Plant chlorophyll morphoses induced by $Co(NO_3)_2$. 2. Chlorophyll content in leaves of intact plants and callus of *Vicia faba*

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The frequency and phenotypical expression of chlorophyll morphoses induced by $Co(NO_3)_2$ in *Vicia faba* are in direct proportion to inductor dose. Despite the fact that chlorophyll decreased very considerably and in general proportionally to plant phenotype, in several plants its content was in disproportion to plant phenotype, determined visually from the plant's colour. An analogous situation was observed with callus from normally green and yellow plants. In both cases the brownish green pigmentation of callus was only optical illusion from behind the other pigments, because in callus only traces of chlorophyll were determined.

Key words: cobalt genotoxicity, chlorophyll morphoses, cobalt induction, *Vicia faba*, chlorophyll content, callus

The significance of metal ions in the life of the plant depends not only on the kind of metal, but also on a plant species, and many other factors such as soil characteristics, stage of plant development, climate conditions take part in it. Cobalt is not an excepion from that rule. Cobalt deficiency is unknown in plants, but Co^{2+} is necessary for nitrogen fixing bacteria in legumes, it is used as a minor ingredient of media for meristematic plant cell cultures. On the other hand, in solution cobalt can be toxic to plants in quantities greater than 0.1 ppm, but there are resistant plants – hyperaccumulators of the cobalt ions [1–5].

Different reaction of various plants to cobalt ions was also confirmed by analysis of morphosis-inducing capacity. Co^{2+} induced severe chlorosis of bush beans (*Phaseolus vulgaris* L. cv. 'Improved Tendergreen') as did titanium but not vanadium, silver or chromium ions. The effect was noticed in solution culture of bush beans [2]. In horse-bean (*Vicia faba* L.) another type of pigmentation alterations was induced by $Co(NO_3)_2$. There were phenocopies to chlorophyll mutations [6–8]. Such effect was induced only by $Co(NO_3)_2$ from 17 metal ions tested. The effect was observed only after seed soaking in $Co(NO_3)_2$ solutions [9].

Chlorophyll morphoses appeared also after such treatment of pea (*Pisum sativum* L., vetch (*Vicia sativa* L.) and lens (*Lens culinaris* Medik.) (unpubl. data) seeds. In pea, a severe reduction of seedling and leaf size was observed additionally [8].

All these plants belonging to the family *Fabaceae*. However, in the same conditions of plant treatment chlorophyll morphoses were absent in lupine (*Lupinus luteus* L.), soya bean (*Glycine hispida* Maxim.), birds'foot (*Ornithopus sativus* Brot.), sainfoin (*Onobrychis viciifolia* Scop.). No induction of chlorophyll morphosis was observed in the plants belonging to other families such as barley (*Hordeum distichon* L.), tobacco (*Nicotiana tabacum* L., *N. rustica* L.), tomato (*Lycopersicon esculentum* Mill.), mustard (*Sinapis alba* L.), rape (*Brassica napus* L. ssp. *napus*) [8].

Polymorphism of individual of horse bean and pea plants was also noticed. In horse beans that phenomenon was expressed more clearly, and plants were divided on the basis of reaction to $Co(NO_3)_2$ into several groups, but the division was based on the phenotype. Analysis of plant pigments was not performed, although the frequency of the various groups of morphoses may also be important in cobalt genotoxicity assessment [6–8].

In the present work, frequency studies of various phenotypical groups of morphosis were accompanied by chlorophyll assessment. Chlorophyll was also determined in callus from leaves of plants differently affected by Co^{2+}.

MATERIALS AND METHODS

Seed material of horse bean cv. 'Aušra', 'Ada', 'Ukko', 'Scirocco 16' was obtained from the Lithuanian Institute of Agriculture (Dotnuva).

Morphosis induction. Several experiments were made in the current work. The seeds were soaked for 15 h in solutions of various or definite concentrations of $Co(NO_3)_2$ (Sigma). All unswollen seeds were removed. The plants were planted in an experimental field of the Botanical Garden of Vilnius University or in a greenhouse of the Department of Botany and Genetics in autumn–winter time. In the greenhouse additional illumination was used. Morphosis types were determined one month following seed soaking in $Co(NO_3)_2$ solutions.

