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Effect of cobalt chloride on soybean seedlings subjected to cadmium stress

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Abstract

Contamination of the environment with heavy metals such as Cd is a serious problem of modern world. Exposure of plants to Cd leads to oxidative stress, inhibition of respiration and photosynthesis, increased rate of mutation and, as a consequence, stunted growth and yield decrease. One of the common reactions of plants to cadmium stress is over-production of ethylene, however the exact role of this hormone in plants response to Cd is still unrecognized. The aim of the present study is evaluation of the impact of an ethylene synthesis inhibitor, Co, on the response of soybean seedlings to cadmium stress. The experiments included measurements of growth, cell viability, ethylene production and expression of genes associated with cellular signaling in soybean seedlings exposed to $CdCl_2$ (with Cd in a concentration of 223 μM) and/or $CoCl_2$ (with Co in concentration of 4.6 μM). Surprisingly, the results show that Co has no effect on ethylene biosynthesis, however, it affects cell viability and expression of Cd-induced genes associated with plant signaling pathways. The affected genes encode mitogen-activated protein kinase kinase2 (MAPKK2), nitrate reductase and DOF1 and bZIP2 transcription factors. The role of Co in plants response to cadmium stress and its potential use as an ethylene inhibitor is discussed.

Keywords: cobalt; heavy metal; gene expression; signaling; *Glycine max*

Introduction

Contamination of the environment with heavy metals, including Cd, is a serious problem of the modern world. Cadmium toxicity in plants leads to the generation of oxidative stress, chlorosis, inhibition of photosynthesis, disturbances in mineral homeostasis, increased rate of mutations and initiation of apoptotic and necrotic processes [1–3]. The described toxic symptoms lead to inhibition of plants growth and decrease in the obtained yield. Moreover, Cd might accumulate in crop plants and enter human organisms through the food chain [4]. One of the common responses of plants to cadmium stress is enhanced production of ethylene [5–8]. However, the exact role of the observed Cd-dependent induction of this hormone's production is still unrecognized. It is known that ethylene constitutes an important stress-related signaling molecule [9]. There are individual reports stating that it mediates Cd-dependent growth inhibition, hydrogen peroxide accumulation and programmed cell death (PCD) [7,10–12]. In mustard plants ethylene has been shown to participate in sulfur dependent alleviation of Cd toxicity through stimulation of antioxidant system [7,13].

Due to the important role of ethylene in flowering, fruit ripening and response of plants to stress factors several inhibitors of its synthesis and perception were developed. One of the commonly used inhibitor is Co, which affects the activity of a key enzyme in ethylene's biosynthesis pathway – 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) [14]. However, although relatively low concentration of Co might be beneficial for plants, higher concentrations exhibit toxic effect [15,16]. This metal has been shown to inhibit plants growth, cause oxidative stress, DNA damage and disturbances in photosynthesis [16–20]. Excess of Co also leads to alterations in germination, sex ratio, photoperiodism and uptake of other elements [16].

In the previous study we have shown that cadmium stress causes induction of ethylene biosynthesis and elevated expression of several genes associated with signaling pathways. Interestingly the promoters of Cd-induced genes contained cis-acting elements connected with ethylene signaling [6]. The aim of present study was to investigate the effect of Co, as a potential ethylene inhibitor, on soybean seedlings subjected to short term cadmium stress. The conducted research includes evaluation of the impact of Co on soybean growth parameters, viability, ethylene production and expression of six Cd-induced genes.

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Material and methods

Plant material, growth conditions and treatment procedures

If not stated differently the reagents were purchased at Sigma-Aldrich company (St. Louis, Missouri, USA). The applied growth conditions, Cd and Co concentrations and treatment periods were based on previous studies [6,21].

Soybean (*Glycine max* L cv. Nawiko) seeds were surface sterilized with 75% ethanol for 5 min and for 10 min with 1% sodium hyperchlorite. Seeds were washed for 30 min, soaked in distilled water for 2 h and germinated on Petri dishes with moistened blotting paper for 48 h. Germinated seedlings were transferred to new Petri dishes and treated with 5 ml of either: distilled water (control), 4.6 μM Co in $CoCl_2$ solution (corresponding 10 μM $CoCl_2$), 223 μM Cd in $CdCl_2$ solution (corresponding to 25 mg l^{-1}) or combined Cd and Co (corresponding to 4.6 μM and 223 μM respectively). After 3 and 6 hours of treatment seedlings root tips (100 mg) were cut off and frozen in −80°C for RNA isolation. Due to the lack of significant effect in shorter treatment periods (data not shown) the measurements of roots growth and cell viability were carried out after 24 hours.

