

SHORT COMMUNICATION

Influence of cadmium on protein profile of flax varieties (*Linum usitatissimum* L.)

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Summary

The aim of the study was the evaluation of the influence of cadmium on protein profile of flax varieties (*Linum usitatissimum* L.). Linola and Norlin explants were cultured on control medium Dorothy and with addition of 25 and 75 mg/l Cd(NO₃)₂. Extracts were separated on DEAE-Cellulose (20 mM Tris-HCl buffer, 0.2-1 M NaCl). Protein content was evaluated by measuring the absorbance at wavelengths 280 and 254 nm. Linola was characterized by proteins occurrence in fractions eluted by 0.4 and 0.6 M NaCl at 25 mg/l of Cd(NO₃)₂, at 75 mg/l and additionally by 0.3 M NaCl. Norlin showed response in the form of proteins appeared at 0.2, 0.4, 0.5 and 0.6 M NaCl gradient at 25 mg/l of cadmium, at higher concentration in fractions eluted by 0.2, 0.5 and 0.6 M NaCl. Electrophoretic analysis showed an increase in the protein bands intensity above 60 kDa and under 52 kDa in extracts from flax cultivated with Cd(NO₃)₂. Studies showed appearance of new proteins during stress condition.

Key words: flax, protein extracts, cadmium

Flax (*Linum usitatissimum* L.) of the *Linaceae* family is an annual plant and one of the oldest fibrous plants in the world (5,000 BCE). Flax is used for food, medicines and textiles and, therefore, has been of great importance for human culture and development for more than 8,000 years. At that time, flax was generally used for fibres production, nowadays, the main production is focused on oil. *Linum usitatissimum* is a member of the largest genus (about 200 species) *Linum* in the family *Linaceae* comprising 22 genera and about 300 species distributed worldwide. It is believed that the center of origin of cultivated flax is the Middle East, although secondary diversity centers were identified in the Mediterranean basin, Ethiopia, Central Asia, and India [1, 2].

Linum usitatissimum is an upright annual plant growing to 1.2 m, with slender stems. The leaves are glaucous green, slender lanceolate, 2–4 cm long and 3 mm broad. The flowers are blue (1.5–2.5 cm in diameter), with five petals; they can also be bright red. The fruit is a round, dry capsule (5–9 mm diameter) with several glossy brown or yellow seeds shaped like an apple pip. The seeds (4–7 mm long) become sticky when wet. The plants have one short, branched taproot (1 m) with lateral roots (30 cm). The life cycle of the plants include vegetative period (60–80 days), flowering period (25–40 days) and maturation period (40–60 days). The *Linum* plants are vulnerable to water stress, high temperatures and diseases [3, 4]. Both linseed and fibrous flax is widely used in the food and pharmaceutical industries, as well as in the textile industry, chemical industry, building and automotive industry [5–7]. Flaxseed and oil obtained by extraction of seeds contain, inter alia, polyunsaturated fatty acids, lignans (phytoestrogens) and cyclolinopeptides which are responsible for the reduction of the risk of cardiovascular diseases and cancer preventive compounds including postmenopausal breast cancer risk [8–10]. Flax seeds contain oil (30–40%) rich in omega-3 and omega-6 fatty acids, high quality proteins (10–37%), mucilage (8%), soluble and insoluble dietary fibre, antioxidants and could be used as a nutritional supplement [8, 11–13]. Flax fibre contain cellulose, hemicellulose, lignin which affect the physical properties of the fibres, as well as pectin and wax [14].

There is also a growing interest in biomedical application of flax fibers as a wound dressing with antioxidant, anti-inflammatory and antibacterial properties [15]. Additionally several studies show the flax tolerance to cadmium contamination. However, due to the toxicity of cadmium, plants ability to accumulate heavy metals prevents their use for pharmaceutical purposes and in food production. Plants which accumulate heavy metals from the soil could be used for phytoremediation of soil and their fibres, containing metals, for geotextile production [5, 7, 16–18]. Flax harvest from polluted areas is a potential source for the energy production. Remaining heavy metals from the ash can be recovered in metallurgical plants [19].

