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Plant chlorophyll morphoses induced by $Co(NO_3)_2$ 3. Cysteine action on survival of *Vicia faba* plants and chlorophyll concentration in leaves

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Chlorophyll morphoses arise after exposure of *Vicia faba* L. seeds to Co^{2+} excess. Chlorophyll concentration is in agreement with the phenotypic expression of morphoses, and normally green plants are really tolerant to Co^{2+} excess. The protective effect of cysteine (Cys) depends on the order of exposure, Co–Cys or Cys–Co, and the 'saving' effect in the plants most sensitive to Co^{2+} is suggested on the ground of the comparative Cys effect on plant survival and chlorophyll concentration in leaves.

Key words: Co^{2+} genotoxicity, cysteine, saving effect, plant morphoses

INTRODUCTION

The evolution of acclimation of a plant to stress factors such as metal excess is an actual problem of plant genetics. Plants differ significantly in their tolerance to heavy metals, and plant species tolerant to metals are divided according to the mechanisms of the tolerance into two groups – those of excluders and hyperaccumulators. Excluders are able to restrict the root uptake and root-to-shoot translocation of heavy metals. Hyperaccumulators have the ability to accumulate large amounts of metals in their shoot tissues to levels by many orders higher that those found in soil, and without remarkable toxic effects on them [1–3]. However, hyperaccumulation itself increases the genotoxic hazard for a plant and is physiologically expensive. Additional mechanisms are necessary to provide for heavy metal transport to shoots, fast immobilization with phytohelatins, metallothioneins, histidine or other chelators, compartmentation into vacuoles, cell walls or trichomes [4–8].

Expression of a heavy metal stress in plants depends on thiol compounds [9–11]. Enhanced demand of cysteine is necessary under heavy metal treatment for the biosynthesis of cysteine-rich polypeptides involved in the plant defense mechanisms against the heavy metal stress. Activation of genes such as *cys-3A* gene coding the cytosolic O-acetylserine(thiol)lyase, which catalyzes the last step of L-cysteine biosynthesis, is induced by a heavy metal stress [9].

A real clue to the evolutional and adaptive fitness of metal hyperacumulation is an intraspecial polymorphism of the individual plants. In that respect the plant coloration variations induced by *Vicia faba* L. seed exposure to elevated concentrations of Co^{2+} is a very convenient test system. The high polymorphism of plants is reproducible and easily fixed, and can be fortified by chlorophyll determination in leaves of individual plants [12, 13].

In the present work, the effect of cysteine on the Co^{2+} stress conditions has been investigated using the *Vicia faba* polymorphism as a test-system. The effect of cysteine is expressed by its action on plant survival and chlorophyll concentration in leaves. Using N,N-dimethylformamide as a chlorophyll solvent allowed to determine chlorophyll *a* and *b* separately.

MATERIALS AND METHODS

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

Morphosis induction. Several experiments were done in the present work. Cysteine was used in equimolar concentrations of $Co(NO_3)_2$. Two conditions of cysteine addition were tested: pretreatment for 12 h with cysteine followed by seed soaking for 8 h in $Co(NO_3)_2$ solution or, *vice versa*, for 12 h in $Co(NO_3)_2$ solution followed by post-treatment of seeds for 8 h with an equimolar concentration of cysteine. For Co^{2+} exposure, seeds were soaked for

12 h in $Co(NO_3)_2$ and then for 8 h in bidistilled water. Control seeds were soaked for (12 + 8) h in bidistilled water. Between replacements from one solution to another, seeds were washed 3 times with bidistilled water.

All chemical reagents used in this work were from Sigma.

All unswollen seeds were removed. The plants were planted in a greenhouse of the Department of Botany and Genetics of Vilnius University. In the greenhouse, additional illumination was used. Morphosis types in plants were determined about one month following seed soaking.