If not stated differently the measurements were performed on samples from 3 independent experimental repetitions, each sample consisted of a pool of 20 seedlings.

Measurements of growth parameters

After 24 hours of treatment the roots of soybean seedlings were straightened and their length was measured with the use of ruler. The fresh and dry weight of roots was measured on the WPS60/C scale (RadWag, Radom, Poland). The fresh weight was evaluated immediately after cutting off, while the dry weight was measured after 72 h of incubation at 55°C.

Estimation of cell viability

The measurements of cell death were carried out according to the modified method described by Lehotai et al. [22]. After 24 hours of treatment with appropriate solutions root tips (200 mg) of soybean seedlings were cut off and incubated 20 min in 0.25% Blue Evans solution. Root tips were washed 3 times with distilled water and homogenized with 1.2 ml of 1% of SDS dissolved in 50% ethanol. The samples were incubated for 15 min at 50°C and centrifuged 15 min at 12 000 *g*. The absorbance of supernatant was measured with Biomate 3S spectrophotometer (Thermo Scientific, Waltham, USA) at λ = 600 nm.

Measurements of ethylene biosynthesis

The ethylene production was measured with the use of ethylene detector ETD-300 (Sensor Sense, Nijmegen, The Netherlands). Soybean seedlings were placed in Petri dishes on two layers of filter paper moistened with 5 ml of either distilled water (control), 4.6 μM Co in $CoCl_2$ solution, 223 μM Cd in $CdCl_2$ solution or combined Cd and Co (corresponding to 4.6 μM and 223 μM, respectively). The bottom part of the dish was covered with a Plexiglas plate with an inlet and outlet for gas flow, and tightly closed. The flow from each cuvette was directed into a photoacoustic cell where ethylene was quantified. The measurements were conducted in the dark during 24 hours, in stop-and-flow mode, with each cuvette being alternatively flushed with a flow of 3 l h^{-1}. The amount of produced ethylene was detected every 12 minutes. As a control from the obtained emission rates the levels of ethylene in a cuvette containing moistened filter papers without seedlings was also measured. A detailed description of the system has been given previously [23]. The obtained results were analyzed with the use of Valve Controller software and expressed as nl per hour per 1 g of roots fresh weight. Measurements were performed on samples from 3 independent experimental repetitions; each sample consisted of a pool of 10 seedlings.

Measurements of genes expression

The RNA was isolated from 100 mg of frozen root tips with the use of TriReagent according to the manufacture's instructions. The concentration of the obtained RNA was evaluated on NanoCell Accessory coupled with spectrophotometer Biomate 3S (Thermo Scientific, Waltham, USA).

For the reverse transcription 1 μg of RNA was purified with Deoxyribonuclease Kit and processed with the use of Reverse Transcription Kit (Thermo Scientific Fermentas, Waltham, USA): incubated with 1 μl oligo dT (100 μM, 0.5 μg/μl) at 65°C for 5 min followed by the incubation with 4 μl of 5× Reaction Buffer, 1 μl of RiboLock™ RNase Inhibitor (20 u/μl), 2 μl of 10 mM dNTP Mix and 1 μl of RevertAid™ Reverse Transcriptase at 42°C for 10 min. The reaction was stopped by incubation at 70°C for 10 min. The obtained cDNA was diluted 5×.

The measurements of genes expression were carried out with the use of real-time PCR reaction performed on Rotor-Gene 6000 Thermocycler (Qiagen, Venlo, The Netherlands). The primers (listed in Tab. 1) were designed on the basis of sequences accessible in Soybase.org with the use of Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). The reaction mixture contained 0.1 μM of each primer, 1 μl of diluted cDNA, 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) and DEPC treated water (BioShop, Burlington, Canada) to the total volume of 20 μl. The real-time PCR reaction started with initial denaturation at 95°C for 5 min, followed by 13 cycles of touchdown PCR (15 s at 95°C, 20 s at 68°C decreasing by 1°C each cycle and 30 s at 72°C) and 45 cycles of 10 s at 95°C, 20 s at 55°C and 30 s at 72°C. The reaction was finalized by denaturation at a temperature rising from 72°C to 95°C by one degree every 5 s.

The relative gene expression was calculated with the use of Pfaffl equation [24] based on the efficiency and Ct values determined by Real-time PCR Miner [25]. Ubiquitin was chosen as reference gene. Measurements were performed on samples from 2–3 independent experimental repetitions; each sample consisted of a pool of 20 seedlings.

