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Cyanogenic glycosides can function as nitrogen reservoir for flax plants cultured under N-deficient conditions

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Abstract: Soil nitrogen (N) deficiency is a common phenomenon that plagues both naturally growing plants as well as agricultural crops, ultimately affecting their growth and productivity. The aim of our study is to determine the effect of short-term N deprivation on secondary metabolites production in developing and mature leaves of flax (*Linum usitatissimum* L.). Two weeks under low-N conditions decrease plant growth, N concentration, and soluble proteins content in leaves. Reduction in photosynthesis intensity was also observed. A decrease of cyanogenic glycoside content under N-deficient conditions was most visible in mature leaves. However, the content of linamarin and lotaustralin was about 10-fold higher in younger than in mature leaves, in which play probably protective role due to the possibility of toxic HCN release. Despite the N deficit, flax plants accumulated nitrogen in cyanoglycosides. N from cyanoglycosides can be used to synthesise amino acids; this possibility is supported by the high activity of β-cyanoalanine synthase, especially in N-deficient leaves. On the other hand, the content of different types of phenolic compounds increased in N-deficient plants, especially in young leaves, possibly replacing cyanoglucosides in protective functions. Our results indicated that cyanogenic glycosides could be an important nitrogen source for flax plants grown under temporary N-deficient conditions.

Keywords: cyanogenesis; linamarase; macronutrient; oxidative stress; plant damage; pigments

Nitrogen (N) is an important nutrient element that builds organic compounds important for plant cell functions, such as proteins, nucleic acids, coenzymes, and various secondary metabolites. Correct N balance in tissues is necessary for the proper functioning of plants during all stages of growth and development (Broadley et al. 2000, Zhao et al. 2005).

Most crop plants take up N from the soil as nitrate ions. Low nitrate levels in arable soils are a common occurrence; therefore, large amounts of N fertilisers are often used to get a good harvest. N deficiency influences metabolism and plant growth – manifested by inhibition of leaf area and shoot elongation, it also reduces flowering and seed production. N deficiency strongly affects photosynthesis apparatus function in leaves (Zhao et al. 2005, Živčák et al. 2014). Plant response to N deficiency is dependent on stress duration (Gao et al. 2018); chronic N starvation causes serious damage and can be lethal. A lot of research has been done into the various responses of plants to N deficiency, but there is still a lot of questions (Gojon 2017). Little is known how N deficit affects secondary metabolism, despite the fact that some metabolites can be an N reservoir in tissue. Among the N-containing secondary metabolites, we distinguish alkaloids, cyanogenic compounds, and glucosinolates. To non-N-containing, phenolic compounds are included, generally divided into:

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phenols, tannins, iso- and flavonoids. Many of them are involved in plant's response to biotic stresses like infection by pathogens or herbivore attacks and have strong allelopathic properties. Phenolic compounds can also protect the plant against oxidative stress that accompanies stresses like drought, cold, or nutrient deprivation, including N deficiency (Harmatha 2005, Cheynier et al. 2013).

Various cyanogenic glycosides (CGs) are known to be synthesised by thousands of plants, including economically important crops like cassava, flax, lima beans, sorghum, or white clover (Gleadow and Møller 2014). CGs are composed of an α-hydroxy-nitrile (aglycone) and sugar moiety, most often D-glucose added at the final step biosynthesis catalysed by UDP-glucose-dependent glucosyltransferases (Kazachkov et al. 2020). All known CGs are synthesised from amino acids like L-valine, L-leucine, or L-tyrosine and L-phenylalanine, in an energy-expensive process. The best known CGs, among several dozen compounds, are amygdalin and prunasin, common in the Rosaceae family, dhurrin in sorghum, linamarin in cassava, lima beans, flax or taxiphyllin in bamboo. The primary function of CGs is potential toxicity against pests and pathogens (dependent on the release of hydrogen cyanide, HCN); CGs are classified to allelochemicals, could also perform other functions, e.g., providing a source of N for germinating seeds (Cuny et al. 2019).