Cadmium contamination, even in small amounts, is harmful not only to humans, animals, but also for plants. Cadmium is not essential for plant metabolism and uptake of this metal affected cell. Plants have developed, like other organisms, control mechanism responding to intake and accumulation of heavy metals. These mechanisms include sequestration and chelation of metal in roots and leaf cells by ligands such as metallothionein and phytochelatin (metallothionein

group III), which form complexes with metals [20, 21]. It is believed that mainly phytochelatins, glutathione derivatives, with the structure of dipeptide (γ -Glu-Cys) $_n$ -Gly, are synthesized by plants in response to the presence of heavy metals in the cytoplasm. Phytochelatins form complexes with heavy metals, which are then transported to the vacuole [22-24]. The molecular weight of the complex increases with the concentration of applied cadmium. In the case of plants, this mass is approximately 30 kDa, although studies indicate the presence of the complexes formed with Fe ions with a molecular mass of 180 kDa [25]. However, studies conducted on different flax varieties showed synthesis of phytochelatins in the form of individual particles of 2 kDa mass and in the form of complexes with a mass of ≥ 9 kDa [7]. The aim of this study was evaluation of changes in protein extracts from flax varieties grown in the presence of cadmium and identification of the variety that can be designed for the production of medicines and food.

MATERIALS AND METHODS

Apical explants of flax varieties Linola and Norlin were collected from plants grown from seed. Then explants were cultivated in photoautotrophic cultures for 6 days on Dorothy medium with addition of 0.4 ml/l kathon as a control and Dorothy medium with addition of 25 mg/l and 75 mg/l $\text{Cd}(\text{NO}_3)_2$ respectively. Isolation of proteins was made from apical explants. Plant material was homogenized with 5 ml of buffer A (20 mM Tris-HCl, pH 8.8), centrifuged at 10,000 rpm/min at 4°C (1 hour) to separate the supernatant, which constituted tested extract. Ion exchange chromatography was used, DEAE-Cellulose column was equilibrated with buffer A. Proteins were eluted using a step gradient of NaCl (respectively 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1 M NaCl). The content of proteins/peptides in all fractions was evaluated by measuring absorbance at wavelengths 280 and 254 nm. In order to identify the proteins in the extract was used electrophoresis. Separation of proteins was carried out in a polyacrylamide gel under denaturing conditions according to Laemmli system (4% thickening gel and 12% separating gel) in the presence of a size marker [26]. Quantitative analysis of separated bands of selected proteins was conducted with an Image Master VDS (Image Master® 1D Elite version 4.00 software). Computations were based on the assumption that the area of a single protein band accounts for a percentage ratio in relation to the area of all separated proteins in a given sample on gel, which constitutes 100 %.

RESULTS AND DISCUSSION

Observations carried out at the 6th day showed no effect of tested concentrations of $\text{Cd}(\text{NO}_3)_2$ on the development of explants, which were characterized by a 100% vitality and multiplication factor 1 (tab. 1). In the experiment, extracts were

prepared from the whole plant, including both roots and stems with leaves. It is important because a number of publications indicates on the accumulation of large amounts of cadmium by flax primarily in roots and stems [5, 7, 17]. These plants during cultivation showed no signs of heavy metals toxic effects such as twisting, chlorotic discoloration and browning of leaves. These changes appeared only after prolonged cultivation, and at higher doses of cadmium. No apparent effect of low concentrations of cadmium on plants was found in the literature to confirm [7].

Table 1.

Photoautotrophic culture of oilseed flax cultivars Linola and Norlin on the control medium Dorothy and medium Dorothy with addition of 25 mg/l and 75 mg/l $\text{Cd}(\text{NO}_3)_2$

Content of $\text{Cd}(\text{NO}_3)_2$ [mg/l]	Flax cultivar	Explant			Multiplication factor
		Vitality [%]	Dying [%]	Infections [%]	
0	Linola	100	0	0	1
	Norlin	100	0	0	1
25	Linola	100	0	0	1
	Norlin	100	0	0	1
75	Linola	100	0	0	1
	Norlin	100	0	0	1

