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Polymorphic response of *Vicia faba* plants to cobalt excess: comparison of intact plants and callus cultures

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A high and unique expression of individual plant polymorphism after a Co-induced stress was observed after exposure of *Vicia faba* seeds. Plant variations were attributed to chlorophyll morphoses. A significant deviation from normal level was observed in chlorophyll, SOD and accumulation of several metals. In regenerants the differences in plant pigmentation disappeared. Alterations in callus cultures were also observed not only for plant pigments but also for SOD isozymes. Interesting differences between intact plants and callus cultures were observes in protochlorophyll concentration.

Key words: plant polymorphism, metal stress, Co^{2+} action, chlorophyll morphoses, regenerants, callus, *Vicia faba*, protochlorophyll

INTRODUCTION

Individual plants differ significantly in their response to heavy metals and other stress-inducing factors [1, 2]. An excellent system for investigation of individual plant polymorphism is the treatment of *Vicia faba* seeds with Co^{2+} excess [3, 4]. Individual polymorphism is expressed in easy noticeable pigmentation variations. These variations are to a high extent similar to dominant chlorophyll mutations. However, they are uninherited and so attributed to chlorophyll morphoses. The effect is specific for Co^{2+} excess, because it has been observed only after $Co(NO_3)_2$ or $CoCl_2$ treatment [5], despite that other 16 metals were tested in chloride and nitrate forms [6]. A high polymorphism of individual *V. faba* plants in SOD (superoxide dismutase) spectrum as also its dependence on plant injurity extent with Co^{2+} have been also revealed [3]. On the other hand, rather informative regarding the nature of the observed plant variations may be a comparison of the same characteristics as chlorophyll content, SOD activity in initially intact plants and regenerants or callus cultures, especially as regards the proposition on the epigenetic nature of the observed variations. The proposition about the epigenetic nature of Co^{2+}-induced variations is based not only on literature data [7–9], but also on our own observations in M_2 generation of Co^{2+}-treated plants: in progenies of Co^{2+}-treated plants the frequency of plants with pigmentation alterations was much higher [10].

The question is: do alterations in chlorophyll content and SOD spectrum preserve in callus culture or regenerants from differently Co^{2+}-affected plants?

MATERIALS AND METHODS

Seeds of the field bean (*Vicia faba* L.) cv. 'Aušra', initially obtained from the Lithuanian Institute of Agriculture, were planted in Botanical Garden of Vilnius University. Only healthy seeds were kept soaking for 15 h in the solutions of $Co(NO_3)_2$ (Sigma). They were washed three times with bidistilled water and planted either in an experimental field of the Botanical Garden or in a greenhouse in pots. Hard seeds were removed, leaving equally well swollen-out seeds. This procedure is very important for a correct evaluation of Co-tolerant plants. The types of plant pigmentation were determined in 30-day plants.

Callus cultures and regeneration experiments were made in Laboratory of Botanical Garden of Vilnius University. The standard methods were used [11]. All reagents were obtained from Sigma.

SOD assay. Superoxide dismutase (SOD, EC 1.15.1.1) electrophoretic fractions were analyzed from 2nd and 3d leaves of intact individual plants classified into groups based on chlorophyll morphosis type [3]. Activity of SOD isozymes was detected in gels by NBT reduction method [12]. SOD isoforms identification was according to Samis et al. [13].

Concentration of pigments was determined according to Inskeep and Bloom [3, 14]. Pigment extraction with N,N-dimethylformamide allows to determine not only chlorophylls *a* and *b*, carotinoids, but also protochlorophyll, which is important for callus cultures.

Determination of metal concentration was made by atomic absorption spectrometry (AAS, model DFS-

13-A) at the Institute of Geology and Geography (Vilnius) [4].

Statistical analysis. The mean values ±SD are given in figures and Table. The significance of differences between the means was analyzed by Student's t test.

RESULTS AND DISCUSSION

Polymorphism of response of the *Vicia faba* individual plants to Co^{2+} excess is an undoubted, well reproducible phenomenon. It was observed in many experiments with that plant species [3–5, 10, 15]. However, variations in the frequency of affected plants has been also observed. Such variations were observed also in the three separate experiments discussed in the present work (Table). According to plant injurity extent, the plants were divided into several groups from normally green (NG), the most tolerant to 7.5 mM $Co(NO_3)_2$, plants to those Co-affected to various extent: brightened green (BG), yellowish green (YG), greenish yellow (GY) and yellow (Y) – the stringently Co-affected plants. Different mosaics were also observed, but only equally colored plants were analyzed for leaf pigments and SOD isoforms.

