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PHENYLALANINE AMMONIA-LYASE ACTIVITY AND ANTHOCYANIN CONTENT IN DIFFERENT VARIETIES OF LETTUCE UNDER THE CADMIUM INFLUENCE

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Cadmium ions influence on the content of anthocyanins as non-plastid pigments and the activity of phenylalanine ammonia-lyase (EC 4.3.1.24) as primary enzyme of their biosynthesis in two lettuce varieties (Lactuca sativa L.) with different anthocyanin content was investigated. The increases in anthocyanin content and enzymatic activity of phenylalanine ammonia-lyase of both lettuce plant varieties during the 14-day exposure in solution of cadmium nitrate (0.1 mmol) were established. The difference in levels of adaptive capacity in two lettuce varieties with different content of anthocyanins under the effect of cadmium ions was revealed. The indexes of tolerance have shown faster adaptive response and higher resistance in red variety plants, compared with plants of the green lettuce variety. These data are consistent with the corresponding changes in anthocyanins content and indicate their active participation in neutralizing the negative impact of cadmium stress.

K e y w o r d s: cadmium ions, anthocyanins, phenylalanine ammonia-lyase, tolerance index, resistance, Lactuca sativa.

The increasing of anthropo-technological load [1] is accompanied with environmental accumulation of cadmium compounds, one of the most common first class toxicity pollutants [2]. This statement necessitates the research of its toxic effect mechanisms on plant organisms and determination of the physiological and biochemical adaptive changes in plant cell metabolism [3, 4].

Plants resistance ability to the high environmental concentrations of cadmium depends on: plant genotype, stage of development and metal concentration, and it is achieved by activation of the induced resistance mechanisms [5]. The support of antioxidant status appropriate level and plant cell ability to the cadmium ions chelation are considered as one of the main intracellular mechanisms of plant resistance to toxic cadmium effect [6]. Such properties are inherent in endogenous low molecular weight phenolic metabolites – anthocyanins that play a significant role in the formation of plant adaptive mechanisms to adverse environmental conditions [6, 7]. Anthocyanins are also important low molecular weight component of the plant antioxidant system. They show both direct and indirect antioxidant action associated with chelation of metal ions with variable valence [8]. Therefore, plant organism is characterized by the increase of phenylpropanoid biosynthesis intensity under the influence of stress factors, and their accumulation level serves as a non-specific indicator of plant stability [9, 10]. Phenylalanine ammonia-lyase (PAL, L-Phenylalanine Ammonia-Lyase EC 4.3.1.24) is a regulatory enzyme of the secondary metabolism compound biosynthesis that catalyzes the primary reaction of reverse deamination of L-phenylalanine amino acid to the trans-cinnamic acid [11, 12]. Therefore the PAL in the plant organism can play an extremely important role to run the resistance mechanisms against the toxic effects of stress factors, including cadmium [10, 13].

It is known that lettuce (*Lactuca sativa* L.) and other leafy vegetables being dietary multivitamin daily cultures of human nutrition [14] with a rapid biomass growth could be considered as potential heavy-metal accumulators [15, 16]. Despite

the known fact about genetic determination of plant ability to heavy metal accumulation at the family, genus and species level, the results of this fact confirmation at the variety level have appeared recently [1].

It was discovered [12, 17] responsible for the secondary phenolic metabolites synthesis R2R3-Myb-domains and bHLH-transcription factors of plant genome that could act as driving factors for differently pigmented varieties selection. The large-scale research of various EST-domains of lettuce (*L. sativa*) plant genome identified the gene expression increase of the enzymatic units in anthocyanin synthesis under stress conditions, in particular the genes responsible for encoding the PAL-biosynthesis [17, 18].

The aim of the study was to assess the impact of exogenous cadmium ions to anthocyanin content and PAL-activity to determine the varietal characteristics of different anthocyanin content varieties of lettuce resistance (*L. sativa* L. *var. crispa*).