The **degree of morphosis** was expressed in an increasing order: phenotypically normal plants; brightened green plants; all leaves yellowish, but green to yellow prevail; all leaves yellowish, but yellow to green prevail; all leaves yellow; various variegated types: slightly brightened with the altered shape of leaf; only part of leaf yellow; only upper leaves yellow; only part of lower leaves yellow, upper leaves yellow; very strongly affected plants: very small, dying, without developed leaves.

For **chlorophyll analysis** in intact plants, two separate experiments were carried out – in field and in greenhouse conditions. Leaves of untreated plants (seeds soaked in distilled water) or after treatment with $0.75 \cdot 10^{-2}$M solution of $Co(NO_3)_2$ were taken fully developed, the second and third from the upper part of a plant. For calculation of pigments per leaf area, the maximal number of leaf rollers (0.4 cm in diameter) was taken from both leaf plates. Chlorophyll content was determined by a standard method with a spectrophotometer at 663 and 644 nm [10]. Special acetone (Sigma) was used for spectrophotometer analysis.

Callus cultivation and evaluation. In a separate experiment, leaf segments (0.36 cm^2) from green untreated plants or green and yellow plants after treatment with $0.5 \cdot 10^{-2}$ M $Co(NO_3)_2$ were used for callus induction. The position of leaves on the plant was the same as for chlorophyll determination. Three separate passages for callus induction were carried out. All manipulations were standard for callus culture [11, 12]. Material was washed in flowing water for 0.5 h, then sterilized for 5 min with Hg_2Cl_2 (Riede-de Haën AG) and washed 3 times for 5 min in distilled sterilized water. Basic Murashige–Skoog media [13] were used (with 2.4-D 4.52 μM added). For determination of explant mass, 50 leaf segments (0.36 cm^2 each) were weighed. The intensity of callus growth was determined according to Frank et al [14] formula $W = (W_t - W_0) / W_0$, where W is the intensity of callus growth, W_0 is the mass of explant, W_t is the common callus+explant mass on the day of determination.

RESULTS AND DISCUSSION

Several experiments conducted in different years [6–8] and in the current work with different sources of $Co(NO_3)_2$ leave no doubt as to a specific action of cobalt ions on horse-bean pigmentation. The high frequency of the altered plants (Table 1) and examination of the progenies of the altered plants [6, 7] showed that the phenomenon must be attributed not to mutations but to morphoses.

A regular decreased of the frequency of normally green plants (Table 1) with increased concentration of $Co(NO_3)_2$ shows also that the phenomenon can be

Table 1. **Dependence of *Vicia faba* cv. 'Aušra' chlorophyll morphosis types and frequency (%) from concentrations of $Co(NO_3)_2$**

Leaf phenotype	$Co(NO_3)_2 \times 10^{-2}$M*							
	0		0.25×		0.5×		1.0×	
	n	%	n	%	n	%	n	%
Green	3433	100	2199	68.0 ± 0.8	1345	39.5 ± 0.8	675	27.0 ± 0.9
Fully altered plant:	0	0	661	20.4 ± 1.6	1489	43.8 ± 1.3	1655	66.1 ± 1.2
brightened green	0	0	82	2.5 ± 0.3	111	3.3 ± 0.3	48	1.9 ± 0.3
yellow	0	0	579	17.9 ± 0.7	1378	40.5 ± 0.8	1607	64.2 ± 1.0
Mosaics:	0	0	375	11.6 ± 0.2	570	16.8 ± 0.1	174	7.0 ± 0.6
yellow only a part of leaf(ves)	0	0	136	4.2 ± 0.4	240	7.1 ± 0.4	77	3.1 ± 0.3
yellow upper and green lower leaves	0	0	239	7.4 ± 0.7	330	9.7 ± 0.5	97	3.9 ± 0.4
Sum total of morphoses	0	0	1036	32.0 ± 0.8	2059	60.5 ± 0.8	1829	73.0 ± 0.9

*$P < 0.001$ to normally green untreated plants.

useful for quantitative evaluation of the negative effect of Co^{2+} ions in the surrounding of seeds of sensitive plants. This phenomenon is noted only after exposing seeds of the sensitive plants to $Co(NO_3)_2$ solutions on. So, seeds of *Vicia faba* and of other *Fabaceae* plants in which that phenomenon was noted [8] may be used as indicators for evaluation of the genotoxicity of Co^{2+} ions in the soils highly contaminated with this metal.