Flax (*Linum usitatissimum* L.) is a well-known cyanogenic plant, commonly used in various areas of human life, but synthesises several CGs, potentially toxic. Fiber flax is used mainly in the textile industry, while linseed oil – in the cosmetics and pharmaceutical industry (Troshchynska et al. 2019). Depending on the amount of sugar molecules, mono-, di- and triglycosides are known among CGs. During the development, the level of CGs is changing. Two main monoglycosides, linamarin (2-(β-D-glucopyranosyloxy)-2-methylpropanenitrile) and lotaustralin (2-hydroxy-2-methylbutyronitrile-β-D-glucopyranoside), were detected in the vegetative organs of flax, including leaves, whereas diglycosides were present mainly in germinated seeds (Zuk et al. 2020). CGs in tissues can be modified by external conditions, including irradiation, temperature, and N nutrition (Niedźwiedź-Siegień and Gierasimiuk 2001, Ebbs et al. 2010).

Hydrolysis of CGs occurs mainly in damaged tissues and is generally a two-step process. The first step is catalysed by one or more β-glycosidases (EC 3.2.1) and leads to the formation of sugar/D-glucose and cyanohydrin, which is unstable and decompose fast into α-hydroxynitrile. Then, α-hydroxynitrile is degraded, spontaneously or in the reaction catalysed by of α-hydroxynitrile lyase (EC 4.1.2.37), into aldehyde/ketone and HCN (Zuk et al. 2020). In unwounded tissues, spatial separation is observed, e.g., in *L. usitatissimum* L., the hydrolysing enzyme linamarase (LIN, EC 3.2.1.21) is located in the cell wall, while CGs are found in the vacuole (Siegień and Bogatek 2006). Released HCN is poisonous for various organisms, including humans, by blocking cellular respiration (as an inhibitor of cytochrome oxidase). Unfortunately, cyanide compounds are waste products of a number of industrial processes, possibly contaminating agricultural areas. Detoxification of HCN relies on its transformation into non-cyanogenic compounds (Ebbs 2004). Living organisms have different HCN detoxification pathways. In plants, main is a reaction catalysed by β-cyanoalanine synthase (CAS, EC 4.4.1.9), which transfers CN- to cysteine and produces β-cyanoalanine (Machingura et al. 2016). In a subsequent reaction, β-cyanoalanine hydrolase converted it to asparagine, the main substrate for other amino acids. CAS is also involved in HCN formation during ethylene synthesis. It is believed that HCN can perform a non-toxic regulatory role in protein synthesis and plant responses to stress (Siegień and Bogatek 2006, García et al. 2019). It was also reported that HCN can participate in the N assimilation process (Machingura and Ebbs 2010, Machingura et al. 2016).

The main purpose of our study is to determine changes of CGs content, metabolism, and function in *L. usitatissimum* L. leaves after short-term culture under low-N stress. We estimated how the N deficiency affects the morphological and physiological parameters of young and mature flax leaves, including total N and photosynthesis rate and phenolic compounds content. The conclusions of this study will broaden the knowledge on the importance of cyanogenic compounds in crop plants under low-N stress conditions.

MATERIAL AND METHODS

Oil flax seeds (*Linum usitatissimum* L. cv. Szafir) were germinated for 7 days, then seedlings were placed in containers with the full nutrient medium as control (+N), as described by Zebrowska et al. (2017), or N-deficient medium with reduced N to 0.1 N

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(–N). Seedlings were grown 14 days under conditions: 16 h photoperiod, light intensity 180 µmol/m^2/s, temperature 22/16 °C day/night, and relative humidity 60%, as described before (Ciereszko et al. 2011). The culture media were adjusted to pH 5.7, continuously aerated, and replaced every 4 days. Plant material (upper "rosette" young and mature leaves from the stem, Figure 1) was collected 3–4 h after the beginning of the light period. Most analyses were performed directly after harvesting; part of the material was frozen in liquid N and kept at –80 °C. The material was weighed (FW), dried in an oven at 105 °C for 24 h, cooled and weighed to determine the dry weight (DW).