Materials and Methods

Two varieties of lettuce plants (*L. sativa* L. *var. crispa*) significantly differing in anthocyanin content – green Lolo and red Lolo Ross were grown over the month by the water culture with half diluted Knop nutrient solution under 25 °C and 16-hour photoperiod conditions. The 0.1 mmol cadmium nitrate solution ($Cd(NO_3)_2 \cdot 4H_2O$) was added in the nutritional environment of lettuce experimental variants. The coeval control and test plants were randomly selected for physiological and biochemical assays in the 1st, 7th and 14th day of exposure.

Anthocyanin pigment content was estimated spectrophotometrically by the method of Beggs and Wellmann [19] with some minor modifications. The crushed leaves sample material (0.5 g) was extracted with 10 ml of HCl:methanol solution (at a ratio of 1:100) during 24 h at 5 °C temperature of the environment. Absorbance was measured by a UV-1800 (Shimadzu, Japan) at 530 nm wavelength. Quantity of anthocyanin was calculated according to Giusti and Wrolstad methodology [20] using cyanidin-3-glucoside coefficients – the major anthocyanin in lettuce plants according to Wu and Prior [21] (molar extinction coefficient of 26 900 $l \cdot cm^{-1} \cdot mol^{-1}$ and molecular weight of 449.2 $g \cdot mol^{-1}$).

PAL activity was determined according to Zucker methodology [22] with some modification [11, 13] and minor additions. The spectrophotometric determination of PAL is based on changes of absorbance at 290 nm compared to the resulting mixture with stopped enzyme reaction at the beginning of heating as blank. For enzyme analysis 0.2 g of fresh leaves were homogenised in 3 ml borate buffer (pH 8.8) containing 23 µl of mercaptoethanol. Extraction was performed at 2 °C. The homogenates were centrifuged for 20 min at 8000 g and the supernatant was used for enzymatic assay. The PAL assay system contained 1 ml of the supernatant, 1 ml of buffer, 1 ml of 0.05 M L-phenylalanine as a substrate was incubated at 37 °C for 1 h. The reaction was stopped with 30% trichloroacetic acid and absorbance of the formed trans-cinnamic acid was measured at 290 nm wavelength. The results of PAL activity were expressed as µmoles of trans-cinnamic acid per minute per milligram of protein (µmoles trans-cinnamic acid $min^{-1}mg^{-1}$). Protein content was determined by the method of Bradford [23].

Cd tolerance index, as one of the main integral indicators of plant resistance [24, 25], was determined by the formula: TI = Dry weight (+ 0.1 mmol Cd^{2+})/Dry weight (control) × 100% [26, 27].

Statistical analysis of the results obtained was carried out with three biological and analytical replicates. The arithmetic mean value, standard deviation (M ± SD) and correlative analysis with Pearson coefficients were performed using the standard program of "Microsoft Office" package – "Microsoft Office Excel". Differences were accepted as significant by the Student's criterion for $P \leq 0.05$.

Results and Discussion

Inhibition of plant growth is one of the first nonspecific reactions and early symptom of a visual display of heavy metal negative effects. Plant growth parameter is most sensitive to changes of nutritional medium composition and most clearly reveals the systemic response to high heavy metals content. Therefore, the stability of the plant growth parameters under the contaminated territories is one of the main integral indicators of tolerance to environment conditions [24].

Using the dry mass, as an integral indicator of the absolute growth, we investigated the tolerance indexes that was based on biomass growth and showed the differences in plant response during the exposure to the presence of Cd^{2+} ions [25]. The data obtained (Fig. 1.) indicate the difference in the levels of adaptive capacity of the various anthocyanin content lettuce varieties to the cadmium ions

