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Polyamines may influence phytochelatin synthesis during Cd stress in rice

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Highlights

- Putrescine pre-treatment increased cadmium toxicity in rice.
- In contrast, putrescine synthesis inhibition alleviated cadmium stress.
- The synthesis of higher polyamines and phytochelatins is antagonistically related.
- Putrescine may decrease phytochelatin synthesis at enzymatic and gene expression levels.

Abstract

Abbreviations: ADC: arginine decarboxylase; DAO: diamine oxidase; DAP: 1,3-diaminopropane; dcSAM: decarboxylated S-adenosylmethionine; DFMO: 2-(difluoromethyl)ornithine; GR: glutathione reductase; *gamma*-Glu-Cys: *gamma*-glutamyl-cysteine; GSH: glutathione; G-POD: guaiacol peroxidase; hmGSH: hydroxylmethyl-glutathione; ODC: ornithine decarboxylase; PCs: phytochelatins; PCS: phytochelatin synthase; PA: polyamine; PAO: polyamine oxidase; PUT: putrescine; SPD: spermidine; SAM: S-adenosylmethionine; SPM: spermine; SPDS/SPMS: spermidine/spermine synthase.

Although the metabolism of phytochelatins and higher polyamines are linked with each other, the direct relationship between them under heavy metal stress has not yet been clarified. Two approaches were used to reveal the influence of polyamine content on cadmium stress responses, particularly with regard to phytochelatin synthesis: putrescine pre-treatment of rice

plants followed by cadmium stress, and treatment with the putrescine synthesis inhibitor, 2-(difluoromethyl)ornithine combined with cadmium treatment. The results indicated that putrescine pre-treatment enhanced the adverse effect of cadmium, while the application of 2-(difluoromethyl)ornithine reduced it to a certain extent. These differences were associated with increased polyamine content, more intensive polyamine metabolism, but decreased thiol and phytochelatin contents. The gene expression level and enzyme activity of phytochelatin synthase also decreased in rice treated with putrescine prior to cadmium stress, compared to cadmium treatment alone. In contrast, the inhibition of putrescine synthesis during cadmium treatment resulted in higher gene expression level of phytochelatin synthase. The results suggest that polyamines may have a substantial influence on phytochelatin synthesis at several levels under cadmium stress in rice.

1. Introduction

The accumulation of heavy metals poses a hazard for the growth and development of plant organisms. The high penetration ability of cadmium gives it easy access to the food chain, causing subsequent damage to animals and humans [1]. One potential defence mechanism, chelation followed by the compartmentalisation of the metal-chelate to the vacuoles, effectively ensures low concentrations of free metals in the cytosol. Metal-chelation can be performed by compounds of thiol origin, such as glutathione (GSH) or phytochelatins (PCs), or non-thiol origin, such as polyamines (PAs) [2]. Both thiol compounds and PAs are reported to play a role in heavy metal stress tolerance. Higher PC accumulation in the roots was responsible for higher Cd tolerance in wheat genotypes [3] and for higher Cu, Zn and Cd tolerance in aquatic plants [4]. The overexpression of phytochelatin synthase (AtPCS) in rice also resulted in Cd tolerance [5], while exogenous GSH has been reported to increase photosynthetic performance under Cd stress in rice [6].

PAs are low-molecular-weight, organic cations which are ubiquitous in all living organisms, and have an indisputable role in plant stress responses and signalling [7]. Cd stress has been reported to increase both the putrescine (PUT) content and the activity of both enzymes involved in PUT biosynthesis, arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) in the leaves of wheat plants. However, the spermidine (SPD) content was not affected and spermine (SPM) was significantly reduced [8]. Cd-induced oxidative stress was alleviated by polyamine treatment in rice [9] and wheat [8]. However, only a few contradictory studies have been published on the relationship between PAs and PCs under heavy metal stress. The protective effect of PUT pre-treatment in mung bean and of SPD or SPM pre-treatments in rice was associated with increased GSH and/or PC content [10; 9], but not after SPM pre-treatment in wheat [8]. After combined SPM+Cd treatment, the thiol and PC content and the GSH synthase activity decreased in the roots of Cd-treated *Canavalia lineatan* plants and an additional PC was detected with lower affinity for Cd [11]. In contrast, the mitigation of Cr stress by SPD treatment in radish was related to increased GSH and PC contents [12]. The situation is also complicated by the fact that cysteine (Cys) is a common precursor for the synthesis of GSH, and thus PCs, and for the formation of S-adenosylmethionine (SAM), which is necessary for the synthesis of higher PAs (SPD and SPM) from PUT (Suppl. Figure 1).

