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Variations in progeny of *Vicia faba* plants differently affected by cobalt

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Co^{2+} excess in *Vicia faba* induced not only chlorophyll morphoses in M_1, but also significanlty increased pigmentation variations in M_2. The effect depended on plant injury extent expressed by chlorophyll morphosis phenotypes in M_1. Superoxide dismutase (SOD) isozyme investigation showed that new types of SOD profiles arose among M_2 altered plants. This observation was associated mainly with FeSOD. The observed phenomena need futher investigations.

Key words: Co-stress, variation in M_2 generation, chlorophyll morphoses, SOD polymorphism

INTRODUCTION

Chlorosis induction is one of the common characteristics of metal stress [1]. However, only Co^{2+} treatment of *Vicia faba* seeds, from 17 metal ions in nitrate and chloride form, induces colour variations which are very similar to chlorophyll mutations. Progeny analysis of such plants has shown that colour variations are uninherited and attributed to chlorophyll morphoses [2, 3]. High individual polymorphism in chlorophyll morphosis expression is the second peculiarity of Co^{2+} effect on *V. faba* seeds [4].

The third peculiarity is that chlorophyll morphosis induction by seed treatment with Co^{2+} is restricted only by several Fabaceae species: *Vicia sativa* L., *Pisum sativum* L., *Lens culinaris* Medik. However, no morphoses are observed in other species of Fabaceae, such as *Lupinus luteus* L., *Glycine hispida* Maxim., *Ornithopus sativus* Brot., *Onobrychis viciifolia* Scop., as well as in plants attributed to other plant families (*Hordeum vulgare* L., *Nicotiana tabacum* L. and *N. rustica* L., *Lycopersicon esculentum* Mill., *Sinapis alba* L., *Brassica napus* L. ssp. *Napus*) [5].

Investigations of the mutagenic effect of Co^{2+} have been renewed, and it has been shown that cobalt increases the level of variations in M_2 and the frequency of plant colour alterations. Such variations were more frequent among progenies of chlorophyll morphoses observed in M_1. Actually, most of pigmentation variations disappeared in the next, M_3–M_5, generations [5].

The temporary character of pigmentation variations observed in plant progenies allows us to suppose the epigenetic character of pigmentation variations. This supposition is supported by the fact that Co^{2+} and several other metals could be actually involved in epigenetic phenomena [6, 7]. Investigation of plants after de-etiolation and especially regenerants from Co^{2+}-induced chlorophyll morphoses shows also a stable character of Co^{2+}-induced alterations of plant pigmentation. However, somatoclonal variation of regenerants has also been determined, and it complicates the interpretation of the results [4].

A feature of epimutations is a relatively high reproducibility of the observed phenomenon. For this reason, it the present work in the plant progenies M_2 were studied according to Co^{2+}-induced plant phenotypes in M_1, and our previous conclusion about the higher level of plant coloration variations among descendants of chlorophyll morphosis plants has been confirmed.

On the other hand, investigation of the mutagenic effect of Co^{2+} on visible mutations of plants is of interest.

MATERIALS AND METHODS

Seed material of field bean *cv.* 'Aušra' was obtained from the Lithuanian Institute of Agriculture (Dotnuva).

Morphosis induction and treatment of plants in M_1. Seeds of field bean were soaked for 15 h in 2.5 mM solution of $Co(NO_3)_2$ (Sigma) and of control seeds in bidistiled water. All unswollen seeds were removed. The plants were grown in the experimental field of Botanical Garden of Vilnius University. Morphosis types were determined one month

after seed soaking in $Co(NO_3)_2$ solutions. The plants were marked according to the phenotype. For progeny analysis, three phenotypic groups, normally green (NG), yellowish green (YG) and greenish yellow (GY), were fixed. The most strongly affected yellow plants comprised a too small group of harvested plants for statistical analysis. Plants from different phenotypic groups were harvested separately.

M_2 analysis was made in the experimental greenhouse of Department of Botany and Genetics in two passages. Results were evaluated 38 days after seed sowing. The dates of sowing were 30 September 2003 (for the 1st passage) and 13 November 2003 (for the 2nd passage). In M_2, only plant pigmentation variations were fixed in the progenies of different in M_1 phenotypic groups. The frequencies of different plant phenotypes were determined in M_2 separately.

Superoxide dismutase (SOD) assay. In several plants of altered pigmentation in M_2, SOD (EC 1.15.1.1) electrophoretic fractions were analyzed. Material from the 2nd leaf of individual plants was analyzed. Fresh leaf tissue (1 g) was homogenized in 1 ml of chilled 50 mM potassium phosphate buffer, pH 7.8. The homogenate was centrifuged at 12,500 g for 15 min at 4 °C. The supernatant was used for non-denaturing PAGE.