Plant injurity extent is in full agreement with chlorophyll *a*, *b* and sum (*a*+*b*) concentration in leaves (Table). This conclusion fully agrees with results of the previous investigations [3, 4, 15]. However, in previous works we were unable to show differences between callus cultures from Co-untreated and Co-treated plants [15]. The use of N,N-dimethylformamide as a solvent for leaf pigments was very effective. In the previous work [15], in calli from green Co-untreated plants the sum *(a+b)* was only 0.045 ± 0.006–0.060 ± 0.006 mg g^{-1} fresh material, while in the present work in calli of the same origin it was 0.078 ± 0.016 mg g^{-1}, i.e. about two times higher. The slight difference observed even between calli arose from both normally green Co-untreated and Co-treated plants (Table).

More intriguing results were observed for protochlorophyll concentration. One of N,N-dimethylformamide advantages is extraction of protochlotrophyll [14]. Results of our work confirm this advantage of N,N-dimethylformamide as a solvent for leaf pigments.

Noticeable is a very strong prevalence of chlorophylls *a* and *b* in leaves of intact plants over protochlorophyll concentration (Table). However, in the most severely Co-affected yellow plants where the concentration of chlorophylls *(a+b)* was very decreased, the ratio of sum *(a+b)* to protochlorophyll was not so strongly contrasting. It was only in the range 1.5–2.7, while for the normally green Co-treated plants it was in the range 14.5–50.7. Several variations were also obvious for various experiments depending on environmental conditions.

In callus cultures the relative concentration of protochlorophyll is significant. The ratio of sum *(a+b)* to

Table. **Chlorophyll concentration in leaves of intact *Vicia faba* plants and callus from plants treated with 7.5 mM $Co(NO_3)_2$**

Leaf phenotype	n[1]	Concentration of pigments, mg g^{-1} fresh material				Ratio	
		Chlorophyll *a*	Chlorophyll *b*	Sum *a+b*	Protochlorophyll	*a/b*	Sum *a+b*/ proto-chlorophyll
Experiment I							
0–green	11	0.882 ± 0.031	0.502 ± 0.021	1.384 ± 0.047	0.071 ± 0.010	1.771 ± 0.057	19.5
Co-green	10	0.885 ± 0.039	0.500 ± 0.024	1.385 ± 0.062	0.075 ± 0.003	1.778 ± 0.029	18.5
Co-yellow	10	0.010 ± 0.002	0.010 ± 0.002	0.020 ± 0.004	0.013 ± 0.002	1.000 ± 0.182	1.5
Experiment II							
0-green	10	0.771 ± 0.079	0.413 ± 0.042	1.184 ± 0.120	0.062 ± 0.007	1.870 ± 0.027	19.1
Co-green	11	0.889 ± 0.065	0.453 ± 0.028	1.254 ± 0.097	0.086 ± 0.031	1.838 ± 0.027	14.6
Co-yellow	10	0.023 ± 0.007	0.015 ± 0.002	0.028 ± 0.006	0.019 ± 0.002	1.279 ± 0.184	1.5
Experiment III							
0-green	10	0.956 ± 0.064	0.597 ± 0.053	1.553 ± 0.115	0.067 ± 0.042	1.640 ± 0.059	23.2
Co-green	10	0.933 ± 0.029	0.536 ± 0.021	1.469 ± 0.048	0.029 ± 0.008	1.748 ± 0.038	50.7
Co-yellow	13	0.024 ± 0.004	0.015 ± 0.002	0.038 ± 0.006	0.014 ± 0.002	1.798 ± 0.334	2.7
Callus							
0-green	5	0.038 ± 0.008	0.040 ± 0.009	0.078 ± 0.016	0.032 ± 0.010	0.967 ± 0.037	2.4
Co-GY[2]	4	0.021 ± 0.003	0.026 ± 0.004	0.047 ± 0.006	0.018 ± 0.002	0.815 ± 0.087	2.6

[1] n – number of tested plants;
[2] – Co-GY – greenish yellow = initial phenotype of plants from which transplants for callus culture were taken.

Fig. 1. Regenerants from *Vicia faba* plants differently affected by Co^{2+} excess. NG – normally green, Y – yellow, C – control

photochlorophyll for calli from normally green plants was only 2.4–2.6, while even in leaves of intact Co-untreated green plants that ratio was in the range 19.1–23.2 (Table).

Regenerants from Co-affected plants were normally green (Fig. 1). This result partially agrees with our previous work [4] and shows that the preliminary pigmentation state disappears in regenerants. It is not inherited by the cloning of plants. This question needs further investigations on a larger material.