A regular decrease of normally green plants with an increased concentration of $Co(NO_3)_2$ was noticed in four different horse-bean cultivars (Figure). So, it is a common phenomenon for genetically different horse-bean material.

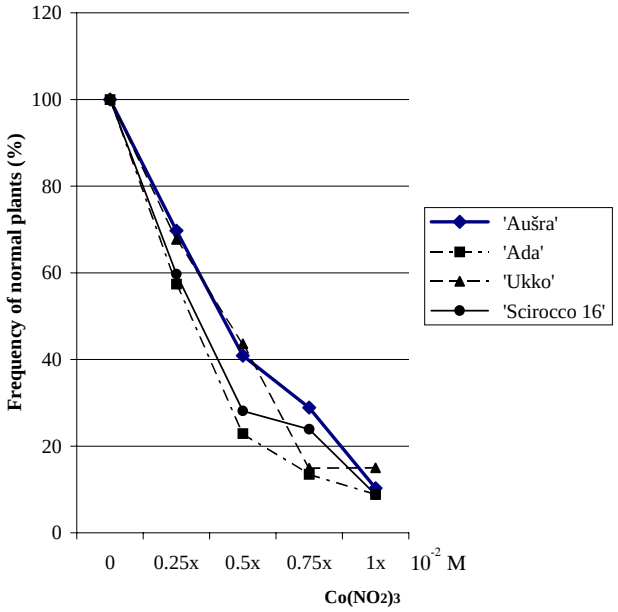

Figure. Comparison of the sensitivity of different *Vicia faba* cultivars according to a decreasing frequency of normally green plants

Relation of this phenomenon to the concentration of Co^{2+} ions was also determined by a regular increase of the degree of injured plants. In the present work it is reflected in the fully or partially yellowish plants (Table 1). Appearance of fully or partially yellow plants was observed only after treatment with $Co(NO_3)_2$ concentrations above $0.25 \cdot 10^{-2}$ M.

A quantitative determination of chlorophyll was carried out in plants grown in different conditions (field and greenhouse). Only fully and equally altered plants after treatment with $0.75 \cdot 10^{-2}$M $Co(NO_3)_2$ were used for chlorophyll analysis (Table 2).

As may be expected, plants of groups 0 and 4 differed very significantly. The chlorophyll content for green plants (group 0) was about 16 times as high as in yellow plants (belonging to group 4). Significant differences among the plants within the same phenotypical groups were also noted. However, variations in chlorophyll content were significant also among plants untreated with $Co(NO_3)_2$. Whether this variation is caused by different reaction of individual plants to $Co(NO_3)_2$ is an open question.

As regards the metal ions, destruction of plant pigments and chlorosis were observed for several metal ions on different plants. However, the effect was specific for both metal ion and plant species. So, Mn^{2+} induced chlorophyll loss and occurrence of visible symptoms in white birch (*Betula platyphylla* var. *japonica*) [15]. Chlorosis was induced also by Cu^{2+} in oregano (*Origanum vulgare* L. subsp. *Hirtum*) [16] and runner bean (*Phaseolus coccineus* L.) [17]. In the runner bean, photosystem I was inhibited and the plant injury was strongly dependent on the plant growth stage in which Cu^{2+} was added to the nutrient solution. Plants treated with Cu^{2+} in the initial stage of intensive leaf

Table 2. **Chlorophyll content in the leaves of chlorophyll *Vicia faba* cv. 'Aušra' by 0.75×10^{-2} $Co(NO_3)_2$**