N content determinations. Total N content in leaves of flax was determined using the Kjeldahl method. Plant material was weighed, then placed into tubes containing 10 mL of concentrated sulfuric acid and a catalyst (K_2SO_4). The samples were mineralised to obtain N as ammonium sulfate, then NaOH was added, and the separated ammonia was distilled off. N content was measured with a Kjel-Foss apparatus automatic (Foss, Hillerod, Denmark) and shown in mg N/g FW. N in CGs was calculated similar to Burns et al. (2002).

Figure 1. Photography of typical flax plant (*Linum usitatissimum*) cultured for two weeks on –N sufficient (+N, control, left) nutrient medium or on a medium with the reduced amount of nitrogen (–N)

Photosynthesis intensity and chlorophyll content. Measurements of photosynthesis rate in leaves were taken using a LI-COR CO_2/H_2O Analyser (LI-6262, LI-COR, Logan, USA) under 200 µmol photon/m^2/s, at atmospheric concentrations of O_2 and CO_2, and 25 °C, as described by Maleszewski et al. (2004). Pigments were extracted from leaves with 100% methanol at 70 °C and determined spectrophotometrically (665.2, 652.4, and 470 nm), according to the Wellburn method, using Cecil CE 2501 (CECIL Instruments, Cambridge, UK).

Determination and analysis of cyanogenic glucosides. Linamarin and lotaustralin were extracted and estimated as described in detail by Siegień (2009). Fresh plant material was homogenised in liquid N, extracted in 80% ethanol, filtered and defatted with chloroform, and the solvent was evaporated at 40 °C. Residues were dissolved in 10% isopropanol. CGs extracts and standard solutions (Sigma-Aldrich, Poznan, Poland) were developed on TLC plates (Kieselgel 60 F254, Merck Chemicals GmbH, Darmstadt, Germany) and localised after spraying with the β-glucosidase solution (from flax seeds) after drying. Then sandwich technique with Feigl-Anger test paper was used (two blue spots for CGs were obtained). Rf values of linamarin and lotaustralin were 0.47 and 0.56, respectively. Chromatographic zones corresponding with individual CGs were removed and eluted with 0.1 mol/L Na-acetate buffer (pH 5.6). CGs content was determined after hydrolysis with β-glucosidase, liberated glucose was estimated in incubation mixture with the glucose oxidase and peroxidase, as described by Siegień (2009).

Enzymes activity: LIN and CAS. Leaf samples (1 g) were homogenised in liquid N_2 with 0.1 mol/L Tris-HCl buffer (pH 8.5). The extracts were centrifuged at 10 000 *g* for 20 min at 4 °C. CAS activity was determined after 15 min incubation at 30 °C with 0.5 mL of 10 mmol/L KCN and 0.5 mL of 4 mmol/L L-cysteine. The reaction was stopped by 0.5 mL of 30 mmol/L $FeCl_3$ and 0.5 mL of 20 mmol/L N,N-dimethyl-*p*-phenylenediamine (both in 1.2 mol/L HCl) similar to the method described by Machingura and Ebbs (2010). Released H_2S was measured at 650 nm (Cecil CE 2501). The activity was shown as nmol H_2S/min/mg protein. For linamarase, the ex-

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traction and incubation methods were as described by Niedźwiedź-Siegień and Gierasimiuk (2001). The liberated glucose was estimated by the glucose oxidase/peroxidase method. Enzyme samples were incubated at 30 °C (0.1 mol/L Na-acetate buffer, pH 5.6) for 20 min. LIN activity was presented as μg glucose/min/mg protein. Proteins content was measured using the Bradford assay (Sigma-Aldrich, Poznan, Poland).