Electrophoresis was conducted at 4 °C using a 4% stacking gel and 9% separating gel (at 200 V, 40 mA) for about 3 h. Tris-glycine was used as a running buffer (pH 8.3). Twenty microliters of the crude leaf extract were loaded into each gel lane. Activity of SOD isozymes was detected in gels by the NBT reduction method [8]. The gel was incubated for an hour at 37 °C in the dark. Molecular mass calibration of SOD isozymes was made in the previous work [1]. Identification of SOD isoforms was made according to Samis [9] and our previous work [1].

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

RESULTS AND DISCUSSION

Numerous experiments [4] show that 2.5 mM $Co(NO_3)_2$ is an almost borderline cobalt concentration for morphosis induction in the most heavily Co^{2+}-affected yellow plants, but it affects rather many plants of the other phenotypic groups of chlorophyll morphosis in M_1. This Co^{2+} concentration was chosen to escape a higher plant sterility of chlorophyll morphoses induced with 7.5–1.0 mM $Co(NO_3)_2$.

Table. **Relation between chlorophyll morphosis induction in M_1 and frequency of plant pigmentation variations in M_2 of *Vicia faba* plants treated in M_1 with 2.5 mM $Co(NO_3)_2$**

Plant phenotype in M_1	n in M_2	Leaf colour phenotype of plants in M_2, %						
		green	various mosaics	chlorotic		with yellow borders	yellow	total
				full plant	with normally green veins			
1st passage								
NG(0)	233	82.4 ± 2.5	9.0 ± 1.9	8.6 ± 1.8	0	0	0	17.6 ± 2.5
NG(Co)	317	80.4 ± 2.2	4.7 ± 1.2	12.9 ± 1.9	1.9 ± 0.8[1]	0	0	19.6 ± 2.2
YG(Co)	659	55.2 ± 1.9[3]	31.0 ± 1.8[3]	11.1 ± 1.2	0	2.7 ± 0.6[3]	0	44.8 ± 1.9[3]
GY(Co)	711	45.6 ± 1.9[3]	37.1 ± 1.8[3]	17.3 ± 1.4[3]	0	0	0	54.4 ± 1.9[3]
Total(Co)	1687	55.9 ± 1.2[3]	28.6 ± 1.1[3]	14.0 ± 0.8[2]	0.4 ± 0.1[1]	1.1 ± 0.3[3]	0	44.1 ± 1.2[3]
2nd passage								
NG(0)	418	87.1 ± 1.6	2.6 ± 0.8	10.3 ± 1.5	0	0	0	12.9 ± 1.6
NG(Co)	374	82.4 ± 2.0	2.7 ± 0.8	15.0 ± 1.8[1]	0	0	0	17.6 ± 2.0
YG(Co)	892	73.2 ± 1.5[3]	1.2 ± 0.4	25.6 ± 1.5[3]	0	0	0	26.8 ± 1.5[3]
GY(Co)	268	60.8 ± 3.0[3]	4.1 ± 1.2	34.0 ± 2.9[3]	0	0	1.1 ± 0.6	39.2 ± 3.0[3]
Total(Co)	1534	73.3 ± 1.1[3]	2.1 ± 0.4	24.4 ± 1.1[3]	0	0	0.2 ± 0.1	26.7 ± 1.1[3]
Sum of 1st and 2nd passages								
NG(0)	651	85.4 ± 1.4	4.9 ± 0.8	9.7 ± 1.2	0	0	0	14.6 ± 1.4
NG(Co)	691	81.5 ± 1.5	3.6 ± 0.7	14.0 ± 1.3[1]	0.9 ± 0.4[1]	0	0	18.5 ± 1.5
YG(Co)	1551	65.6 ± 1.5[3]	13.9 ± 0.9[3]	19.4 ± 1.0[3]	0	1.02 ± 0.3[3]	0	34.4 ± 1.2[3]
GY(Co)	979	49.7 ± 1.6[3]	28.1 ± 1.4[3]	21.9 ± 1.3[3]	0	0	0.3 ± 0.2	50.3 ± 1.6[3]
Total(Co)	3221	64.2 ± 0.8[3]	16.0 ± 0.6[3]	19.0 ± 0.7[3]	0.2 ± 0.1[1]	0.6 ± 0.1[3]	0.1 ± 0.1	35.8 ± 0.8[3]

n – number of tested plants; NG(0) – normally green Co-untreated plants (control); phenotypes of plants in M_1 treated with Co^{2+}: NG(Co) – normally green; YG(Co) – yellowish green; GY (Co) – greenish yellow; 1 – $P < 0.05$; 2 – $P < 0.01$; 3 – $P < 0.001$ in comparison to normally green Co-untreated [NG(0)] plants