Statistical analysis

For evaluation of statistically significant differences the obtained data was analyzed with the use of ANOVA (α = 0.05). In the case of the measurements of genes expression, due to the non-normal distribution of data, Mann–Whitney *U* post-hoc test has been used. In all the other cases Scheffe's test has been applied. Results, which showed no statistically significant differences, are marked with the same letter.

Tab. 1 Sequences of primers used for the real-time PCR reaction.

Gene number in Soybase.org	Primers	Encoded protein
Glyma05g37410	Left: TGTGCTATGCCAACATGGAT Right: GAGGTATGGGGGAGTGAGGT	1-aminocyclopropane-1-carboxylate synthase (ACS)
Glyma17g06020	Left: AGCAGGTGCTGAAGGGTCTA Right: TTCCTGGCTTCCATTGATTC	mitogen-activated protein kinase kinase 2 (MAPKK2)
Glyma13g02510	Left: AAATCCCATGCAAGCTCATC Right: GGTGCACCCCTTTGAAGTAA	nitrate reductase (NR)
Glyma13g42820	Left: AAGCCAAAACTTGGAGCAGA Right: CCTTGTCGACGGAGGAATTA	DOF1 transcription factor
Glyma11g11450	Left: GAATCGACCCTGCAACTCAT Right: ACCCAAACTGCAAACGAAAC	MYBZ2 transcription factor
Glyma06g08390	Left: GCCCCATTGCTGTTCCTCATGT Right: GCTGAGACTGGGCTCCCAACA	bZIP62 transcription factor
Glyma20g27950	Left: GAAGTCGAAAGCTCCGACAC Right: TGTT TTGGGAACACATCCAA	ubiquitin – reference gene

Results

Treatment with Cd for 24 hours caused inhibition of roots length and dry weight (Tab. 2). In turn Co had no effect on any of the growth parameters. At this time point there were also no differences in the viability of cells in the roots of control seedlings and seedlings treated with Co (Fig. 1). However, treatment with Cd caused increase in the Evans Blue uptake providing a significant increase in cells mortality. The cells mortality increased even stronger in the case of the roots of seedlings treated simultaneously with Cd and Co.

Tab. 2 Growth parameters of the roots of soybean seedlings.

Experimental variant	Roots length (mm)	Roots fresh weight (mg)	Roots dry weight (mg)
Control	43 ±1[a]	54 ±4[a]	2.9 ±0.2[a]
$CoCl_2$	47 ±5[a]	62 ±8[a]	3.3 ±0.3[a]
Cadmium	30 ±2[b]	53 ±3[a]	2.7 ±0.1[b]
Cadmium+$CoCl_2$	28 ±1[b]	50 ±3[a]	2.7 ±0.2[b]

The results are means of 3 independent experiments ±*SE*. Results which showed no statistically significant differences are marked with the same letter (a or b).

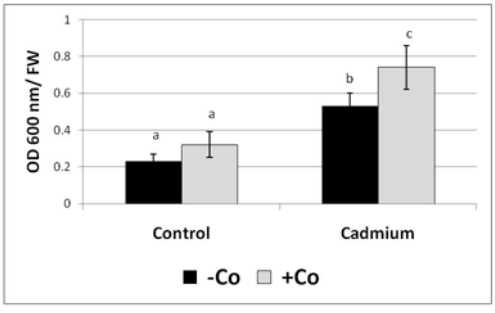

Fig. 1 Mortality of cell of soybean seedling roots represented as uptake of Evans Blue dye. The results are means of 4 independent experiments ±*SE*. Results, which showed no statistically significant differences, are marked with the same letter (a, b or c).

Application of Cd lead to augmented ethylene biosynthesis starting from the 5th hour of treatment (Fig. 2). After 24 h the levels of ethylene were four times higher in the roots of seedlings exposed to Cd than in the roots of control seedlings and reached 8 nl/h × fresh weight. Co had no significant effect on ethylene production neither in control nor Cd-treated seedlings.

Accordingly to our previous study [6] treatment with $CdCl_2$ for 3 h caused increase in the expression levels of genes encoding aminocyclopropane-1-carboxylic acid synthase (ACS), mitogen-activated kinase kinase2 (MAPKK2) and DOF1 and MYBZ2 transcription factors (Fig. 3). Present research shows that Co diminished the Cd-dependent induction of *MAPKK2* and *DOF1* expression (Fig. 3b,c).