In leaves of intact plants the three SOD isoforms, like in the previous work [3], were also observed. It alleviated significantly the identification of SOD isoforms and isozymes and the interpretation of the electrophoregrams (Fig. 2): the most heavy electrophoretic fraction (85 kDa) was MnSOD (Fig. 2, N 3) located in the matrix of mitochondria and peroxysomes; the lighter (48 kDa) Fe SOD (Fig. 2, N 2) was located in chloroplasts [13, 16, 17]. The most polymorphic was Cu/Zn SOD presented by three main isozymes [3, 17] (Fig. 2, N 1a, 1b, 1c; mol. weight 1c-32 kDa) and located in the cytosol and chloroplasts. In leaves of yellow plants, additional Cu/Zn SOD isoforms appeared (Fig. 2, 26Y). Additional bands were also observed for Mn SOD (Fig. 2, 25Y). In callus cultures, more monotonous electrophoregrams were observed despite the initial plant characteristics, and Cu/Zn SOD was less polymorphic (Fig. 2).

The effects observed in the present work on plant pigments and SOD polymorphism may be due not only to a significantly higher accumulation of cobalt in the leaves of Co-treated plants [4]. The concentration on several other metals increased also in the leaves of Co-affected yellowish green (YG) and yellow (Y) plants in comparison to normally green plants after seed-treatment with 7.5 mM $Co(NO_3)_2$. In the previous work [4] we

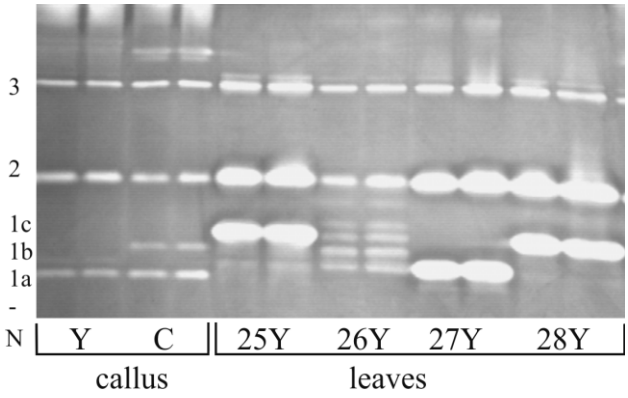

Fig. 2. Polymorphism of *Vicia faba* plants according to SOD spectra. Comparison of intact plants with callus cultures. Y – yellow, C – control, N – number of fraction, 25–28 – number of plants

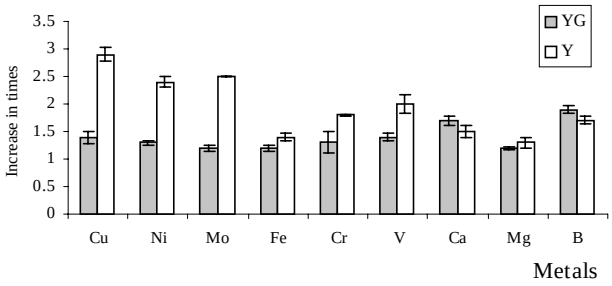

Fig. 3. Accumulation of various metals in leaves of Co-affected *Vicia faba* plants: YG- yellowish green; Y – yellow; I, II, III – metal groups (see in text)

distinguished two groups of metals in this respect. An analysis of the Co-treated material (Fig. 3) shows that even four groups of metals may be distinguished, three of them being for metals whose concentration increased in leaves of Co-affected plants. In Fig. 3 it is shown in comparison to unaffected normally green plants. Only for metals of the first group (Cu, Ni, Mo and, of course, Co) an increase of metal concentration proportionally depended on the Co-injurity extent of *V. faba* plants; to the second group (Fe, Cr, V) there were attributed metals whose accumulation depended not so clearly on the plant injurity extent. The third group (Ca, Mg, B) comprised elements whose concentration increased after seed treatment with 7.5 mM $Co(NO_3)_2$, but the effect did not depend on the plant injurity extent. The fourth group (Zn, Mg, Ba, Pb, Sr, did not shown in Fig. 3) consisted of metals whose concentration in Co-treated plants did not chang or even decreased.

In conclusion, differences among differently Co-affected plants and their comparison with callus cultures or regenerants offer a promising tool for the further investigations.

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Vicia faba AUGALŲ ATSAKAS Į KOBALTO PERTEKLIŲ: INTAKTINIŲ AUGALŲ IR KALIAUS PALYGINIMAS

Santrauka

Unikalus reiškinys stebimas pupų (*Vicia faba* L.) sėklas paveikus augalų stresą sukeliančiomis kobalto koncentracijomis. Išryškėja individualus augalų polimorfizmas, kuris pasireiškia įvairiomis chlorofilinėmis morfozėmis. Ryškūs skirtumai tarp šių morfozių nustatyti pagal chlorofilų kiekį, superoksido dismutazės (SOD) izozimus, įvairių metalų sankaupas lapuose. Regenerantuose skirtumai tarp pradinių augalų išnyko. Kaliaus kultūrose pastebėti skirtumai pagal chlorofilų, ypač protochlorofilo, koncentraciją ir SOD izospektrus.