Electrophoretic separation of extracts obtained from explants varieties Norlin and Linola shows changes with respect to stress condition induced by cadmium in comparison to extracts obtained from control group (fig. 1). Electrophoretic analysis of extracts Linola varieties grown in media supplemented with either 25 mg/l or 75mg/l $\text{Cd}(\text{NO}_3)_2$ showed a reduction in the intensity of protein bands with a molecular weight of about 60 kDa from 15.56% to 2.17% at the highest concentration of cadmium. There has been shown the increase of the band intensity of approximately 52 kDa for Linola variety grown in the control medium from 19.38% to 23.67%, and there was a slight increase in the intensity of bands less than 50 kDa from 43.55% to 46.91%. In the second variety electrophoretic separation of protein extracts becomes apparent. The reduction of the intensity of protein of approximately 60 kDa in the explants cultured on medium with a higher cadmium concentration (75 mg/l) was from 21.42% to 4.12%, while at 25 mg/l of cadmium band representing 18.08% of the total protein. At the same time was observed reduction of the protein bands intensity of approximately 52 kDa from 21.19% to 19.80% at 75 mg/l of cadmium nitrate. In the case of protein with mass lower than 50 kDa showed an increase in intensity of the bands from 38.13% to 47.18% was shown. Furthermore, for both varieties an intensity increase in the protein bands of 60 kDa under stress due to the presence of cadmium was shown.

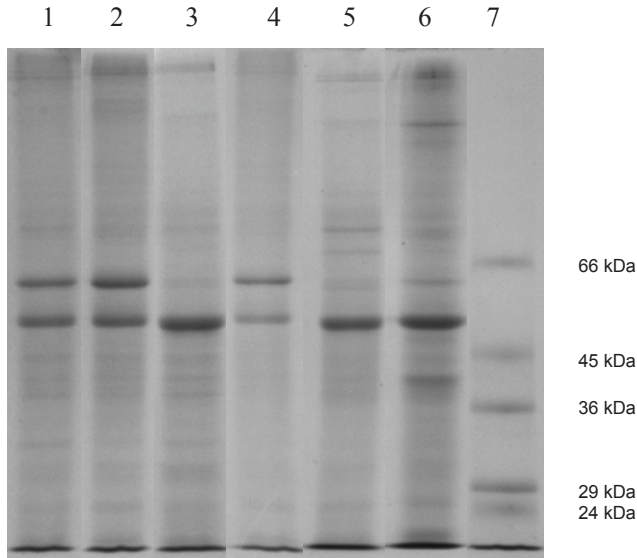


Figure 1.

Electrophoretic separation of protein extracts obtained from explants (SDS – PAGE by Laemmli). Lane 1, extract from explants Linola variety in the control medium; Lane 2, extract from explants Norlin variety in the control medium; Lane 3, extract from explants variety Linola on a medium with 25 mg/l Cd(NO₃)₂; Lane 4, extract from explants variety Norlin on a medium with 25 mg/l Cd(NO₃)₂; Lane 5, extract from explants Linola variety on a medium with 75 mg/l Cd(NO₃)₂; Lane 6, extract from explants Norlin variety on a medium with 75 mg/l Cd(NO₃)₂; Lane 7, size marker

Electrophoretic analysis showed a reduction of the protein content in the range 60-52 kDa in extracts of flax variety Linola from 34.94% to 25.84% and from 42.61% in Norlin variety to 21.74% under the influence of cadmium. Addition of cadmium nitrate resulted in the appearance of new proteins, peptides or their complexes as indicated by the increase in intensity of the bands of the two varieties higher than 60 kDa. However, a similar trend in the case of bands, less than 52 kDa, suggests a formation of new peptides/proteins or degradation products of higher molecular weight proteins. More intense bands in the range of 60 kDa and less than 52 kDa in control extracts may also indicate a phytochelatin synthesis of approximately 30 kDa, which can form complexes with a higher weight [25].

Spectrophotometric analysis of the fractions obtained by separation of extracts of variety Linola cultivated on medium containing 25 mg/l Cd(NO₃)₂ on a column DEAE - Cellulose (fig. 2a) showed an increase in the absorbance in the fractions 16 and 19 (elution with 0.4 M NaCl), 33 and 35 (elution with 0.6 M NaCl), indicating the presence of proteins that are not found in the extract of the control variety Linola. Cultivation on medium containing 75 mg/l cadmium (fig. 2b), caused an

increase in absorbance in fraction 12 eluted by 0.3 M NaCl solution as well as at lower concentrations of cadmium in the fractions eluted by 0.4 M (fraction 18 and 21) and 0.6 M (fraction 29) NaCl solution. These data indicate changes in the flax variety Linola extracts in the presence of different concentrations of cadmium.