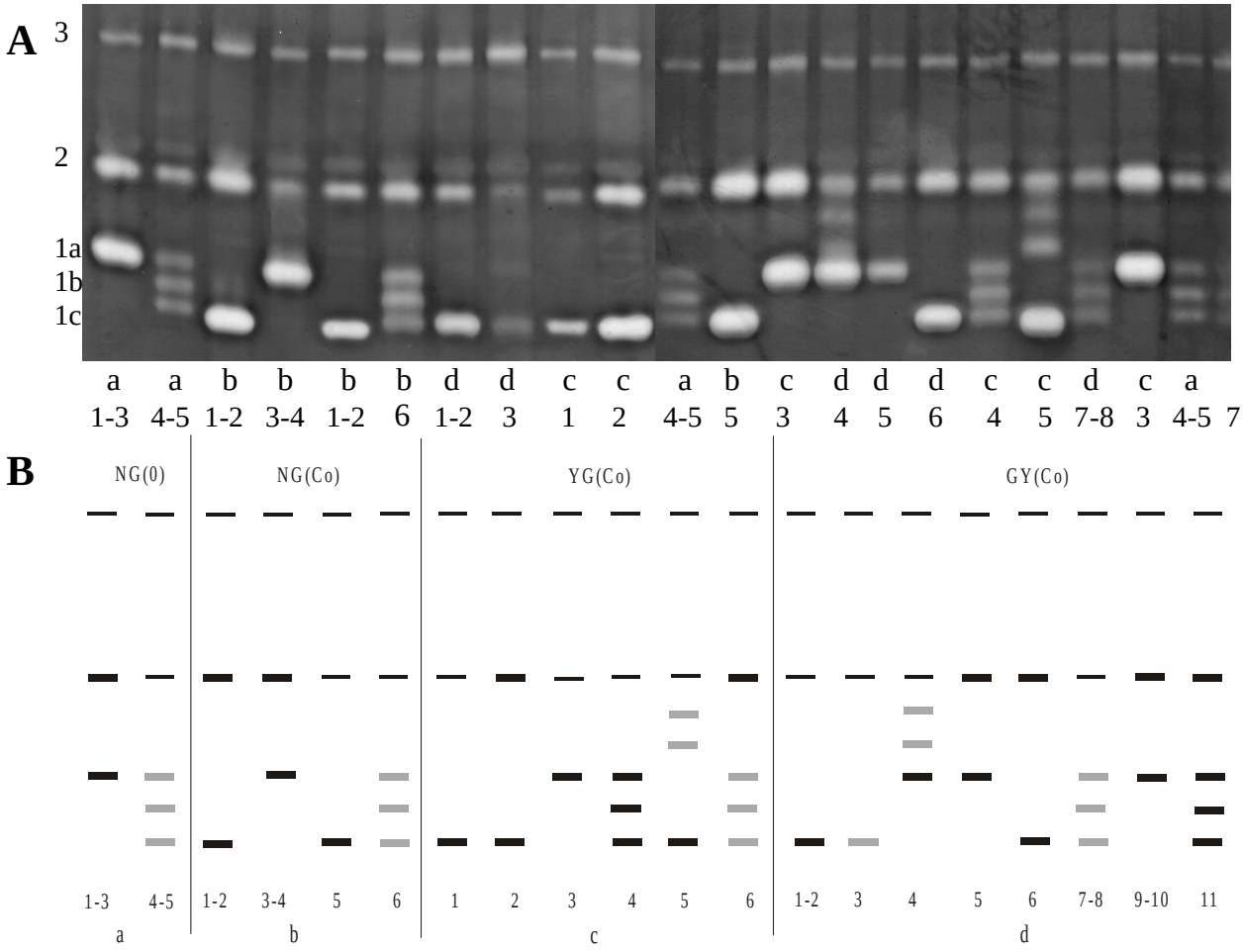

Figure. SOD isozyme profiles from *Vicia faba* plants differently affected in M_1 by Co^{2+} excess and of various phenotypes in M_2:
A – SOD electrophoretic profiles. On the left – SOD isoforms: 1a, 1b, 1c – Cu/ZnSOD isozymes; 2 – FeSOD; 3 – MnSOD; in the horizontal line – characteristics of tested plants in M_1: a – NG(0) – untreated normally green ; b – NG(Co) – Co-treated normally green; c – YG(Co) – yellowish green; d – GY(Co) – greenish yellow.
The number of plants in the group and in definite profile.
B – plants grouped according phenotype in M_1. Designations are the same as in A. The gray colour means the lesser expression of isozyme.

Induction of chlorophyll morphosis in M_1 plants, their marking and the harvesting of seed material were made in the same field conditions. However, the M_2 generation was grown in a greenhouse, and technically it was impossible to test all the material for M_2 together; two separate passages were necessary.