Phenotype[a]	Frequency of morphoses, %		Number of plants for chlorophyll analysis		Variation of chlorophyll content mg/dm² min–max		Average chlorophyll content[b], mg/dm²		After correction in field conditions[b]
	in field	in greenhouse	in field	in greenhouse	in field	in greenhouse	in field	in greenhouse	
Control	0	0	17	7	2.77–6.86	3.49–7.77	4.60 ± 0.33	4.89 ± 0.57	–
0	20.3 ± 0.7	27.9 ± 1.5	18	7	2.72–9.14	2.90–5.75	4.31 ± 0.38	4.00 ± 0.41	–
1	19.2 ± 0.7	12.0 ± 0.6	5	6	1.66–2.91	0.87–2.92	2.45 ± 0.21*	1.90 ± 0.38*	–
2	20.8 ± 0.7	14.3 ± 0.8	5	7	0.80–1.88	0.29–1.91	1.22 ± 0.20**	1.18 ± 0.10**	1.08 ± 0.10**
3	11.9 ± 0.7	5.3 ± 0.3	8	8	0.18–0.92	0.20–1.13	0.46 ± 0.08**	0.56 ± 0.23**	0.67 ± 0.08**
4	4.5 ± 0.2	0.3 ± 0.02	17	–	0.10–0.99	–	0.42 ± 0.08**	–	0.21 ± 0.02**
Others	23.3 ± 0.8	40.2 ± 2.1	–	–	–	–	–	–	–

Correction only in groups 2, 3 and 4;
a) 0 – treated 'normal' green plants; 1–4 – degree of morphoses: 1 – brightened green leaves;
2 – yellowish leaves, but prevailing green to yellow; 3 – yellowish leaves, but prevailing yellow to green; 4 – all leaves yellow or even pale;
b) comparison of 0 with other phenotypical groups: * $P < 0.01$, ** $P < 0.001$.

growth showed a reduction in leaf area, while treatment at the end of the intensive growth stage of primary leaves showed chlorosis [17]. The same relation was observed in chlorophyll morphosis induction by Co^{2+} ions in our work with *Vicia faba* L.: the effect was induced only after treatment of seeds [6–8 and the present work]. However, Cu^{2+} ions had a little effect on photosynthetic system I and chlorophyll a in duckweed (*Lemna gibba* L.) [18] and no effect on chlorophyll in tobacco callus (*Nicotiana tabacum* L.) [19]. Chlorosis of *Silene vulgaris* (Moench) Garcke was associated with excess of Zn, but not of Cu [20].

Different effect of Co^{2+}, Ti^{2+}, Ag^{+}, Cr^{3+} was observed on bush bean (*Phaseolus vulgaris* L.). Co^{2+} induced severe chlorosis, Ti^{2+}-chlorosis, necrotic spots on leaves and stunting, while the other metal ions had no effect on the pigmentation of leaves [2]. Co^{2+} effectively decreased the content of total, a and b chlorophyll and reduced drastically the yield of tomato (*Lycopersicon esculentum Mill.*) [4]. Chlorophyll accumulation was also inhibited by Cs^{+} in barley [21].

However, in most of the works the question of the causes of chlorophyll content decrease remains open. Metal ions and other stress-inducing factors can block pigment synthesis or chlorophyll function or enhance chlorophyll degradation. The latter mechanism of the action was shown for several metal ions by R. Abdel-Basset et al. [22] in *Chlorella fusca* Beij. and *Kirchneriella lunaris* (Kirchn.) Schmidle algae. The chlorophyll-degrading enzyme, chlorophyllase, was enhanced to various extent by Cd^{2+}, Pb^{2+}, Mn^{2+}, Ni^{2+} and, what is important for the interpretation of our data, by Co^{2+}.

Plants were divided into phenotypical groups 0, 1, 2, 3 and 4 (Table 2) on the grounds of exterior appearance. Quantitative chlorophyll analysis allowed us to verify the attribution of the individual plants to these phenotypical groups.

Correction of the attribution was made on the basis of chlorophyll content (Table 2). The correction was significant only in the phenotypical group 3 and especially in group 4. Several plants, previously attributed to chlorophyll content group 4, could be transferred to group 3 or even to group 2 on the basis of chlorophyll content. For groups 0 and 1 no correction was necessary: the exterior attribution of the plants to those groups corresponded to the results of chlorophyll determination.