Determination of secondary metabolites. Extraction and analysis of phenylpropanoids anthocyanins were performed as described by Kubo et al. (1999). Samples were homogenised with 100 mmol/L K-phosphate buffer and 1 mmol/L ascorbate at pH 7.8, centrifuged for 30 min at 4 °C at 22 000 *g* (MPW 351R, Warsaw, Poland). The supernatant was diluted and measured at 600 nm for anthocyanin compounds and 320 nm for phenylpropanoid flavonoids (Cecil CE 2501); contents were expressed as relative units (U) per mg protein. Protein content was measured by the Bradford method. Extraction and determination of the sum of phenolic compounds and tannins were carried out according to Makkar et al. (1993). 0.05 g of dried leaves were homogenised with 70% acetone, incubated 1 h in the dark (room temperature) and centrifuged (10 min, 3 000 *g*). To determine the total polyphenol content, Folin-Ciocalteu reagent (Sigma-Aldrich, Poznan, Poland) and Na_2CO_3 were added and incubated for 30 min. Then, the absorbance at 725 nm was measured. The calibration curve was prepared for tannic acid. To determine non-PVPP-related compounds, 100 mg of PVPP was added to the extract. The samples were incubated for 15 min at 4 °C and centrifuged (10 min, 3 000 *g*). Then, the aforementioned procedure was followed. Tannin content was calculated by subtracting compounds not related to PVPP from total polyphenols. Polyphenols content was expressed in μg/g DW.

Statistical analysis. Experiments were performed in 3–5 independent series, and all assays were carried out in at least triplicate. Standard deviation (SD) was calculated. The data were analysed by one-way analysis of variance (ANOVA). In addition, Tukey's *HSD* (honestly significant difference) test was carried out (Statistica 6, StatSoft, Palo Alto, USA). The significance level in comparisons was $P \leq 0.05$.

RESULTS AND DISCUSSION

Impact of N deficit on flax growth and photosynthesis. The growth of *L. usitatissimum* L. for two weeks on –N media resulted in decreased growth and smaller leaves when compared to the control (+N) plants (Figure 1). The fresh mass of mature leaves of –N plants was about 41% of control, whereas the mass of younger leaves – about 62% (Table 1). Total N content was significantly higher in the leaves of control plants than those under low-N stress (Table 1). Younger leaves of flax were generally characterised by higher N content than older, which could be explained by N mobility and effective release in metabolic processes. N is an easily remobilised nutrient, detected in the phloem and transported to growing plant organs (Gojon 2017). Water content was not affected by N deficiency in young leaves, but in –N mature leaves was slightly lower than in control. Soluble protein content in N-deficient flax was smaller by about 22% and 18% compared to the control in young and mature leaves, respectively (Table 1). Photosynthesis intensity was lower in the leaves of –N plants; in mature leaves, it was about 81–82% of that found in control (Table 2). Chlorophyll *a*, chlorophyll *b* and carotenoids contents in leaves were not significantly affected by low N, but their content was lower in older leaves than younger ones (Table 2), indicating that N deficiency can promote senescence of mature leaves.

Table 1. Nitrogen (N) content, leaf fresh mass and proteins content in flax (*Linum usitatissimum*) cultured on N-sufficient (+N) or N-deficient (–N) nutrient medium

Parameter	Young leaves		Mature leaves	
	+N	–N	+N	–N
Total N content (mg N/g FW)	11.54 ± 0.42^{a}	7.78 ± 0.26^{b}	6.95 ± 0.24^{c}	4.823 ± 0.11^{d}
Fresh mass (g)	0.047 ± 0.01^{c}	0.029 ± 0.003^{d}	0.64 ± 0.08^{a}	0.26 ± 0.01^{b}
Water content (g H_2O/g FW)	0.88 ± 0.02^{ab}	0.86 ± 0.04^{ab}	0.91 ± 0.01^{a}	0.85 ± 0.02^{b}
Soluble proteins (mg/g FW)	23.73 ± 1.38^{a}	16.99 ± 1.09^{b}	$16.\ 19 \pm 0.78^{b}$	13.19 ± 1.34^{c}

Different letters indicate significant differences ($P \leq 0.05$) among the treatments. FW – fresh weight

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Table 2. Photosynthesis intensity, chlorophyll, and carotenoids content in leaves of flax (*Linum usitatissimum*) plants cultured on N-sufficient (+N) or N-deficient (–N) nutrient medium