Only progenies from three phenotype groups of chlorophyll morphosis were tested for variation frequency in M_2. The first group was from the most tolerant to Co^{2+} excess normally green (NG–Co) plants, and their progenies were compared with progenies of Co^{2+}-untreated, also normally green plants (NG-0). The second group comprised Co^{2+}-affected yellowish green (YG–Co), and the third group contained greenish yellow (GY–Co) plants in M_1 (Table).

Comparison of results from two passages is advantageous, because it shows that despite differences in environmental conditions which are impossible to escape in different passages, the general conclusions are common for both passages.

Only plant colour variations were examined in a greenhouse. Two main types of colour variations, mosaic or fully chlorotic (brightened green) plants, were fixed. Other types of pigmentation variation in M_2 were rare, and their distribution was irregular among the test plant groups.

A significant level of both main variation types (mosaic and fully chlorotic plants) was fixed also among control, in M_1 Co^{2+}-untreated plants. Descendants from Co^{2+}-treated normally green (NG) plants did not differ significantly from control NG plants in both variation types. Only in the 2nd passage the frequency of fully chlorotic plants was noticeably higher in comparison to descendants from NG but Co^{2+}-untreated plants.

Environmental conditions contributed to the frequency of both main types of variations. In the se-

cond passage, mosaic plants were relatively rare in all test groups, and no noteable differences among plants of different origin according to that variation type were observed, while in the first passage the increase of mosaic frequency among descendants of YG and GY plants was obvious. On the other hand, the differences in the frequency of fully chlorotic plants were more pronounced only in the second passage.

Two values express very well the variation dependence in M_2 on plant characteristics in M_1. Those values are the dynamic change of normally green plants or, *vice versa*, of the sum of all variations in M_2 depending on plant phenotype in M_1 (Table). In M_2 the summarized frequency of altered plants after Co^{2+} treatment in M_1 was 2.4 times higher (t = 13.1) than in the progeny of Co^{2+}-untreated plants. The results of plant progenies from M_1 differently Co^{2+}-injured plants may be expressed in the following order of pigmentation alterations observed in M_2: 14.6% NG(0) < 18.5% NG(Co) < 34.5% YG(Co) < 50.3% GY(Co). The level of coloration changes was clearly dependent on the extent of plant injurity by Co^{2+} in M_1.

The progeny analysis of altered plants was not carried out and a high frequency of pigmentation variations was observed in the progeny of control plants in greenhouse conditions, nevertheless the permanent action of Co^{2+} stress on plant progeny was obvious. We suppose that although the main part of alterations disappear in the next generations as it was observed in previous work [5], *V. faba* pigmentation variations in M_2 are a reproducible phenomenon, and it is promising to examine the nature of altered plants. It is our nearest task in future.

Very useful markers for *Vicia faba* plant polymorphism and for expression of different reactions of *V. faba* to Co^{2+} ions are superoxide dismutase (SOD) isozyme spectra [1]. The differences among the plants are mainly defined by Cu/ZnSOD. According to them three main groups of plants were established [1].

The same main three groups of SOD-plant types have also been fixed among plants in M_2 differentiated into groups according to plant phenotypes in M_1. In M_2 those plants had various phenotypes (Figure). Attracts attention also plant polymorphism in the FeSOD expression. It was not so clear in our previous work [1]. This form of plant polymorphism was determined in M_1 phenotypic groups of Co^{2+}-affected plants. Such profiles have two chlorotic in M_2 plants. Interestingly, FeSOD is localized in chloroplasts while Cu/ZnSOD isozymes are localized mainly in the cytosol, but also in chloroplast [9–11]. It shows that examination of different phenotype plants can be more informative about plant polymorphism according to SOD profiles, and several relations between plant pigmentation variations and FeSOD may take place. It is perspective to carry out more extensive investigations of SOD profiles in Co^{2+}-induced plant variations.

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T. Èësnienë, V. Kleizaitë, V. Ranèelis

SKIRTINGAI PAÞEISTØ KOBALTU *VICIA FABA* AUGALØ KITOS KARTOS KINTAMUMAS

Santrauka

Co^{2+} perteklius *Vicia faba* sukelia M_1 ne tik chlorofilines morfozes, bet ir padidina augalø pigmentacijos pokyèiø daþná M_2. Ðis efektas priklauso nuo augalø paþeidimo stiprumo M_1. Superoksido dismutazës (SOD) izozimø tyrimais nustatyta, kad tarp M_2 pakitusiø augalø atsiranda nauji SOD profiliai. Ðis reiðkinys susijæs su FeSOD. Perspektyvu toliau tirti pastebëtus reiðkinius.