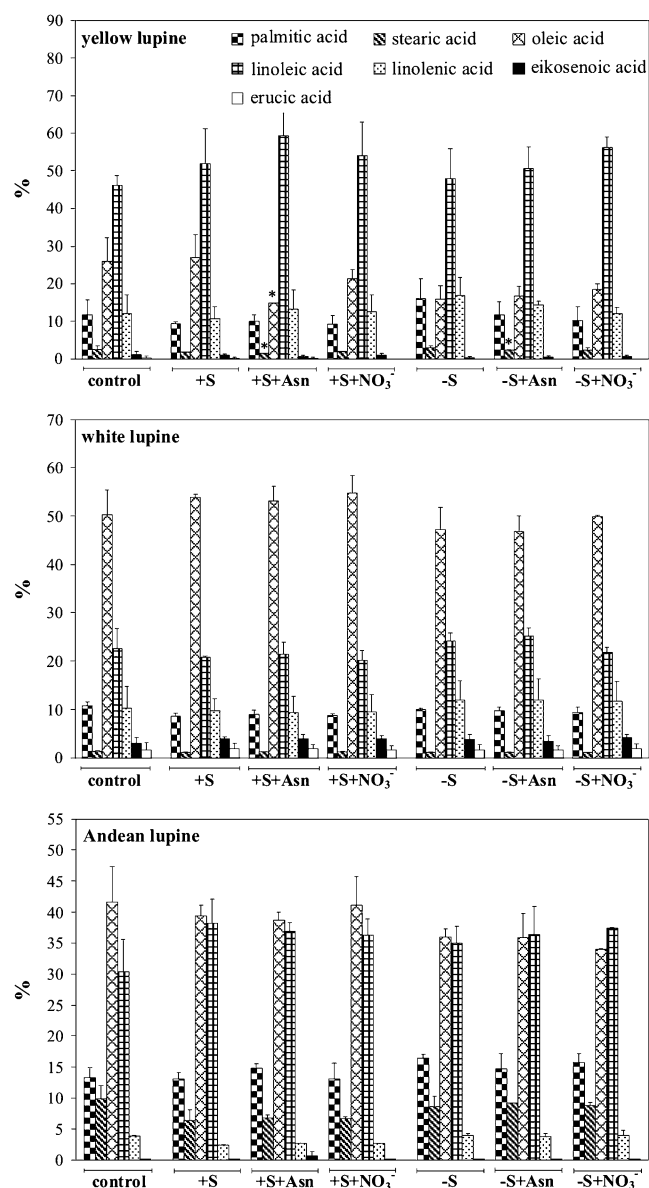


Fig. 12. Fatty acid composition (percentage of total integrated peaks on chromatographs) in cotyledons isolated from seeds from developmental stage III, grown *in vitro* for 96 h. Control, cotyledons isolated from seeds used for preparation of *in vitro* culture; +S, medium with 60 mM sucrose; -S, medium without sucrose; +Asn, medium with 35 mM asparagine; +NO₃⁻, medium with 35 mM NaNO₃. Statistical significance at **P* < 0.05.

analysed (Fig. 12), which may be a favourable feature for crop production.

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