Parameter	Young leaves		Mature leaves	
	+N	–N	+N	–N
Photosynthesis (μmol CO_2/min/g FW)	6.5 ± 0.3^{a}	5.87 ± 0.8^{ab}	4.9 ± 0.36^{bc}	3.97 ± 0.06^{c}
Chlorophyll *a* (mg/g FW)	1.19 ± 0.07^{a}	1.23 ± 0.03^{a}	0.72 ± 0.04^{b}	0.70 ± 0.04^{b}
Chlorophyll *b* (mg/g FW)	0.45 ± 0.1^{a}	0.44 ± 0.08^{a}	0.25 ± 0.05^{b}	0.27 ± 0.03^{b}
Carotenoids (mg/g FW)	0.37 ± 0.03^{a}	0.39 ± 0.06^{a}	0.18 ± 0.01^{b}	0.19 ± 0.01^{b}

Different letters indicate significant differences ($P \leq 0.05$) among the treatments. FW – fresh weight

In our study, the leaves' growth was affected more than photosynthesis intensity after 2 weeks of N deprivation. Under short-term N deficit, pigments content was not affected. However, under long-term stress, a significant decrease of both chlorophyll content and photosynthesis activity was reported (Broadley et al. 2000, Zhao et al. 2005). Photosynthesis activity depends on environmental factors and biochemical processes, e.g., the inhibitory effects of N starvation on Calvin-Benson cycle enzymes, including Rubisco, was reported for various plants (Zhao et al. 2005, Gao et al. 2018). Wheat cultivars tolerant to N deprivation more efficiently assimilated N and activated Rubisco to improve photosynthesis under short-term N deficit (Gao et al. 2018). Zhao et al. (2005) reported that a stomatal conductance decrease is the main cause of photosynthesis reduction in sorghum leaves under N deficit. N deficiency also affected transpiration and water use efficiency, which is in agreement with our observation of lower water content in mature flax leaves.

Secondary metabolism modifications in flax leaves under N deficiency. Two main CGs present in flax leaves, linamarin and lotaustralin, were analysed. The content of both compounds in young leaves was several times higher than in mature (Figure 2). In young leaves, the content of linamarin was lower by 33% under low-N-stress; in mature, it was at a similar level (Figure 2A). Lotaustralin content in mature –N leaves was lower by 36%, whereas in young – by 20% lower when compared to control (Figure 2B). Other studies indicated a high linamarin and lotaustralin content in flax leaves as well. CGs composition was dependent on plant age and stage of development (Zuk et al. 2020). In our study, we calculated N content in CGs as affected by N deficit. In young –N leaves, N content in linamarin decreased by about 33%, while N in lotaustralin decreased by 20% when compared to the control (Table 3). Mature –N leaves accumulated similar N amounts in CGs like control; however, N in lotaustralin was 14–15 times higher in young leaves. The amount of N in CGs in relation to the total N content was slightly higher under the N-deficient condition; in addition, it was about 6–7 times higher in young leaves than mature