The degree of morphosis was expressed in an increasing order: phenotypically normal green plants (NG); brightened green plants (BG); all leaves yellowish green and green to yellow prevail (YG); all leaves greenish yellow and yellow to green prevail (GY); all leaves yellow (Y); various variegates types: slightly brightened, with the altered shape of leaves; only part of a 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.

Chlorophyll was extracted from leaves of individual plants with 2 ml N,N-dimethylformamide in the dark for 48 h at 4 °C and measured using a spectrophotometer according to Inskeep and Bloom [14]. In all experiments the second and third fully developed leaves were used for analysis. Leaf segments 0.36 cm^2 for chlorophyll extraction were used to determine chlorophyll concentration per leaf area unit.

RESULTS AND DISCUSSION

To determine the reproducibility of the modifying action of cysteine on Co^{2+} effect on *Vicia fab*a plant morphosis induction and chlorophyll synthesis, three separate experiments were done in a greenhouse at different periods of the year: the 1st lasted from 4 April to 16 May; the 2nd from 23 September to 30 October the and 3rd from 3 to 29 November. In the 1st and 2nd experiments the same concentration of Co^{2+} and the equimolar concentration of cysteine were used (2.5 mM). In the 3rd experiment the test concentrations were 7.5 mM.

An important index of the action of the modifying factors on stress situations is the survival of plants. Despite of the high concentrations of Co^{2+} and cysteine used, a low level of plant lethality was observed. A stronger effect of Co^{2+} on seed germination was observed only in the 1st experiment. Even 7.5 mM Co^{2+} didn't decrease the germination capacity of seeds. The effect of cysteine was positive, but on the background of such low lethality of Co^{2+} it was not statistically significant (Table 1).

Table 1. **Variations of *Vicia faba* plant survival after interaction of Co^{2+} with cysteine**

Exposure	Plant number in separate experiments[1]			Survival in separate experiments, %		
	1st	2nd	3rd	1st	2nd	3rd
0	218	437	231	95.6 ± 1.4	92.0 ± 1.3	94.3 ± 1.5
Co	230	308	341	80.7 ± 2.6	91.9 ± 1.6	96.1 ± 1.1
Cys	250	307	310	89.9 ± 1.9	97.5 ± 1.0	96.9 ± 1.0
Co–Cys	204	312	305	72.9 ± 3.1	93.1 ± 1.4	95.3 ± 1.2
Cys–Co	232	301	333	86.6 ± 2.2	94.1 ± 1.4	95.1 ± 1.2

[1] In the 1st and 2 nd experiments the concentration of $Co(NO_3)_2$ and equimolar cysteine was 2.5 mM; in the 3rd experiment the concentration of $Co(NO_3)_2$ and cysteine was 7.5 mM.

On the ground of the results presented in Table 1, the 2nd experiment was taken as the basic. The results observed in that experiment show that the effect on lethality is more pronounced for the older, 37-d plants (Fig. 1). If in 16-d plants the Co^{2+} effect was slightly stimulating, in older plants the lethality exceeded 4%. The same effect was observed if cysteine was used after seed exposure to Co^{2+}. Pretreatment with cysteine increased the viability of plants after Co^{2+} exposure independently of the plant's age.

The low lethality of Co^{2+} seems to be a peculiarity of *Vicia faba*. Co^{2+} ions were the least toxic

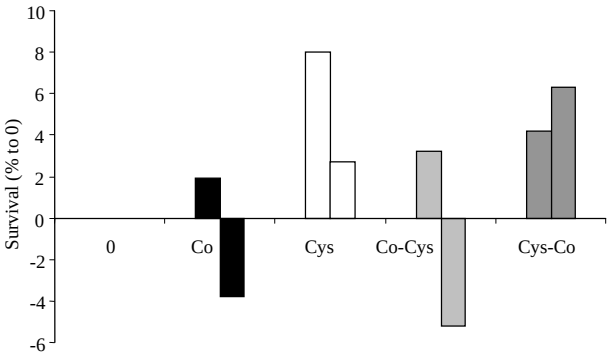

Fig. 1. Effect of cysteine on survival of *Vicia faba* plants exposed to 2.5 mM $Co(NO_3)_2$: deviation from control level; from the left the first column – 16-d plants after exposure to Co, Cys, Co–Cys or Cys–Co; the second column – 37-d plants after the same exposure

among the four metals (Co, Cd, Ni and Zn) tested [15].