Longer (6 h) exposure to Cd resulted in elevated levels of mRNA encoding nitrate reductase (NR), ACS and MYBZ2 and bZIP62 transcription factors (Fig. 4). Co caused augmentation of Cd-dependent induction of NR gene (Fig. 4b) and at the same time decrease in Cd-dependent stimulation of gene encoding bZIP62 transcription factor (Fig. 4d).

Discussion

Although Cd caused significant reduction of roots length and dry weight after 24 hours of application, Co did not affect any of the measured growth parameters (Tab. 2). Treatment with Cd also led to the increase in the amount of dead cells in the roots soybean seedlings (Fig. 1). Application of Co additionally increased Cd toxicity as the amount of dead cells was significantly higher in seedlings treated with both Co and Cd than in the seedlings treated with only Cd (Fig. 1). Co is known to have beneficial effects on plant growth at moderate levels [15], however, it is possible that the combined effect of Co and Cd leads to the aggravation of the metals' toxicity. Indeed, combination of Cu and Zn was shown to be more toxic to black lentil than the separate effect of both heavy metals, while combined Cd and Pb stress was found to be more harmful to mustard plants than application of Cd or Pb alone [26,27]. In higher concentrations Co is toxic. It was shown to inhibit plants growth, germination rate, cause leaf fall, hamper photosynthesis and respiration and lead

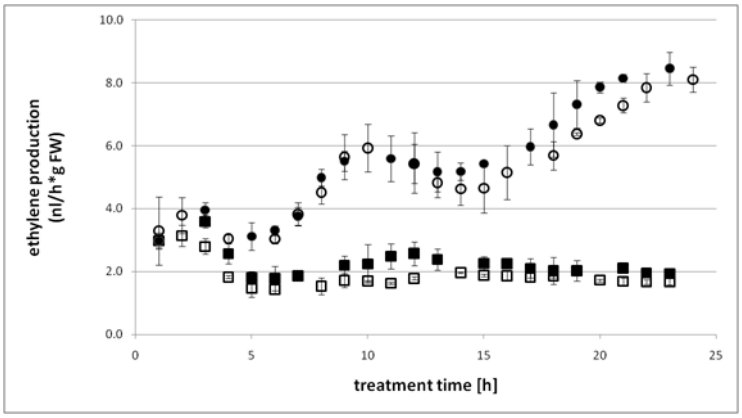

Fig. 2 Production of ethylene in roots of control soybean seedlings (dark square), seedlings treated with Co (light square), seedlings treated with Cd (dark circle) and seedlings treated with Cd and Co (light circle). The ethylene production is presented in nanoliters per hour in one gram of roots fresh weight. The results are means of 3 independent experiments ±*SE*.

to DNA damage as well as decrease in RNA levels [16–20]. One admitted function of Co is to impair ethylene synthesis at various concentrations, treatment durations and plant species, by inhibiting ACC oxidase [28–32]. Surprisingly in the applied experimental conditions Co had no effect on hormones production (Fig. 2). It is possible that the inhibitory action of Co requires longer treatment periods or higher concentrations. Indeed, in the majority of studies performed by other researchers Co was applied for several days [28,30–32]. In the research conducted by Koehl et al. [29] tobacco suspension culture was also treated with Co for short time periods (3, 9 and 24 h), however, the applied $CoCl_2$ concentration was much higher (100 μM) and the inhibitory effect on ethylene biosynthesis was noticed only after 9 h of treatment [29]. It is also possible that ethylene is synthesized without the participation of ACC oxdiase – a target gene for Co inhibitory action. It has been shown that ACC might be oxidized nonenzymatically through superoxide anion generated in response to wounding [33].

In our previous study we have demonstrated that Cd causes induction of several genes associated with plant signaling pathways [6]. Application of Cd for 3 h induced expression of genes encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS), mitogen-activated kinase kinase2 (MAPKK2) and DOF1 and MYBZ2 transcription factors, while 6 h long treatment led to the increase in the expression of *nitrate reductase* (*NR*), *ACS*, *MYBZ2* and *bZIP62* genes. In the present study the influence of Co on the expression of mentioned, signaling associated genes after the same treatment has been evaluated.