One way to increase endogenous PA contents is to apply PA exogenously. In the PA cycle, PAs are able to interconvert to each other and the PA pool is thus dynamic, changing over time [7]. Difluoromethylornithine (DFMO) has been capable of covalently binding to and thus irreversibly inhibiting the PUT synthesis enzyme, ODC [13]. DFMO has been shown to protect plants from pathogenic fungi [14] and larval attacks [15], with no negative effects to the plants [16; 15]. However, the effects of DFMO vary in different organic systems, ranging from inhibition to stimulation of PA levels and plant development, depending on the concentration

[17-20], and the existence of compensatory mechanisms, such as the induction of the arginine decarboxylase (ADC) pathway [21] or an increase in the ODC protein turnover, as ODC is a short-living enzyme in plant cells [22].

Since the crosstalk between the PA- and PC-related heavy metal protection mechanisms is still poorly understood, answers were thus sought to two questions: 1. how exogenous PUT influences the PA metabolism and plant development in rice, and 2. what relationship exists between changes in PA content and the thiol-origin part of the heavy metal detoxification system in rice plants? To this end, exogenous PUT pre-treatment was applied followed by Cd stress, or DFMO treatment was combined with exposure to Cd. The results indicate that PUT and DFMO have a substantial influence on the PC synthesis in Cd-stressed rice.

2. Materials and methods

2.1. Plant material and growth conditions

After germination, rice seeds (*Oryza sativa* L. var. Janka) were grown in glass pots containing hydroponic solution [23] under controlled growth conditions (Conviron PGV-36, Controlled Environments Ltd., Winnipeg, Canada) with a 13/11 h light/dark photoperiod, 250 μmol m^{-1} s^{-2} photosynthetic photon flux density (PPFD), 28/26 °C, and 70% relative humidity. The nutrient solution was renewed every 2 days in all the treatments. The 7-day-old plants divided into six groups; four of which continued to grow under control conditions, while two were treated with 0.5 mM PUT for 7 days. One each of the control and PUT pre-treated groups were then exposed to 7 days of 50 μM Cd(NO$_3$)$_2$ treatment (Cd and PUTpre+Cd). The other group treated with PUT was given a 7-day recovery period without PUT (PUTpre). Of the other three groups grown for 14 days under control conditions, one continued to receive no treatment (Control), one was treated with 0.5 mM DFMO alone (DFMO) and one with DFMO combined with Cd (DFMO+Cd). The plants were sampled for gene expression measurements on the 14th

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and 21st days, while samples for other analyses were collected after 21 days of growth as described in Suppl. Fig.2.

2.2. Determination of growth biomarkers, Cd and hydrogen peroxide content

Measurements were made of the root and shoot length and the fresh weight of the roots and shoots. The Cd content was measured according to Hegedűs et al. [24]. The ferrous ammonium sulphate/xylenol orange (FOX-1) method was used to determine the H_2O_2 content of the samples [25].

2.3. Enzyme assays

Measurements were performed as described in Pál et al. [26]. The guaiacol peroxidase (G-POD)(EC 1.11.1.7.) activity was determined at 470 nm and the glutathione reductase (GR)(EC 1.6.4.2.) activity at 412 nm and expressed in nkatal g^{-1} fresh weight (FW).

2.4. Polyamine and 1,3-diaminopropane (DAP) analysis

The analysis was carried out as described by Németh et al. [27]. The polyamines, namely PUT, cadaverine (CAD), SPD, SPM, and DAP, the product of SPD and SPM terminal catabolism, were analysed as dansylated derivatives via HPLC using a W2690 separation module on a reverse phase column (Kinetex C18, 5μ, 100 x 4.6 mm, Phenomenex, Inc.) and a W474 scanning fluorescence detector with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA).

2.5. Diamine oxidase and polyamine oxidase enzyme activities

The enzyme activities of diamine oxidase (DAO, EC 1.4.3.6.) and polyamine oxidase (PAO, EC 1.5.3.3.) were estimated by the method of Takács et al. [28].

2.6. Determination of cysteine, gamma-glutamyl-cysteine (gamma-Glu-Cys) and GSH content

The thiol content of the samples was determined as described by Kocsy et al. [29]. HPLC analysis was carried out using an Alliance 2690 system (Waters, Milford, MA, USA)

equipped with a W474 fluorescence detector (Waters, USA), with excitation at 380 nm and emission at 480 nm on a Hyperprep HS C18 column (250x4.6 mm, 8 μm) (ThermoFisher Scientific Inc.).