Induction of plant colour morphoses is a highly reproducible effect after exposure of *V. faba* seeds to Co^{2+} excess [12, 13]. It was confirmed also in three separate experiments done in the present work. In all experiments, a polymorphism of plant reaction to Co^{2+} excess was noted. The plants can be divided into several phenotypic groups from normally green as the most Co-tolerant plants to yellow as the most sensitive to Co-exposure (Fig. 2).

In Fig. 3, the plant polymorphism is shown by dividing plants into two groups – normally green and with chlorophyll morphoses. The dependence on the exposure conditions is also evident, especially for the cysteine action. A contradictory effect was observed on the morphosis frequency after pretreatment with cysteine. In the first experiment Cys enhanced the effect of Co^{2+} by lowering the frequency of normally green plants, while in the second experiment a slight protective effect of cysteine was observed in the same order of cysteine usage.

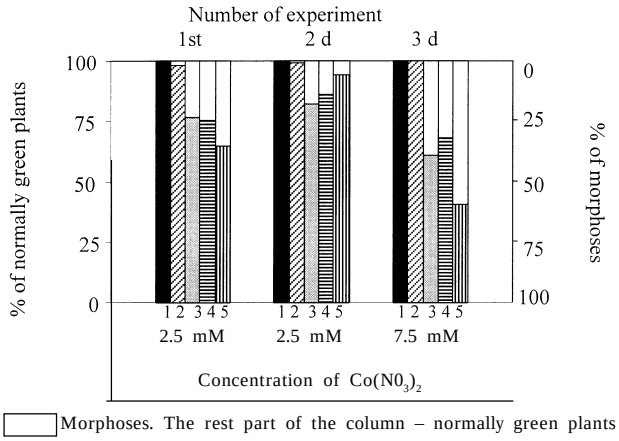

Fig. 3. Relation of *Vicia faba* morphoses to normal plants after exposure to: 1 – no exposure (control plants); 2 – Cys, 3 – Co, 4 – Co–Cys, 5 – Cys–Co

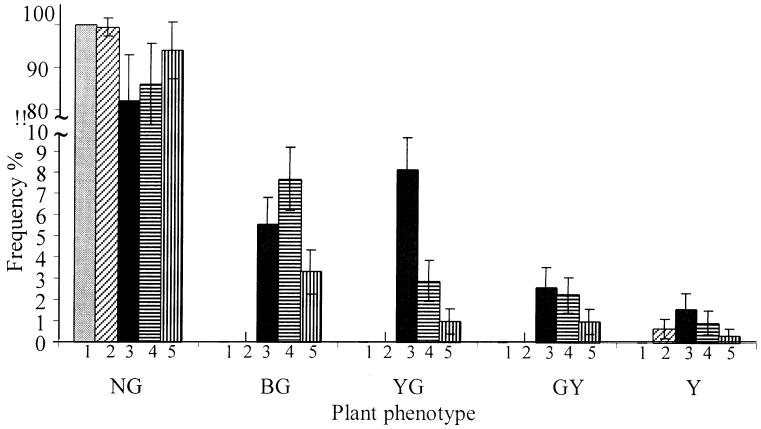

Fig. 2. Effect of cysteine on frequency of phenotypic groups of plant coloration morphoses induced with 2.5 mM $Co(NO_3)_2$: NG – normally green plants, BG – brightened green, YG – yellowish green, GY – greenish yellow, Y – yellow. 1 – control, Co – unexposed plants, 2 – Cys, 3 – Co^{2+}, 4 – Co–Cys, 5 – Cys–Co

The effect of cysteine on plant polymorphism is more evident after exposure of plants to a higher, 7.5 mM concentration of Co^{2+}. The effect of cysteine pretreatment on the frequency of morphosis induced with 7.5 mM Co^{2+} was unexpected. In such conditions, cysteine pretreatment increased the frequency of chlorophyll morphoses (Fig. 3).

