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Original article

Nitrate simultaneously enhances lipid and protein accumulation in developing yellow lupin cotyledons cultured *in vitro*, but not under field conditions

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ABSTRACT

The research was conducted on yellow lupin (*Lupinus luteus* L.) mature seeds, developing cotyledons, developing pods, and seedlings. The main storage compound in yellow lupin seeds is protein, whose content may reach up to 45%. Oil content in seeds of yellow lupin is about 6%. In such protein-storing seeds there is a strong negative relationship between accumulation of storage lipid and protein. An increase in protein content causes a decrease in lipid level, and vice versa. However, simultaneous increase in lipid and protein content is possible in developing lupin cotyledons (the main storage organs of lupin seeds) cultured *in vitro*. Such an effect was obtained by feeding the cotyledons with nitrate (35 mM). The same positive relationship in storage lipid and protein accumulation was also obtained in developing lupin pods fed with nitrate (35 mM), detached from the mother plant, and maintained under *quasi in vitro* conditions. Fertilization of lupin plants with nitrate under field conditions (40 or 80 kg N ha^{-1} applied before sowing, at the nodulation stage or at the flowering and pod formation stage) did not cause significant changes in lipid and protein contents in mature seeds. Experiments performed on lupin seedlings cultivated hydroponically showed that nitrate added to the medium was accumulated mainly in roots, and at a remarkably lower level in shoots. We hypothesize that the lack of stimulatory effect of nitrate on storage lipid and protein accumulation in seeds under field conditions is due to inefficient transport of nitrate from the root to developing pods in lupin plants. This causes that the level of nitrate inside the developing lupin seeds is not elevated under field conditions.

1. Introduction

Lupins are one of the most important leguminous crops grown worldwide (Duranti and Morazzoni, 2011; Lucas et al., 2015). Cultivation of leguminous crops is especially important from an economical point of view, because they thrive in symbiosis with soil symbiotic bacteria fixing N_2. This means that the need for nitrogen fertilization of such crops is significantly reduced or it is even not necessary at all. Additional benefits of cultivation of leguminous crops are obtained in subsequent growing seasons, because soil is naturally enriched in nitrogen. N_2 is reduced by the bacteria in root nodules, and next is used by the plant. The reduced nitrogen is transported within leguminous plants as amino acids (mostly asparagine, for example in lupin or pea) or as ureides (for example in soybean or bean) (Amarante et al., 2006; Lea et al., 2007; Sulieman and Tran, 2013). The most common lupin symbiotic bacteria belong to the genus *Bradyrhizobium*, which may naturally occur in soil of many regions of the word. The symbiosis means that nitrogen fertilization of lupins is not necessary, and only low doses of nitrate fertilizer (up to 30 kg N ha^{-1}) need to be applied before sowing to improve seedling establishment. In legumes, an elevated level of nitrate inhibits nodulation, whereas N-limitation can stimulate nodulation (Mohd-Radzman et al., 2013).

The main storage compound in lupin seeds is protein, the level of which, depending on the species or weather conditions, may reach up to 40–50% of seed dry matter. An example is seeds of yellow lupin and Andean lupin, which contain the highest amount of protein among

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plants (Borek et al., 2015). Because of their high protein content, lupin seeds are very valuable as a fodder for animals and also as a human food. However, seeds of some lupins also contain quite a high amount of storage lipid. A good example is Andean lupin, which accumulates oil in seeds up to a level of 20%. Nevertheless, lupin seeds usually contain much less oil; for example, seeds of yellow lupin contain only about 6% oil (Borek et al., 2015). For comparison, oil content in seeds of soybean may range from 12% to 26% (Borek et al., 2015), but typically it is about 20% (He and Chen, 2013). It is estimated that seeds should contain at least 15% oil to be deployed industrially to produce oil. Using lupin seeds as an oil source may be beneficial because of the fatty acid composition. For example, oil of yellow lupin is rich in linoleic acid (similarly to oil of soybean and sunflower), and contains no erucic acid (Borek et al., 2009), which is very harmful for mammals. Total carbohydrate content in lupin seeds is about 36%, but 26% is fiber (Borek et al., 2015). Mature lupin seeds contain no starch (Borek et al., 2013). Lupins are dicotyledonous plants, and the storage compounds in mature seeds are located in the embryo. The majority of them are accumulated in cotyledons. However, storage compounds are also accumulated in the embryo axes (Borek et al., 2011; Borek et al., 2012a; Borek et al., 2012b). Developing lupin embryos are dark green (Borek et al., 2009), similar to soybean embryos, where photosynthetic activity was confirmed (Borisjuk et al., 2005; Rolletschek et al., 2005; Allen et al., 2009). Such green developing seeds are called 'photoheterotrophic seeds'. Lupin embryos probably develop under hypoxic conditions. Such conditions inside developing lupin seeds have not been confirmed yet, but they have been evidenced in other plants, for example in leguminous plants such as soybean, pea, vetch (Rolletschek et al., 2002; Rolletschek et al., 2005; Weber et al., 2005) and in rape (Vigeolas et al., 2003).