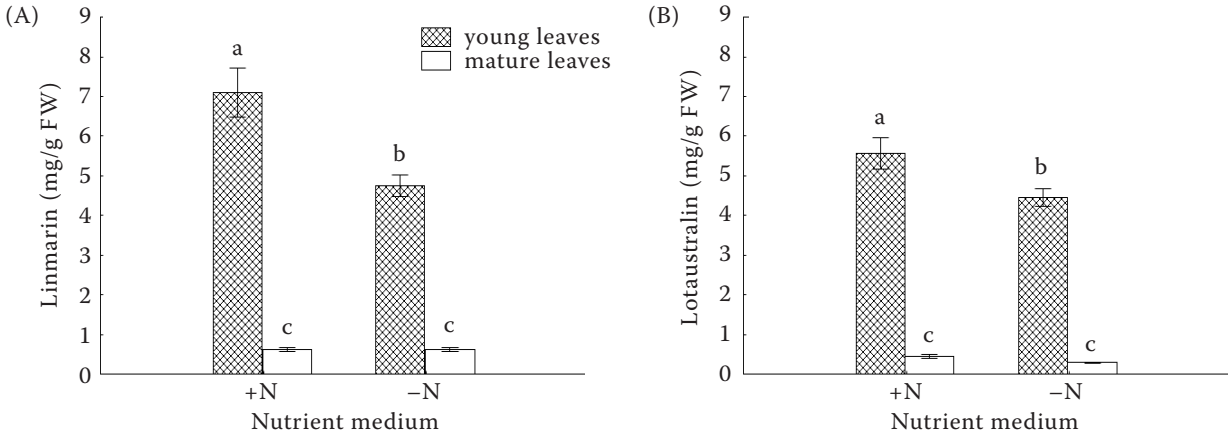

Figure 2. Cyanogenic glucosides (CGs): linamarin and lotaustralin content in young or mature leaves of the flax plant (*Linum usitatissimum*) cultured on N-sufficient (+N) or N-deficient (–N) medium. Different letters indicate differences statistically important ($P \leq 0.05$). FW – fresh weight

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Table 3. Nitrogen (N) content in cyanogenic glucosides (CGs): linamarin and lotaustralin) in leaves of flax (*Linum usitatissimum*) cultured on N-sufficient (+N) or N-deficient (–N) nutrient medium

	Young leaves		Mature leaves	
	+N	–N	+N	–N
N in linamarin (mg N/g FW)	0.40 ± 0.06^a	0.27 ± 0.03^b	0.04 ± 0.01^c	0.04 ± 0.005^c
N in lotaustralin (mg N/g FW)	0.29 ± 0.04^a	0.23 ± 0.02^b	0.02 ± 0.004^c	0.015 ± 0.001^c
Nitrogen in CGs/total N (%)	6 ± 0.65^a	6.45 ± 0.36^a	0.85 ± 0.11^b	1.05 ± 0.24^b

Different letters indicate significant differences ($P \leq 0.05$) among the treatments. FW – fresh weight

ones (Table 3). CGs could be a good source of N for plants under short-term N starvation. Developing leaves are characterised by higher activity of anabolic processes, including photosynthesis, and generally need more N so that they could draw this missing element from CGs. N remobilised from older leaves can be used for growth, metabolism, and synthesis of new/necessary compounds, including pigments or protective metabolites. Enhanced transport of N-containing CGs from older to young leaves under low-N stress cannot also be excluded. Ebbs et al. (2010) study on wheat and sorghum indicated an increase in cyanide transport in response to decreased ammonium supply, also suggested that cyanogenic N in the rhizosphere might be a source of N under limiting conditions.

The observed changes in the level of CGs were accompanied by similar trends in the activity of the LIN and CAS. CAS activity, the enzyme involved in cyanide assimilation, was higher in –N flax. For younger –N leaves, the activity was about 1.7 times higher than the control, whereas for mature –N leaves – 2.6 times higher (Figure 3A). Under N deficit, LIN activity significantly increases in all leaves. For young –N leaves, it was about 2.4 times higher, whereas for mature –N leaves – about 1.4-fold more than in control. LIN activity was generally higher in mature leaves than in younger ones (Figure 3B). High LIN activity in older N-deficient leaves corresponded to a lower linamarin content in these leaves (Figures 2–3). A recent study by Zuk et al. (2020) indicated that fluctuations of CGs during flax development are due to LIN gene expression increase. Previous studies demonstrated that various stress factors can affect CGs content/metabolism in tissues. The light was shown to be important for the stable content of CGs and *β*-glycosidase activity in flax seedlings. Water stress also significantly affected CGs content and metabolism (Niedźwiedz-Siegień and Gierasimiuk 2001).