However, on the ground of comparison of data presented in Figs. 1 and 4, we can hypothesise that pretreatment with cysteine increases the survival of plants most sensitive to Co exposure, which usually don't survive, but such 'saved' plants are still affected and display morphoses. So, formally cysteine pretreatment increases the relative part of morphoses.

In the experiment numbered as 2nd, the Co effect was significantly slighter than in the 3rd experiment. So, the part of 'saved' plants turned into a group of the phenotypically normal plants. It is in agreement with a decrease of the frequency of plants in all morphosis groups and an increase of the number of normally green plants (Fig. 2).

Determination of chlorophyll in plants is a subject of prolonged discussion. The classical method of Arnon [16] has been subjected to a severe criticism [17]. Two moments of that method the target of criticism: acetone as the solvent for chlorophyll extraction and the formula for calculation of chlorophylls *a* and *b*. In the previous work [13] we also used acetone as a solvent. In agreement with the critical comment, in the present work as a solvent N,N-dimethylformamide and the respective formula have been used. It allowed a more exact determination of chlorophyll *a* and *b* separately and calculation of chlorophyll *a/b* ratio (Table 2). This ratio can show on which photosystem, PSI or PSII, the stress factors [19] (as also Co^{2+} excess) exert their action.

If to compare the normally green plants exposed to Co, Cys, Co–Cys or Cys–Co in various experimental conditions, statistically significant differences among these plants in their *a/b* ratio were not observed.

It is more important to compare the total chlorophyll concentration in the normally green control, Co-unexposed plants with plants exposed to Co, Cys, Co–Cys and Cys–Co. Analysis of the sum of chlo-

rophylls *a* + *b* shows that these plants are really normal in chlorophyll content as determined only from the external phenotypical appearance of the plants.

The same result was noted in our pervious work [13] with the green plants that remained after exposure to 7.5 mM $Co(NO_3)_2$. So, it can be concluded that the normally green plants remaining after exposure to Co excess are really the plants most tolerant to Co^{2+}, and *Vicia faba* shows a high polymorphism to Co^{2+} excess.

An attractive fact is that in the normally green plants after exposure to Co–Cys or, *vice versa*, Cys–Co the total chlorophyll concentration in leaves is higher than in normally green plants without any exposure or after exposure to Co^{2+} or Cys alone (Table 2 and Fig. 4). However, such effect was observed only in one (2nd) experiment. The effect may

Table 2. **Cysteine effect on chlorophyll concentration (μg/cm²) in leaves of *Vicia faba* plants differently sensitive to Co^{2+} excess**