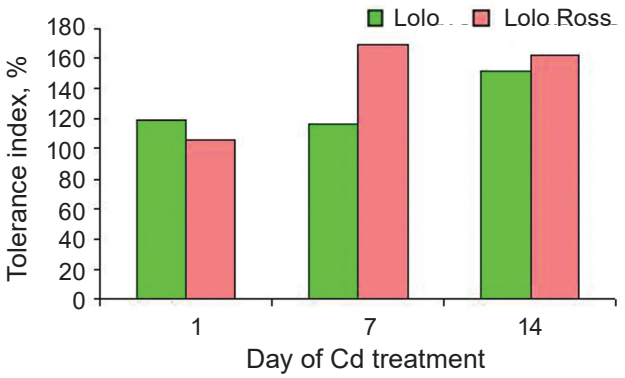

Fig. 1. Tolerance indexes of L. sativa green Lolo and red Lolo Ross varieties at the 0.1 mmol Cd^{2+} influence

influence. In particular, the test plants of red Lolo Ross variety showed faster and higher resistance occurred at the 7th exposure day compared to experimental green Lolo variety plants, which has reached the relevant level only at the 14th exposure day. It can be explained by the involvement of anthocyanins, low molecular weight endogenous antioxidants and chelators, in the plant adaptive response processes [6, 12]. Because it is known that this group of phenolic compounds have the ability to form chelate complexes with heavy metals [4], transporting them into the vacuole for detoxification, and also affect the membrane fluidity, reducing the flow of hazardous ions into the cell [9, 28].

The results indicated a significant increase in the pool of anthocyanin pigments in the experimental plants of both varieties under the influence of cadmium ions (Fig. 2). However, we identified significantly higher anthocyanin levels in the experimental plant of red Lolo Ross lettuce variety, which grew throughout the exhibition, reaching maximum values on the 7th day and surpassing control plants value by 80% (Fig. 2, *B*).

A higher level of non-plastid pigment accumulation in red lettuce is a varietal characteristic. However, its significant increase throughout the experiment could obviously testify to developed chelator metabolism and non-plastid pigment active participation in the negative impact of this heavy metal neutralization. The consistently high anthocyanin content during prolonged stressor exposure may also indicate the activation of its synthesis at the time of the previous reserves depletion and is consistent with scientist assertions about the antioxidant role of phenolic compounds in oxidative stress combating [3, 9].

At the same time, the experimental plants of Lolo lettuce variety showed a significant increase in the anthocyanin content by 30% only on the first day of exposure with cadmium ions (Fig. 2, *A*). Further anthocyanin reduction in treated plants throughout the exposition can testify to their active opposition to the Cd^{2+} toxic effects by mass deposition of the formed metal complexes in vacuoles, which is one of the main manifestations of non-plastid pigment protective functions in the plant organism under the influence of stress factors [7].

Thus, our results confirm the important role of phenolic compounds (anthocyanins) as antioxidants and chelators in the formation of adaptive plant response to the adverse effects of heavy metal ions.

As PAL is a key enzyme in a pool of secondary fenolic metabolite biosynthesis [13], changes in its activity levels under Cd toxicity could be regarded as the non-specific indicator of plant adaptive capacity,