Interestingly the results of present research show that Co influences expression of a key enzyme engaged in ethylene synthesis: 1-aminocyclopropane-1-carboxylic acid synthase. The genes expression was slightly repressed after 3 h (Fig. 3a)

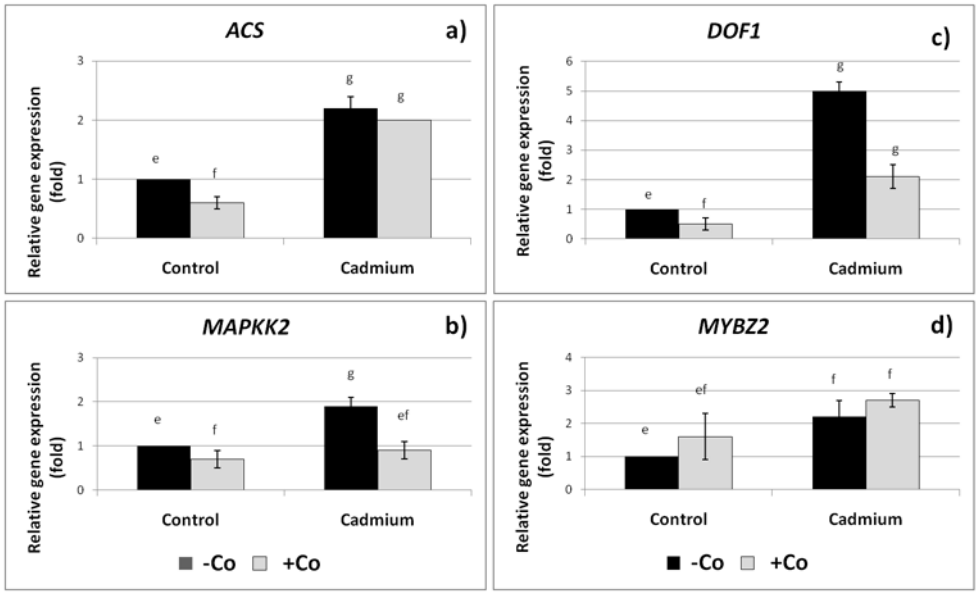

Fig. 3 Relative expression of gene encoding 1-aminocyclopropane-1-carboxylic acid synthase (**a**), mitogen-activated kinase kinase2 (**b**), DOF1 transcription factor (**c**) and MYBZ2 transcription factor (**d**) in soybean seedlings treated with appropriate solutions for 3 h. The results are the means of 2–3 independent experiments ±*SE*. Results, which showed no statistically significant differences, are marked with the same letter (e, f or g).

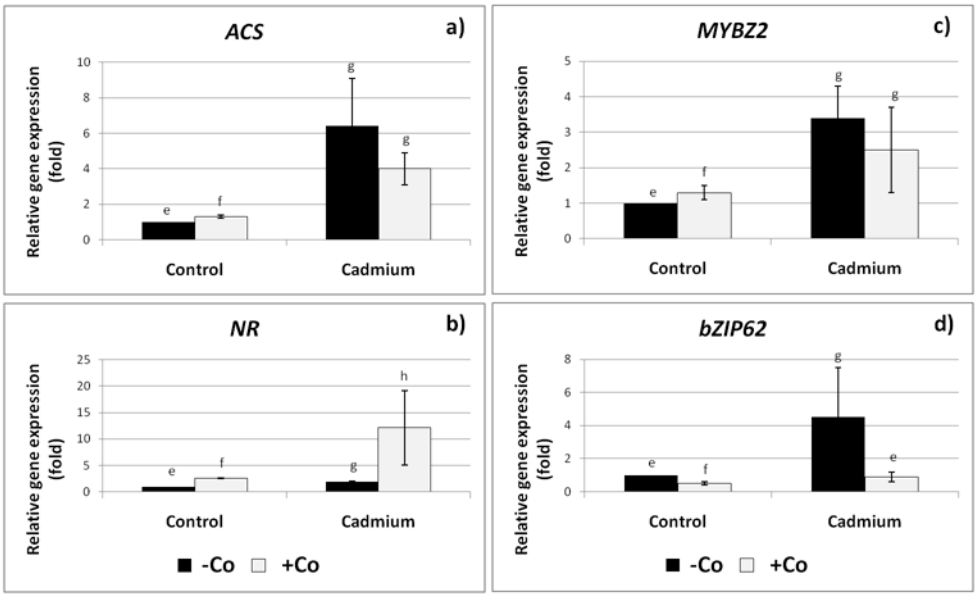

Fig. 4 Relative expression of gene encoding 1-aminocyclopropane-1-carboxylic acid synthase (**a**), nitrate reductase (**b**), MYBZ2 transcription factor (**c**) and bZIP62 transcription factor (**d**) in soybean seedlings treated with appropriate solutions for 6 h. The results are the means of 2–4 independent experiments ±*SE*. Results, which showed no statistically significant differences, are marked with the same letter (e, f, g or h).

and up-regulated after 6 h of treatment (Fig. 4a). Therefore it is possible that Co affects ethylene biosynthesis not solely by inhibiting ACC oxidase.