2.7. Determination of phytochelatin content and phytochelatin synthase (PCS) activity

HPLC analysis of the *in vivo* PC concentrations and *in vitro* PCS activity was carried out according to Szalai et al. [30] using an Alliance 2690 system equipped with UV W996 photodiode array detector (Waters, Milford, MA, USA) on a reverse phase column (Hypersil ODS, 100x2.1 mm, 5 μm, Thermo Scientific). The specific activity of PCS was expressed as nmol PC min^{-1} g^{-1} FW.

2.8. Gene expression analysis

To analyse the expression of the ODC, ADC, spermidine/spermine synthase (SPDS/SPMS) and PCS genes, fully expanded 2nd leaves of 14-day-old and 3rd leaves of 21-day-old rice plants were sampled according to Suppl. Fig.2. RNA isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and was treated with DNase I and cleaned with a Direct-Zol RNA MiniPrep Kit (Zymo Research, USA). 1000 ng RNA was reverse transcribed with oligo dT18 primer using a RevertAid first strand cDNA synthesis kit (Thermo Fisher Scientific, USA). Real-time PCR was performed on an Applied Biosystems 7500 instrument using SYBR Green detection chemistry (Applied Biosystems) and gene-specific primers. The rice ubiquitin5 (AK061988.1) gene was used as an endogenous control, (forward: ACCACTTCGACCGCCACTACT; reverse: ACGCCTAAGCCTGCT GGTT). The primers for ODC3 (LOC_Os02g28110; forward: TCTCCACGTCCAACATGAAGAC; reverse: GGCACTTTCCCAGTGATCTAGC), ADC3 (LOC_Os08g33620; forward: AATCATCCCAATCCAGTGCCTT; reverse: TGCCTCCCGCTGATGAAGT) and SPDS/SPMS3 (LOC_Os02g15550; forward: AGAGCATGTGGTTGCATACGC; reverse:

AACCCTTGAATGTCTCACGGC) were designed according to Do et al. [31], and for PCS9 (forward: ATGGGGGCGGAGGTCCATGA; reverse: TCAATGCAAGGTTCTAGGAGTGA) according to Shen et al. [32]. Real-time PCR was performed on an Applied Biosystems Fast 7500 instrument using SYBR Green detection chemistry (QuantiFast SYBR Green PCR kit; Qiagen) [33]. The relative ratio of the threshold cycle (Ct) values of the endogenous control to that of the specific genes was calculated for each sample.

2.9. Statistical analysis

The results were the means of at least twenty replicates for each treatment for biomass parameters, and of five replicates for the determination of Cd content, H_2O_2 measurement, enzyme activity and HPLC analysis. The data were statistically evaluated using the standard deviation and *t-test* methods.

3. Results

3.1. Biomass parameters and cadmium content

The biomass parameters were decreased by 0.5 mM PUT pre-treatment alone, more severely by 50 μM Cd treatment and to the greatest extent by the combined PUTpre+Cd treatment, but were not influenced significantly by DFMO alone (Table 1). DFMO combined with Cd resulted in higher root length and shoot FW values than in Cd-treated plants, while all the biomass parameters had higher values than in the PUTpre+Cd treatment (Table 1).

Exposure to Cd either alone or in combination with PUTpre or DFMO resulted in Cd accumulation, especially in the roots, though Cd was also translocated to the leaves. However, significantly lower Cd uptake was found in the roots of DFMO+Cd-treated plants than in the Cd and PUTpre+Cd treatments (Table 1).

3.2. Hydrogen peroxide content and antioxidant enzyme activities

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Cd treatment either alone or in combined treatments (PUTpre+Cd and DFMO+Cd) induced oxidative stress, manifested as H_2O_2 accumulation, with the highest amount in the leaves of PUTpre+Cd-treated plants (Table 1).

In the same way the GR (Fig. 1A, B) and G-POD enzyme activities (Fig. 1C, D) showed that the most severe oxidative stress was induced by the PUTpre+Cd treatment in both the leaves and roots, suggesting that PUT and Cd stress had an additive effect. The application of DFMO with Cd, however alleviated the damage caused by Cd. Taken together, these results suggest that PUT pre-treatment aggravated the adverse effect of Cd, while the application of DFMO reduced it to a certain extent.