The discrepancy between the results concerning the exterior colour of several plants and the chlorophyll content may be explained by the contribution of other coloured compounds, not only chlorophyll, to the colour phenotype of a plant, and the levels of those compounds may be also decreased in plants strongly affected by Co^{2+} ions.

This suggestion is corroborated by chlorophyll analysis in meristematical cultures of leaf cells from the plants treated with $Co(NO_3)_2$ (Table 3). Despite the extremely low chlorophyll content in the callus cultures, they seem coloured. The colour is possibly produced by the phenolic compounds as the callus looks greenish brown.

Only one parameter attracted our attention: in callus cultures from yellow plants the chlorophyll a/b ratio decreased significantly (Table 3). The intensity of mass growth of callus from yellow plants was also significantly lower.

After correction of plant phenotype on the grounds of chlorophyll analysis, a phenomenon opposite to cal-

Table 3. **Chlorophyll content in callus from green and yellow *Vicia faba* cv. 'Aušra' plants treated by 0.5×10^{-2}M $Co(NO_3)_2$**

Growth duration[3], days	Initial plant[1]	n	Chlorophyll[2] content mg/fresh callus	Variation in chlorophyll content min-max	Chlorophyll a/b ratio[2]
73	0-green	25	0.045 ± 0.006	0.016–0.137	0.96 ± 0.07
	Co-green	25	0.050 ± 0.005	0.024–0.102	1.11 ± 0.06
	Co-yellow	25	0.061 ± 0.010	0.012–0.143	0.71 ± 0.06**
79	0-green	17	0.060 ± 0.006	0.025–0.093	0.93 ± 0.07
	Co-green	17	0.081 ± 0.014	0.026–0.131	0.83 ± 0.07
	Co-yellow	17	0.033 ± 0.006*	0.010–0.148	0.66 ± 0.07***
85	0-green	5	0.058 ± 0.025	0.008–0.312	0.78 ± 0.08
	Co-green	5	0.061 ± 0.009	0.012–0.167	0.80 ± 0.10
	Co-yellow	11	0.063 ± 0.012	0.013–0.412	0.68 ± 0.12
Average	0-green	47	0.052 ± 0.005	0.008–0.312	0.93 ± 0.05
	Co-green	47	0.063 ± 0.007	0.012–0.167	0.93 ± 0.05
	Co-yellow	53	0.051 ± 0.006	0.010–0.412	0.70 ± 0.05**

[1] Initial plants: 0 – green–untreated green plants; Co green – treated with $Co(NO_3)_2$ normally green plants; Co – yellow – treated with $Co(NO_3)_2$ yellow leaf plants;
[2] Comparison of Co-yellow with Co-green: $P < 0.01$; ** $P < 0.02$; *** $P < 0.001$. [3] Results from different passages.

lus culture was noted. Several plants had a higher chlorophyll content than could be suggested by their phenotype. In the phenotypical group of yellow seedlings (number 4) there were four plants which were transferred to the higher, more intensive phenotypical group 3 and, *vice versa*, only one plant from group 3 was transferred to group 4 as could be expected from the mimicking effect of phenolic or other coloured compounds. That phenomenon requires further studies.

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AUGALŲ CHLOROFILINĖS MORFOZĖS, INDUKUOTOS $CO(NO_3)_2$. 2. CHLOROFILO KIEKIS INTAKTYVIŲ *VICIA FABA* AUGALŲ LAPUOSE IR KALIUJE

S a n t r a u k a

Pupų chlorofilinių morfozių, kurias sukelia $Co(NO_3)_2$, dažnis ir raiška yra tiesiai proporcingos induktoriaus dozei. Nors chlorofilo kiekis pakitusiuose augaluose labai sumažėjęs ir yra proporcingas fenotipinei raiškai, ne visuose augaluose jo kiekis atitinka pagal lapų spalvą nustatomą fenotipą. Panašiai yra kaliuje iš normaliai žalių ir geltonų augalų. Rudai žalia jo pigmentacija yra apgaulinga dėl kitų pigmentų, nes chlorofilo kaliuje aptikti tik pėdsakai.