The obtained results also show that Co reversed the Cd-dependent induction of genes encoding MAPKK2 (Fig. 3b) and DOF1 transcription factor (Fig. 3c) after 3 h as well as gene encoding bZIP62 transcription factor (Fig. 4d) after 6 h of treatment. A possible explanation of the described phenomenon is the inhibitory effect of Co on Cd uptake. Indeed, Co was shown to reduce Cd uptake in bush beans and green alga *Chlamydomonas reinhardatii* [16,34]. However, in the referenced experiments the treatment times were much longer (21 and 60 days respectively). In the present study Co was applied for short time (3, 6 or 24 h) and even after 24 h did not reverse the Cd-dependent inhibition of roots growth (Tab. 2). Moreover, application of Co for 24 h led to the intensification of Cd toxicity expressed by higher cells mortality (Fig. 1). Therefore, it is unlikely that Co caused a significant reduction of Cd uptake. The measurements of genes expression also showed that, after 6 h of treatment Co led to strong augmentation of Cd-dependent induction of *NR* gene (Fig. 4b).

The impact of Co on the expression of analyzed genes might lead to altered Cd sensing. There are several reports implying that MAPK cascades are involved in the transduction of Cd signals. Stimulation of various MAPKs by Cd has been observed in rice, alfalfa and *Arabidopsis* plants. Moreover, MPK6 identified in *Arabidopsis* plants, was shown to participate in Cd-dependent initiation of programmed cell death (PCD) [10,35–38]. Therefore, observed in the present research alterations in *MAPKK2* expression in response to Co might lead to disorders in the transduction of Cd signal and contribute to exacerbation of Cd toxicity.

There are reports stating that both applied metals, Cd and Co, cause inhibition of nitrate reductase activity after long treatment times [17,39–41]. Interestingly, the present study shows that short-term cadmium stress causes induction of *NR* gene, which is strongly augmented by Co (Fig. 4b). Nitrate reductase is an important enzymes engaged in nitrogen metabolism – it catalyses reduction of nitrates to nitrites [42]. Therefore, the observed stimulation of *NR* expression might constitute a defense mechanism, which aims to sustain nitrogen homeostasis. Nitrate reductase is also one of the enzymes engaged in NO formation [42]. Accumulation of NO in response to Cd has been observed in various plant species [43], however, its role is still debatable. The treatment with nitric oxide donor, SNP, caused attenuation of chlorophyll degradation and oxidative stress in plant exposed to Cd [44,45]. Nitric oxide also mediates induction of several signaling associated genes in response to short-term cadmium stress [46]. On the other hand NO contributes to Cd toxicity through increase in Cd uptake [1, 47]. The observed Co-dependent induction of *NR* gene might lead to over-production of NO, however, the exact role of such induction would need further investigation.

Increase in *DOF1* and *bZIP62* mRNA levels in response to Cd [6] and the fact that DOF1 was induced by drought stress in amaranth roots and bZIP62 conferred tolerance to low temperatures and salt stress in transgenic *Arabidopsis* plants, suggests that both transcription factors are involved in the regulation of genes expression under various stress conditions [48,49]. Therefore, observed in the present study Co-dependent decrease in *DOF1* and *bZIP62* expression might lead to alterations in the expression pattern of Cd-responsive genes.

It can be concluded that Co may increase Cd toxicity, at least partly, through alterations in MAPK and NO signaling as well as disorders in regulation of genes expression mediated by DOF1 and bZIP62 transcription factors. The observed effects are independent from ethylene action. Therefore, another important conclusion is that $CoCl_2$ should be used as an ethylene inhibitor with cautions, as it might be difficult to distinguish if the observed effects of $CoCl_2$ are dependent on changes in ethylene production or on the action of Co itself.

Acknowledgments

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Authors' contributions

The following declarations about authors' contributions to the research have been made: designed the experiments: JD, JCB; supervised the research: JD, SL; conducted the experiments: JCB, AK; analyzed the results: JCB, IL; wrote the manuscript: JCB; revised the manuscript: JD, SL, IL.

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