3.3. Changes in polyamine metabolism

As expected, PUT pre-treatment increased the polyamine content in rice, suggesting that the exogenous PUT was taken up and used for the further synthesis of higher PAs (SPD and SPM). At the end of the 7-day PUT pre-treatment the PUT, SPD and SPM contents in the leaves increased by 287, 127 and 229%, respectively (data not shown). Even after the plants were moved to control conditions for an additional 7-day recovery period, higher leaf PUT, root SPD, and leaf and root SPM contents were detected (Fig. 2). In contrast, treatment with DFMO (which inhibits PUT synthesis) resulted in lower PUT content in the leaves, but did not influence the other PAs. Cd alone also induced PA synthesis, which was more pronounced in the roots. The highest PA accumulation was observed in the PUTpre+Cd treatment, as an additive effect. In comparison, the combined DFMO+Cd treatment caused a less pronounced increase in the amount of PAs (Fig. 2). The level of the other diamine, cadaverine (CAD), which is synthesised independently from PUT, also increased in the roots of rice during Cd stress, particularly in PUTpre+Cd-treated plants (Fig. 3).

The DAO activity, which is responsible for the catabolism of PUT and CAD increased in the roots of PUTpre+Cd treated plants, where the highest PUT and CAD accumulation was

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found (Table 2). The PAO activity, which is responsible for the catabolism of higher PAs (SPD and SPM), was induced in the leaves after Cd treatment either alone or combined with PUT pre-treatment or DFMO, while in the roots all the treatments increased it, with the highest increment in the case of PUTpre+Cd (Table 2).

Changes in the PUT/(SPD+SPM) ratio were correlated with the PAO activity. PUT pre-treatment increased the leaf PUT/(SPD+SPM) ratio due to the increased PUT level, but decreased it in the roots, as the amount of SPD and SPM increased despite the activation of PAO. Cd alone increased this ratio both in the leaves and roots, due to the parallel increase in PUT level and PAO activity. The highest ratio was found in the PUTpre+Cd treated plants, where both PAO and DAO were induced in the roots, and higher DAP accumulation was found in both the leaves and roots. DFMO alone resulted in the lowest leaf and root PUT/(SPD+SPM) ratio because of the decreased PUT level, while after DFMO+Cd treatment the higher PUT level, combined with increased PAO activity, caused a higher ratio than in the control, but lower than in PUTpre+Cd plants (Table 2). However, this elevated PAO activity was not associated with higher DAP content, but correlated with the H_2O_2 content in the leaves.

3.4. Thiol and phytochelatin content, phytochelatin synthase activity

The most abundant thiol compound was hydroxymethyl-glutathione (hmGSH), followed by GSH, Cys and *gamma*-Glu-Cys. PUT pre-treatment did not influence the thiol content in either the leaves or the roots (Table 3), while Cd induced slight but statistically significant thiol accumulation in the leaves of rice plants. Compared to Cd treatment alone or DFMO+Cd, lower thiol levels were observed in the leaves and roots in the PUTpre+Cd treatment.

The initial *in vivo* PC level in the leaves did not differ significantly in the PUTpre-treated plants compared to the control (only PC_2 was detected), while PC_2 and PC_3 accumulated not only after Cd stress alone, but also during DFMO treatment, applied alone or in combination

with Cd (Fig. 3A). Like thiol, a significantly lower accumulation of PC_2 was induced by PUTpre+Cd treatment than by Cd alone, while PC_3 synthesis was similar in the Cd, DFMO and DFMO+Cd treatments. HMW PCs, defined as $PC_{5-10,}$ could not be detected.

A similar pattern was detected in the roots. PUT pre-treatment alone did not cause pronounced changes, while Cd and DFMO+Cd treatments induced the accumulation of PC_2, PC_3 and PC_4. Although PUTpre+Cd treatment slightly induced PC_4 synthesis, a lower level of PC_3 was found and no PC_2 could be detected in rice roots (Fig. 4B).

The *in vitro* PCS activity corresponded to the PC contents, with the lowest activity in the leaves and roots of PUTpre+Cd-treated plants (Fig. 4C).

3.5. Gene expression

Even 7 days of pre-treatment with PUT induced a slight increase in ODC, ADC and SPDS/SPMS gene expression, which became more pronounced after a 7-day recovery period (PUTpre) compared to the same day control (Fig. 5A-C). In contrast, PUT induced a substantial decrease in the gene expression level of PCS in the leaves of rice plants. Although this effect ceased after the plants were moved to control conditions (PUTpre), a lower gene expression level of PCS was measured in PUTpre+Cd-treated leaves than after Cd or DFMO+Cd treatments (Fig. 5D), resulting in the lowest PC level in the leaves. Not surprisingly, DFMO treatment stimulated ODC and ADC transcription in order to compensate for the inhibited PUT synthesis (Fig. 5A-B). However, DFMO also induced PCS transcription (Fig. 5C), which was in accordance with the increased PC content of the leaves. The highest level of PCS gene expression was observed in the leaves of DFMO+Cd-treated rice plants (Fig. 5D).