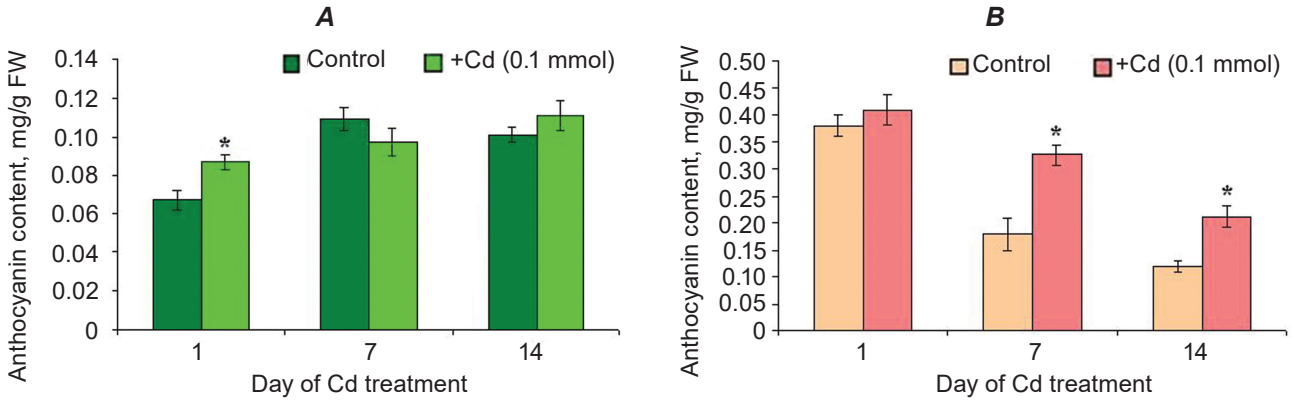

*Fig.2. Anthocyanin content in leaves of Lolo (A) and Lolo Ross (B) varieties of L. sativa at the 0.1 mmol Cd^{2+} influence. *Difference compared with control statistically significant at $P < 0.05$, FW – fresh weight*

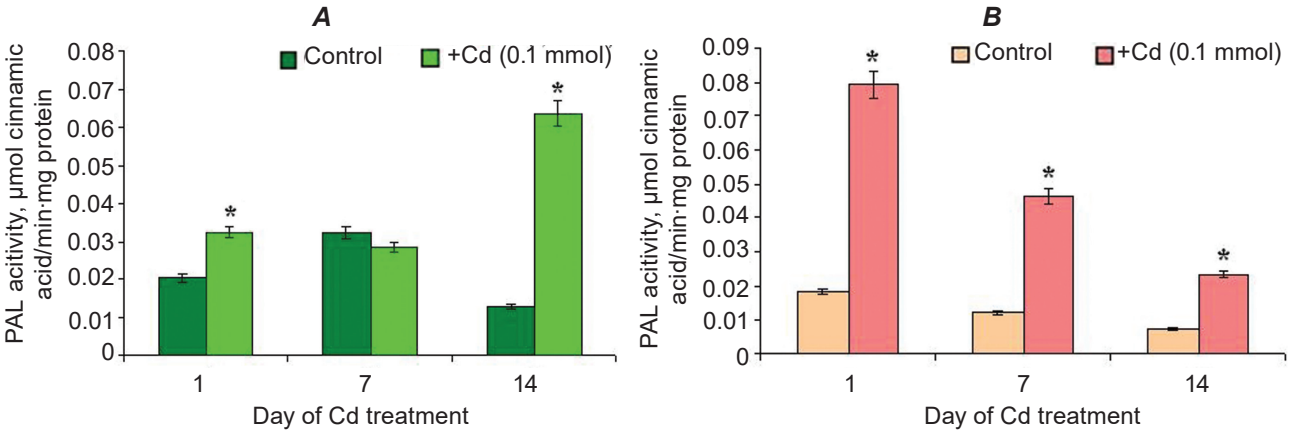

*Fig. 3. PAL-activity in leaves of Lolo (A) and Lolo Ross (B) varieties of L. sativa at the 0.1 mmol Cd^{2+} influence. *Difference compared with control statistically significant at $P < 0.05$*

which is manifested as increased synthesis of low molecular weight antioxidants and chelators [4].

According to our research, high anthocyanin accumulation levels in plants of both lettuce varieties under the cadmium influence during the experiment are consistent with a significant increase in the PAL activity. It was confirmed by the correlative analysis, according to which the high positive correlation with Pearson coefficients for green Lolo variety – 0.851 and red Lolo Ross variety – 0.987 was traced.

According to the data obtained a significant (60%) increase of PAL activity above control was identified in the experimental green Lolo plants on the first day of cadmium exposure, which confirmed by the data presented in the literature [11, 12] about the fast enzyme response and was consistent with the increase of anthocyanin content in this variety plants at the first exposure day. The reducing of enzymatic activity in test samples by 12% below control was consistent with corresponding anthocyanin content decrease and could be explained by the inclusion of additional plant protection systems because of toxic effects of heavy metals. According to experimental data the maximum level of PAL activity for green Lolo variety was identified on the 14th exposure day and reached up to six-time control values (Fig. 3, *A*).