Our results show that flax plants grown under N deficit conditions accumulate phenolic compounds in leaves to a greater extent than control (Figure 4). The anthocyanins content was higher for –N mature leaves by about 90% than in control (Figure 4A). N deficit increased the content of phenylpropanoid

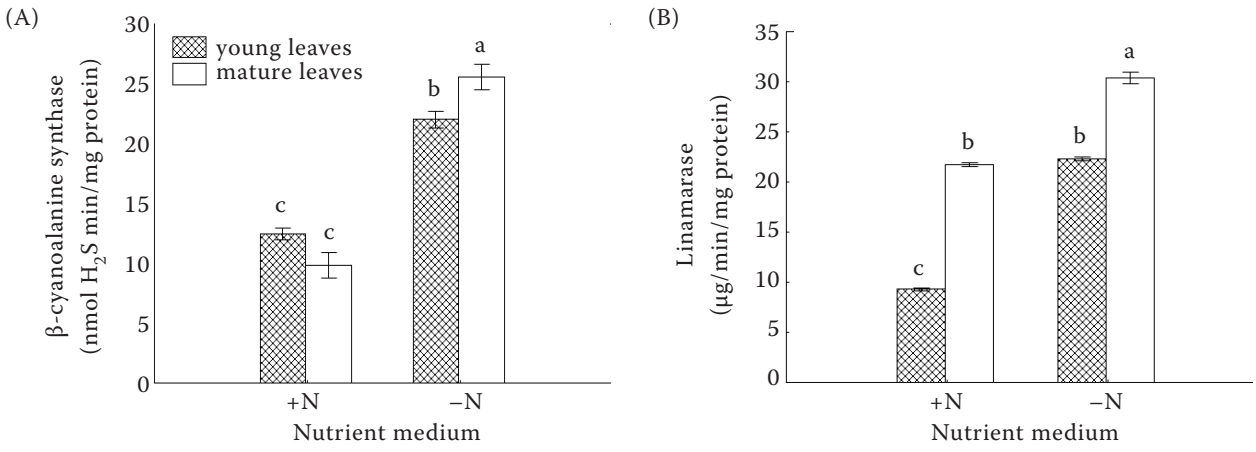

Figure 3. (A) β-cyanoalanine synthase and (B) linamarase activity in young or mature leaves of the flax plant (*Linum usitatissimum*) cultured on N-sufficient (+N) and N-deficient (–N) medium. Different letters indicate differences statistically important ($P \leq 0.05$)

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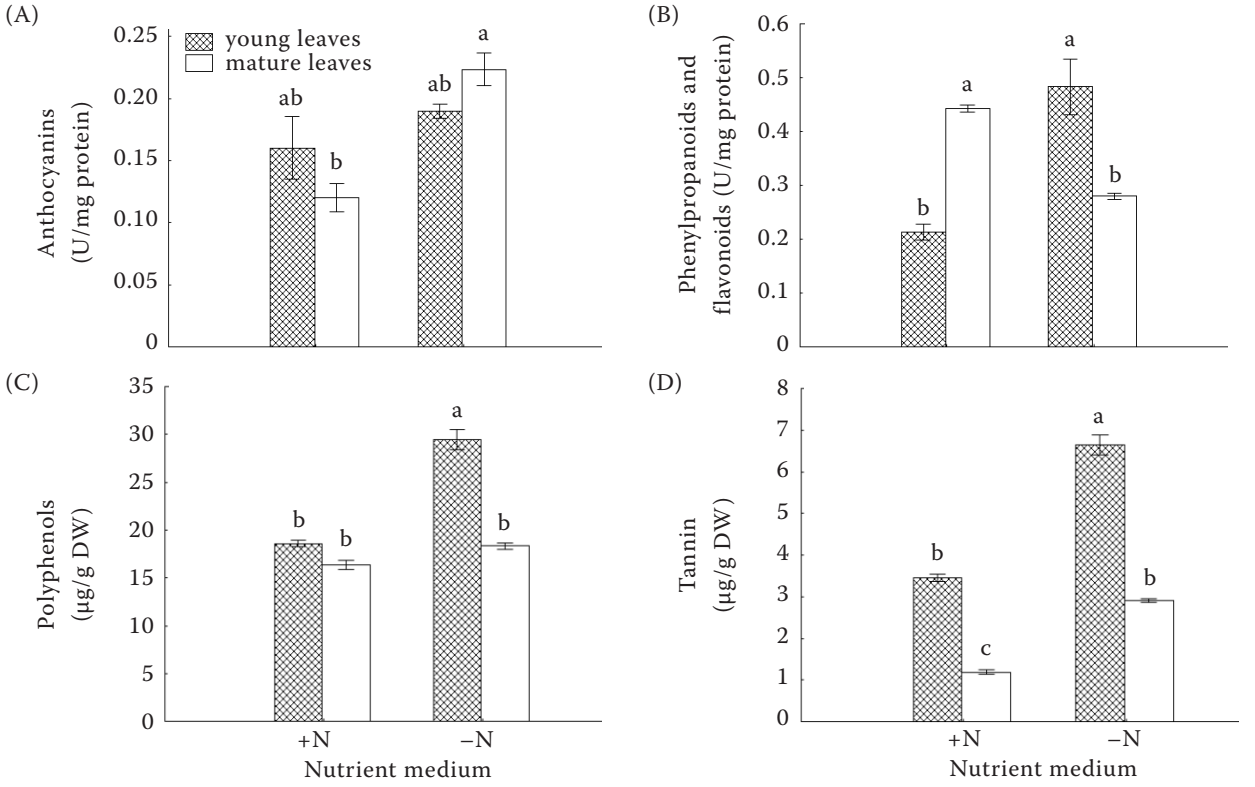