Exposure conditions	Phenotype of plants[4]	Number of plants	Chlorophyll			Ratio *a/b*
			a	*b*	Sum *a* + *b*	
1st experiment, plants 25 days old						
0	NG	10	26.2 ± 1.8	15.2 ± 1.8	41.4 ± 2.2	1.72 ± 0.53
Co[1]	NG	10	25.0 ± 0.9	18.7 ± 0.9	43.7 ± 1.7	1.33 ± 0.03
	BG	6	14.2 ± 1.0	10.0 ± 0.9	24.2 ± 1.1	1.42 ± 0.17
	YG	2	12.3	7.1	19.4	1.73
	GY	4	10.3 ± 1.4	6.6 ± 1.0	16.9 ± 2.4	1.58 ± 0.09
	Y	5	4.7 ± 1.2	3.0 ± 0.9	7.7 ± 2.1	1.57 ± 0.15
Cys	NG	10	23.6 ± 1.1	17.7 ± 0.9	41.3 ± 1.8	1.33 ± 0.05
Co–Cys	NG	9	24.7 ± 0.8	17.8 ± 0.8	42.5 ± 1.7	1.39 ± 0.03
	BG	1	16.2	20.8	37.0	0.78
	YG	5	13.6 ± 2.7	12.9 ± 6.0	26.5 ± 8.3	1.05 ± 0.30
	GY	6	8.9 ± 0.8	7.5 ± 2.0	16.4 ± 1.9	1.18 ± 0.23
	Y	1	8.0	5.4	13.4	1.48
Cys–Co	NG	9	24.6 ± 0.7	17.2 ± 0.8	41.8 ± 1.4	1.43 ± 0.04
	BG	5	10.3 ± 1.8	7.4 ± 0.4	17.7 ± 1.7	1.39 ± 0.26
	YG	3	11.6 ± 4.0	7.4 ± 2.1	19.0 ± 6.1	1.57 ± 0.09
	GY	6	6.6 ± 1.5	4.4 ± 1.5	11.0 ± 2.8	1.50 ± 0.30
	Y	4	4.3 ± 0.9	3.7 ± 0.9	8.0 ± 1.3	1.16 ± 0.25
2nd experiment, plants 17 days old						
0	NG	7	25.8 ± 1.1	22.2 ± 0.8	48.0 ± 1.8	1.16 ± 0.04
Co[2]	NG	3	27.2 ± 0.8	21.7 ± 1.9	48.9 ± 1.4	1.25 ± 0.13
	BG	3	20.2 ± 0.5	13.6 ± 0.6	33.8 ± 1.1	1.49 ± 0.04
	YG	14	13.5 ± 0.6	9.0 ± 0.5	22.5 ± 1.1	1.50 ± 0.04
	GY	3	7.0 ± 1.3	3.7 ± 0.7	10.7 ± 2.0	1.89 ± 0.05
	Y	1	1.3	0.8	2.1	1.63
Cys	NG	3	24.5 ± 0.6	21.7 ± 0.9	46.2 ± 1.4	1.13 ± 0.03
	Y	2	0.5	0.3	0.8	1.67
Co–Cys	NG	3	30.7 ±2.5	23.7 ± 1.3	54.4 ± 3.8	1.30 ± 0.04
	BG	11	19.3 ± 0.5	12.9 ± 0.4	32.2 ± 0.9	1.50 ± 0.02
	YG	4	18.7 ± 2.4	12.2 ± 1.4	30.9 ± 3.8	1.53 ± 0.06
	GY	8	6.4 ± 0.9	4.0 ± 0.6	10.4 ± 1.5	1.60 ± 0.03
Cys–Co	NG	2	31.6	22.4	54.0	1.41
	BG	5	20.6 ± 1.7	13.9 ± 1.0	34.5 ± 2.7	1.48 ± 0.02
	YG	2	13.2 ± 1.3	8.1 ± 1.0	21.3 ± 2.4	1.63 ± 0.05
	GY	3	9.2 ± 3.2	5.7 ± 2.1	14.9 ± 5.3	1.61 ± 0.05
	Y	3	1.4 ± 0.3	1.1 ± 0.1	2.5 ± 0.4	1.27 ± 0.11
3rd experiment, plants 22 days old						
0	NG	10	26.6 ± 0.7	20.3 ± 0.5	46.9 ± 1.1	1.31 ± 0.03
Co[3]	NG	10	26.6 ± 0.7	21.6 ± 0.6	48.2 ± 1.2	1.23 ± 0.02
	Y	12	2.7 ± 0.4	1.8 ± 0.3	4.5 ± 0.7	1.50 ± 0.11
Cys	NG	10	26.6 ± 1.1	21.1 ± 1.0	47.7 ± 2.1	1.26 ± 0.02

1 and 2 – $Co(NO_3)_2$ and equimolar cysteine concentration was 2.5 mM; 3 – $Co(NO_3)_2$ and cysteine concentration was 7.5 mM; 4 – plants: NG – normally green, BG – brightened green, YG – yellowish green, GY – greenish yellow, Y – yellow.