Accumulation of storage lipid and accumulation of protein in oil-storing seeds are independent processes. It means that a decrease or an increase in the amount of oil or protein in seeds does not generally cause a compensating increase or decrease in the other major storage compound. Such a lack of relationship in storage lipid and protein accumulation was observed in seeds of *Arabidopsis* (Focks and Benning, 1998) and *Brassica napus* (Li et al., 2006). Contrary to oil-storing seeds, in protein-storing seeds there is a strong negative relationship in accumulation of storage lipid and protein. Such a negative relationship was detected in soybean seeds already in the 1950s, and it was confirmed many times later (Chung et al., 2003). However, experiments performed on developing lupin cotyledons cultured *in vitro* showed that simultaneous increase in lipid and protein accumulation is possible (Borek et al., 2009). Such a positive relationship in accumulation of these two main storage compounds in lupin seed storage organs was obtained in isolated cotyledons cultured *in vitro*. Sucrose, as the main substrate for lipid biosynthesis in plants, significantly enhanced lipid accumulation in lupin cotyledons. Also protein content in sucrose-fed cotyledons was slightly increased. But the most important effect was caused by nitrate, which significantly and simultaneously enhanced lipid and protein content, in lupin cotyledons both fed and not fed with sucrose. Nitrate caused an increase in lipid content by 7.8% and in protein content by 16.1% in sucrose-fed yellow lupin cotyledons (Borek et al., 2009). Such a result was very important, because it was shown for the first time that a positive relationship in accumulation of storage lipid and protein in lupin protein-storing seeds may be achievable. Contrary to nitrate, asparagine, as a 'central' amino acid in nitrogen metabolism of lupin seeds, significantly enhanced the accumulation of protein, but simultaneously reduced the lipid content. Asparagine caused an increase in protein content by 28.1% and a decrease in lipid content by 6.2% in sucrose-fed yellow lupin cotyledons (Borek et al., 2009).

The main aim of the research was to check whether the positive effect of nitrate on accumulation of lipid and protein observed in isolated lupin cotyledons cultured *in vitro* (Borek et al., 2009) is possible to achieve also under field conditions. To this end, nitrate fertilizer was applied under field conditions at different stages of lupin plant development, and lipid and protein content was measured in mature seeds. To explain the mechanism of nitrate action, additional experiments under laboratory controlled conditions were performed on developing pods, developing isolated cotyledons, and seedlings. In laboratory experiments the effect of sucrose (the main substrate for lipid biosynthesis in plants; Weber et al., 2005) and asparagine (a 'central' amino acid in lupin plant metabolism; Lehmann and Ratajczak 2008) was also investigated.

2. Materials and methods

2.1. Plant material

Plants of yellow lupin (*Lupinus luteus* L.) cultivar Juno were obtained and grown on experimental plots at the Plant Breeding Station Smolice Division in Przebędowo (Poland). Experiments were conducted on: *i*) mature lupin seeds collected from the experimental plots, *ii*) developing lupin pods maintained under *quasi in vitro* conditions, *iii*) cultured *in vitro* cotyledons isolated from developing lupin seeds, and *iv*) 4-week-old lupin seedlings cultivated hydroponically.

i) Mature seeds of yellow lupin were obtained from the experimental plots (8.0 m x 1.3 m = 10.4 m^2 each). The weather conditions and content of N-NO_3 and N-NH_4 in soil are given in Supplementary Fig. 1 and Supplementary Table 1, respectively. Plants were grown during two consecutive growing seasons (2011 and 2012). Seeds were sown at the beginning of April. During the growing seasons plants were fertilized with $NaNO_3$. The doses of nitrogen were 40 kg N ha^{-1} (2011) or 80 kg N ha^{-1} (2012). The fertilizer was applied as follows: Plot A, without nitrate fertilizer (control); Plot B, application of nitrate fertilizer (40 or 80 kg N ha^{-1}) before sowing; Plot C, application of nitrate fertilizer (40 or 80 kg N ha^{-1}) at the nodulation stage (about 2 weeks after germination); Plot D, application of nitrate fertilizer (40 or 80 kg N ha^{-1}) at flowering and pod formation stage. No pesticide or herbicide was used. Weeds were removed by hand. Mature seeds were collected in July. They were weighed and stored under low humidity conditions for analysis.

ii) Yellow lupin plants with pods containing seeds at developmental stage III were collected from the field. Five stages are distinguished during lupin seed development, and at stage III the lipid content reaches about 45–50% of the maximal level (Borek et al., 2009). Pods were carefully and thoroughly washed with tap water and next they were cut away from the mother plant. Pods were cut off under water to prevent air lock of vascular bundles. The pods were maintained under *quasi in vitro* conditions (not fully sterile conditions) for 96 h. The pods were placed vertically in open, small beakers containing 15 ml of autoclaved, above-described media (+S, +S + Asn, +S + NO_3, −S, −S + Asn and −S + NO_3). Pods were immersed to a depth of about 1.5 cm (Supplementary Fig. 2). Each medium was exchanged each 24 h. It was done to avoid fungal or microbial infections. This also allowed refilling of the media which were used by the pods. The pods were maintained at 27–28 °C and under illumination (photosynthetically active radiation) of 225 μM light quantum m^{-2} s^{-1}. The photoperiod 17 h light and 7 h darkness was applied. After 96 h of cultivation the pods were divided into wall of pod, coat of seed, and embryo. The samples were weighed, frozen in liquid nitrogen, and stored at −80 °C for analysis.

iii) The cotyledons were isolated from seeds which were at developmental stage III. The seeds were obtained from developing pods which were collected from the field. Pods were thoroughly washed with tap water and next were surface sterilized. They were immersed in 96% (v/v) ethanol for 30 s and next in 0.2% $HgCl_2$