In red Lolo Ross lettuce plants a gradual drop in PAL activity was observed during the Cd^{2+} treatment and consistent with an anthocyanin content decrease and could be explained by the secondary metabolite biosynthesis system sensitivity. The maximal level of red Lolo Ross PAL activity was identified on the first day of treatment and reached up to four-fold control values (Fig. 3, *B*). Further cadmium exposure showed the three-fold increasing of enzyme activity on the 14th day.

Thus, according to the data obtained, we can say that the influence of cadmium ions activates this enzyme in red lettuce plants as evidenced by the significantly higher PAL activity level, which is stored within the experiment.

Thus, the different levels of phenylalanine ammonia-lyase activity in the plants of two lettuce varieties significantly differing in anthocyanin content during prolonged exposure with 0.1 mmol Cd^{2+} was noted. The high tolerance index of experimental red lettuce plants allows us to suggest a more intense involvement of their adaptation processes to detoxify cadmium ions compared to experimental plants of green variety. Therefore, it is assumed that due to the presence of significant anthocyanin amounts, which effectively neutralized the negative effects of this toxicant exposure, red Lolo Ross variety showed a higher level of system stability under the cadmium influence compared to experimental plants of green Lolo variety. At the same time, a significant increase of the level of anthocyanin content at the Cd^{2+} impact in the test samples of both lettuce varieties both is consistent with the high enzyme activity of phenylalanine ammonia-lyase – the key enzyme in a low molecular weight antioxidants and fenolic chelator biosynthesis and also could be regarded as the non-specific indicator of plant adaptive capacity.

АКТИВНІСТЬ ФЕНІЛАЛАНІН АМІАК-ЛІАЗИ ТА ВМІСТ АНТОЦІАНІВ У САЛАТІ РІЗНИХ СОРТІВ ЗА ДІЇ ІОНІВ КАДМІЮ

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Досліджено вплив іонів кадмію на вміст непластидних пігментів – антоціанів та активність основного ензиму їх біосинтезу – фенілаланін аміак-ліази (4.3.1.24) в двох сортах салату посівного (*Lactuca sativa* L.), що містять різну кількість антоціанів. Встановлено підвищення вмісту антоціанів та зростання активності фенілаланін аміак-ліази впродовж 14-добової експозиції рослин салату обох сортів у розчині нітрату кадмію (0,1 ммоль). Виявлено різницю в рівнях адаптаційної спроможності салату різних сортів за вмістом антоціанів до дії іонів кадмію. За індексами толерантності відзначено швидшу адаптивну відповідь та вищу резистентність у рослин червоного сорту салату, що узгоджується із відповідними змінами вмісту антоціанів та свідчить про їх активну участь у зниженні негативного впливу кадмієвого стресу.

Ключові слова: антоціани, фенілаланін аміак-ліаза, індекс толерантності, іони кадмію, стійкість, *Lactuca sativa*.

АКТИВНОСТЬ ФЕНИЛАЛАНИН АММИАК-ЛИАЗЫ И СОДЕРЖАНИЕ АНТОЦИАНОВ В САЛАТЕ РАЗЛИЧНЫХ СОРТОВ ПОД ДЕЙСТВИЕМ ИОНОВ КАДМИЯ

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Исследовано влияние ионов кадмия на содержание непластидных пигментов – антоцианов и активность основного энзима их биосинтеза – фенилаланин аммиак-лиазы (4.3.1.24) в двух сортах салата посевного (*Lactuca sativa* L.), с различным содержанием антоцианов. Установлено повышение антоцианов и активности фенилаланин аммиак-лиазы при 14-суточной экспозиции салата обоих сортов в растворе нитрата кадмия (0,1 ммоль). Показано, что уровни адаптационной способности к действию ионов кадмия у обоих сортов салата различны. Оценка индексов толерантности показала более быстрый адаптивный ответ и более высокий уровень резистентности у растений салата красного сорта, что согласуется с соответствующими изменениями содержания антоцианов и свидетельствует об их активном участии в снижении негативного влияния кадмиевого стресса.

Ключевые слова: антоцианы, фенилаланин аммиак-лиаза, индекс толерантности, ионы кадмия, устойчивость, *Lactuca sativa*.

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