Figure 4. Content of anthocyanins, phenylpropanoid flavonoids, polyphenols, and tannin in young or mature leaves of flax (*Linum usitatissimum*) plants cultured on N-sufficient (+N) or N-deficient (–N) nutrient medium. Different letters indicate differences statistically important ($P \leq 0.05$). DW – dry weight

flavonoids in young leaves by 2.3 times when compared to control (Figure 4B). Polyphenols content increased by about 1.6 times in young –N leaves (Figure 4C). In young –N leaves, tannin content was about 1.9 times higher than in control but in mature –N leaves – around 2.5 times more (Figure 4D). Analysed phenolic compounds most probably perform protective functions against stress in leaves, partly compensating for the loss of N-containing CGs caused by N deficit. Higher accumulation of sum of phenolic compounds and tannins in N-deficient young leaves may be due to their need for better protection against various stress factors, including secondary oxidative stress also observed under N starvation. Zhou et al. (2019) showed that low N supply change phenolic composition in lettuce, increases their content, and improve antioxidant capacity. However, more research is needed to accurately explain the role of individual fractions of phenolic compounds under N deficiency. Recently, Zuk et al. (2019) characterised phenolics composition, indicating for apigenin and luteolin glycosides as the major group of flavonoids in flax. In addition, research by Zuk et al. (2019, 2020) suggested an inverse correlation between flavonoids and CGs content at the flowering stage of flax. Previous studies also indicated the role of various phenolic compounds in plant development and response to various stresses (Cheynier et al. 2013).

Our results suggest a trophic role of CGs and/or HCN in young leaves of flax. The CAS activity was high under N deficiency, N accumulation in CGs was observed, which indicated that CGs are useful under low-N stress. It is believed that HCN can play a dual role, toxic and positive, but the signal transduction pathway is not fully understood. Recent studies by García et al. (2019), however, demonstrate that that *S*-cyanylation is involved in metabolic regulation, including glycolysis and the Calvin cycle. The action of HCN depends on its quantity in the cell, external factors and development stage; high HCN synthesis may lead to plant damage (Siegień and Bogatek 2006, Gleadow and Møller 2014). Recently, the dual function of CGs was studied by Cuny et al. (2019). Their results suggested that CGs in *Phaseolus lunatus* L. seeds are most likely associated with seedling defense but do not improve germination. In our study, CGs decrease was more visible in mature leaves than young ones. High activity of CAS and N accumula-

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tion in CGs indicate that these compounds can be used under low-N conditions to synthesise amino acids and other metabolites in developing leaves. In addition, the higher accumulation of some phenolic compounds in N-deficient young leaves may be due to replacing of decreased CGs and their need for better protection against various stress factors.

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