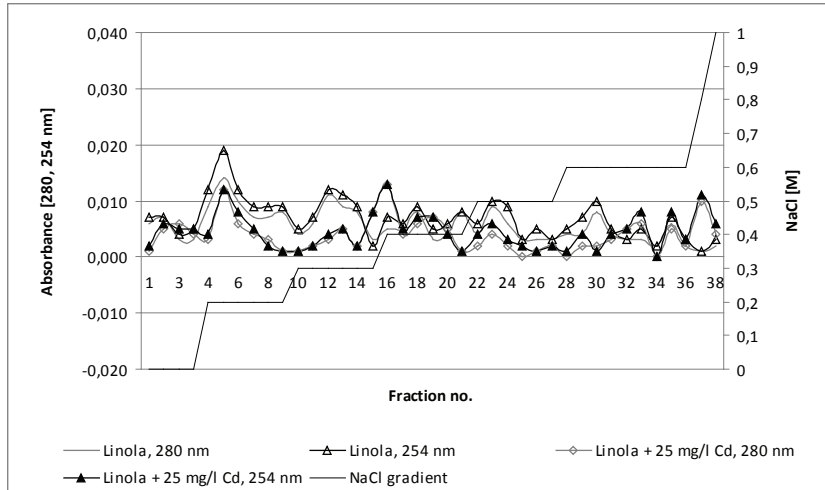


Figure 2a.

Separation of proteins extracts from explants variety Linola cultured on the control medium Dorothy and medium Dorothy with addition of 25 mg/l $\text{Cd}(\text{NO}_3)_2$ on ion exchange column DEAE-Cellulose

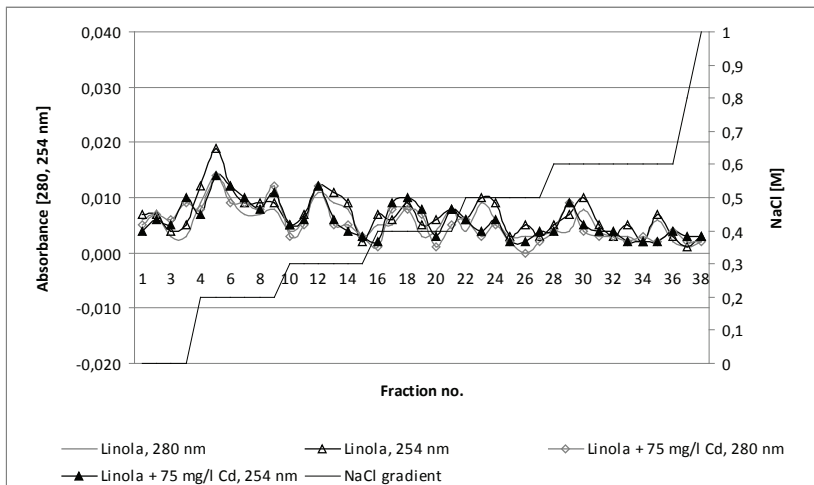


Figure 2b.

Separation of proteins extracts from explants variety Linola cultured on the control medium Dorothy and medium Dorothy with addition of 75 mg/l $\text{Cd}(\text{NO}_3)_2$ on ion exchange column DEAE-Cellulose