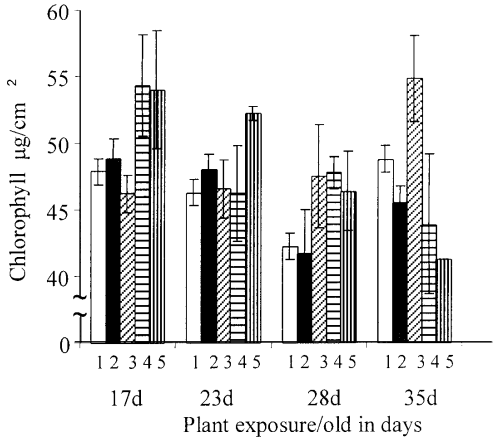

Fig. 4. Dependence of chlorophyll concentration in leaves of normally green *Vicia faba* plants on their age: 1 – unexposed (control) plants; exposed to: 2 – Cys, 3 – Co, 4 – Co–Cys, 5 – Cys–Co. $Co(NO_3)_2$ and cysteine concentration is 2.5 mM

be explained by the influence of environmental conditions on the interaction of Co^{2+} and Cys if investigation of plants of various age (from 17 to 35 days) were not compared (Fig. 4). This comparison showed that the effect was observed only in leaves of young plants. In leaves of 35-d plants even an opposite effect was noted: total chlorophyll concentration was lowest in the plants that had been exposed to Co^{2+} in combination with Cys.

However, we are prone to explain such effect by the 'saving' effect of cysteine and the appearance of several parts of such 'saved' plants in the group of the normally green plants. This conclusion was confirmed also by the results of analysis of the 28-d plants. The normally green 28-d plants after exposure to Cys, Co–Cys or Cys–Co had a significantly higher concentration of chlorophyll in leaves than did the normally green plants after exposure to Co^{2+} or control, Co-untreated plants. Besides, the 28-d plants after exposure to Cys, Co–Cys or Cys–Co did not differ in chlorophyll concentration.

The protective effect of cysteine on Co^{2+}-injured plants deserves further studies.

As could be expected, plants belonging to the BG, YG, GY and Y phenotypic groups differed very significantly from normally green (NG) plants, which remained in the same experimental conditions and the differences increased with the plant injury extent (Fig. 2).

The externally determined level of chlorophyll morphoses was fully confirmed by chlorophyll levels in leaves of the Co-affected plants (Table 2 and Fig. 2). In leaves of the yellow plants the chlorophyll concentration decreased 3.0–23.3 times. In such plants, more significant deviations were observed also in the *a/b* ratio. The general tendency, with several exceptions, was a relative increase of the proportion of chlorophyll *a* to chlorophyll *b* in plants with altered coloration.

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$CO(NO_3)_2$ SUKELTOS AUGALØ CHLOROFILINËS MORFOZËS. 3. CISTEINO POVEIKIS *Vicia faba* AUGALØ IÐGYVENIMUI IR CHLOROFILO KONCENTRACIJAI LAPUOSE

S a n t r a u k a

Co^{2+} jonø perteklius sukelia *Vicia faba* L. augalø chlorofilines morfozes. Chlorofilo koncentracija augalø lapuose atitinka fenotipinæ morfoziø raiðkà, ir normaliai atrodantys þali augalai ið tikrøjø yra labiausiai tolerantiðki Co^{2+} stresui. Cisteino (Cys) apsauginis poveikis priklauso nuo sekos, kuria derinama ekspozicija Co^{2+} jonams ir cisteinui – Co–Cys ar Cys–Co. Daroma prielaida, kad Cys nulemia jautriausiø Co^{2+} stresui augalø „gelbëjimo" efektà. Ði iðvada daroma palyginus cisteino poveiká augalø iðgyvenimui ir chlorofilo kieká augalø lapuose.

Raktaþodþiai: Co^{2+} genotoksiðkumas, cisteinas, „gelbëjimo" efektas, augalø morfozës