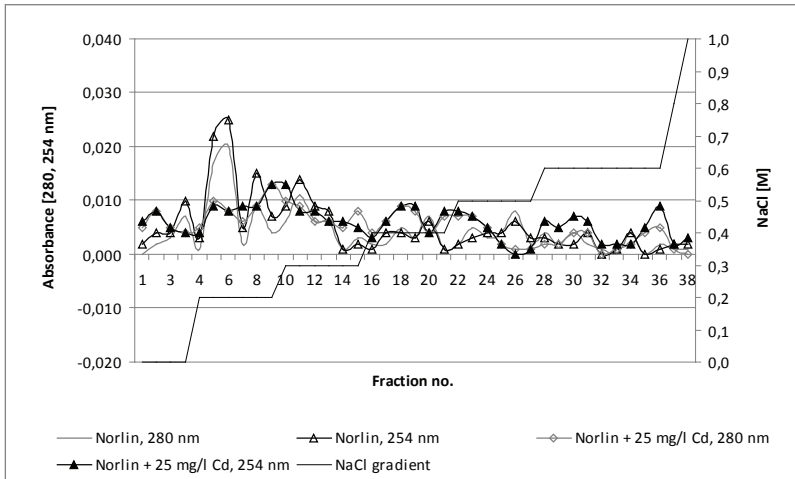


Figure 3a.

Separation of proteins extracts from explants variety Norlin cultured on the control medium Dorothy and medium Dorothy with addition of 25 mg/l $Cd(NO_3)_2$ on ion exchange column DEAE-Cellulose

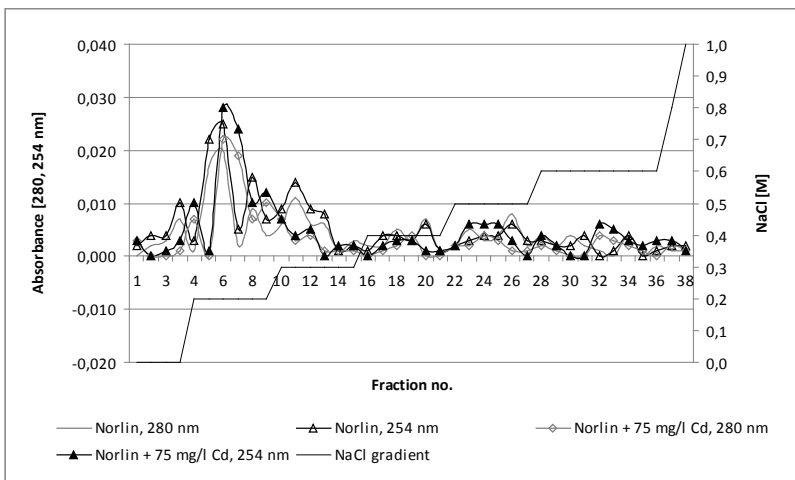


Figure 3b.

Separation of proteins extracts from explants variety Norlin cultured on the control medium and medium Dorothy with addition of 75 mg/l $Cd(NO_3)_2$ on ion exchange column DEAE-Cellulose

Extracts obtained from variety Norlin cultivated on control medium and medium supplemented with cadmium were also evaluated. Separation on a column of DEAE - Cellulose Norlin extract derived from a culture of 25 mg/l of cadmium showed an increase in absorbance in the fractions 9 and 10 (eluted by 0.2 M NaCl), 18, 19 and 21 (eluted by 0.4 M NaCl), in the fraction 22 (eluted by 0.5 M NaCl),

and fractions 28, 30 and 36 (eluted with 0.6 M NaCl) (fig. 3a). In the case of the variety Norlin, absorbance related to presence of protein fractions eluted at 0.2 and 0.5 M NaCl solution, demonstrates the higher protein synthesis in response to cadmium stress than in the case of Linola variety. Analysis of fractions obtained by column separation of the Norlin extract originating from a medium containing 75 mg/l of cadmium showed an increased absorbance in the fraction 9 (eluted by 0.2 M NaCl), in fractions 23–25 (eluted with 0.5 M NaCl solution) as well as in fractions 28 and 32 (eluted with 0.6 M NaCl) (fig. 3b).

Previous studies carried out on the flax variety Cristal, where the seeds were germinated in the presence of 0.5 mM $\text{Cd}(\text{NO}_3)_2$ also showed the appearance of proteins in the eluted fractions by 0.3, 0.4 and 0.5 M NaCl gradient, which were not present in extracts from control seedlings [27]. Research carried out by Li-Chan et al. [28] on flax seed also showed appearance of complexes similar to phytochelatins in fractions eluted respectively with 0.45 and 0.5 M NaCl solution. The presence of cadmium binding complexes in several fractions eluted by 0.10, 0.25 and 0.50 M NaCl in different varieties of flax confirmed Oomah et al. [29]. In addition, flax varieties and their distance to the contamination source is important for heavy metals accumulation, migration and movement in plants. Najmanova et al. [7] demonstrated the synthesis of phytochelatins in the form of complexes with a mass of 2 kDa and higher than 9 kDa, by flax plants in the presence of cadmium. Research conducted by Bjelkova et al. [5] on different varieties of fiber flax and oil flax showed the greatest accumulation of metals in the roots which was confirmed in this study. These studies may explain the observed differences in this experiment between the two flax varieties. In addition, electrophoretic analysis indicates the presence of proteins/peptides in the extract of flax varieties Linola and Norlin cultured with cadmium. These proteins/peptides could be phytochelatins and their complexes with increased molecular weight. The occurrence of flax varieties resistant to cadmium points to the potential use in the process of phytoremediation. Differentiated response of varieties under the influence of stressor indicates the possibility of the occurrence of flax varieties more resistant to cadmium contamination. Further studies on the changes of flax protein profile during phytoremediation process requires usage of additional analysis including Western blotting and two-dimensional electrophoresis.

CONCLUSIONS

1. During the photoautotrophic cultivation of flax the influence of cadmium on the explants development was not observed. Cadmium stress symptoms such as twisting, chlorotic discoloration and browning of leaves were visible after prolonged culture at higher concentrations of cadmium.
2. Electrophoretic analysis showed an increase in the intensity of the protein bands 60 kDa and 52 kDa under the influence of cadmium in two varieties of flax. These changes may be due to degradation of proteins or phytochelatins synthesis by plants, which can form complexes with higher molecular weight.

3. Spectrophotometric analysis of the extracts obtained from explants grown on media supplemented with 25 mg/l of cadmium nitrate showed the presence of proteins in the fractions eluted mainly by 0.4 and 0.6 M NaCl. However, in the case of Norlin, fractions were eluted additionally with 0.2 and 0.5 M NaCl. These proteins were not observed in the extracts of plants grown in controlled conditions.
4. Higher concentrations of cadmium nitrate (75 mg/l) in the case of a variety Linola affected the appearance of protein absent in extracts from control plants in the eluted fraction of 0.3, 0.4 and 0.6 M salt solution. However, Norlin variety was characterized by the presence of proteins in the fractions eluted by 0.2, 0.5 and 0.6 M NaCl solution.
5. The studied varieties of flax are characterized by a diverse response to the presence of cadmium.

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WPLYW KADMU NA ZMIANĘ PROFILI BIAŁKOWYCH ODMIAN LNU (*LINUM USITATISSIMUM* L.)

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Streszczenie

Celem badań była ocena wpływu kadmu na zmianę profili białkowych odmian lnu (*Linum usitatissimum* L.). Eksplantaty Linola i Norlin hodowano na podłożu kontrolnym Dorota oraz z dodatkiem 25 i 75 mg/l $\text{Cd}(\text{NO}_3)_2$. Ekstrakty rozdzielano na kolumnie DEAE-Celuloza (20 mM Tris-HCl bufor, gradient 0,2-1 M NaCl). Zawartość białek oceniano przez pomiar absorbancji przy długości fali 280 i 254 nm. Linola charakteryzowała się pojawieniem białek we frakcji wymywanej 0,4 i 0,6 M NaCl przy niższym stężeniu kadmu, natomiast przy 75 mg/l azotanu kadmu dodatkowo we frakcjach wymywanych 0,3 M NaCl. Norlin wykazała obecność białek we frakcjach wymywanych 0,2, 0,4, 0,5 i 0,6 M NaCl w obecności 25 mg/l $\text{Cd}(\text{NO}_3)_2$ w podłożu, przy większym stężeniu kadmu pojawiły się białka we frakcjach wymywanych 0,2, 0,5 oraz 0,6 M NaCl. Analiza elektroforetyczna wykazała wzrost intensywności pasm białek w zakresie powyżej 60 kDa i poniżej 52 kDa pod wpływem kadmu w obu odmianach lnu. Przedstawione badania potwierdziły pojawienie się nowych białek w warunkach stresu kadmowego.

Słowa kluczowe: *len, ekstrakty białkowe, kadm*