4. Discussion

When Cd enters the cytosol, the number of metal-binding ligands, e.g. the system linked to the sulfur metabolism, is activated in plants, resulting in the formation of important complex-forming agents. The enzyme responsible for the synthesis of PCs is *gamma*-glutamyl cysteine

dipeptidyl transpeptidase (PCS), which is also expressed without heavy-metal stress, but is primarily activated by the presence of heavy metals [1]. Recently reviewed findings indicate that polyamines, which are key compounds in the signalling of cadmium stress responses [34], could also act as metal chelators [35]. The metabolisms of PCs and higher polyamines are linked with each other, due to the common precursor, Cys, but little research has been done on the direct relationship between them under heavy metal stress (Suppl. Fig.1.). Two approaches were used to study the influence of PAs on cadmium stress responses, in particular with regard to PC synthesis: rice plants were treated either with exogenous PUT or with DFMO, the inhibitor of endogenous PUT synthesis, with or without Cd stress. In a preliminary experiment 10, 25 and 50 μM $Cd(NO_3)_2$ concentrations were applied. Only 50 μM Cd treatment was effective enough to induce oxidative stress, furthermore this concentration already induced polyamine synthesis, which is necessary if we would like to study the competition of polyamine and phytochelatin synthesis. However, higher Cd concentrations may result in so severe stress that damage processes would be dominant rather than defence mechanism.

Enhanced metal stress tolerance has been reported after PA treatment as a result of the role of PAs in the regulation of antioxidants and the interaction of PAs with endogenous plant hormones [2; 9]. In addition it was suggested that a higher SPD level contributed to enhanced heavy metal tolerance for example in a SPDS-overexpressing transgenic European pear, possibly by exerting antioxidant activity as well as having a metal chelator function [36]. The present results showed that 7 days of 50 μM Cd treatment resulted in Cd uptake, especially in the roots, inducing growth inhibition and oxidative stress, accompanied by a decrease in biomass parameters and an increase in H_2O_2 content and GR and G-POD antioxidant enzyme activities. 0.5 mM PUT pre-treatment alone also caused slight growth inhibition and induced the antioxidant enzymes GR and G-POD in the roots. Although 0.5 mM PUT treatment had no negative effect on biomass or photosynthesis parameters in wheat or maize plants [37], the same

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concentration of higher polyamines (SPD or SPM) [37] or 1 mM PUT [8] decreased the root length and increased the malondialdehyde content or H_2O_2 content in wheat plants. However, the exogenous application of PUT (0.01-1 mM) has also been reported to enhance the root length of rice, while SPD and SPM inhibited it [18]. In the present study, as an additive effect, the greatest growth inhibition and the highest H_2O_2 content and antioxidant activities were detected in the PUTpre+Cd-treated rice plants, suggesting that under these conditions PUT pre-treatment provided no protection against Cd stress. In contrast DFMO treatment alone had hardly any negative effect. In addition, DFMO alleviated the Cd-induced growth inhibition to a certain extent, lowering the root Cd content and the antioxidant enzyme activities in both leaves and roots. In wheat plants the simultaneous addition of 0.1 mM Cd and 1 mM DFMO prevented the Cd-induced increase in PUT level, but did not facilitate normal root growth [8].

Many studies have reported an increased level of PAs when plants are exposed to heavy metal stress [8; 38]. In accordance with this, a higher gene expression level was also detected in several plant species for the enzymes involved in PA synthesis [39]. Due to the interconversion of PAs, the exogenous application of one PA also increased the content of other endogenous PAs in wheat and maize plants [7]. Both the PUTpre and Cd treatments increased the endogenous PA contents, and the PUTpre+Cd treatment induced high PA accumulation, especially in the case of PUT. The ODC and ADC gene expression levels also increased. DFMO treatment alone decreased the PUT content in the leaves, and when applied in combination with Cd, it substantially lowered the root CAD and SPD contents of rice compared to Cd treatment alone. DFMO was also reported to inhibit the key enzyme of CAD synthesis, lysine decarboxylase, in *Leguminosae* [40]. Parallel with these changes, all the treatments increased the root PAO activity, while only treatments involving Cd (Cd, PUTpre+Cd or DFMO+Cd) increased it in the leaves. DAO activity was only influenced in the roots of PUTpre+Cd-treated plants. These results showed that an excess of PAs (accumulation of PAs due to exogenous

PUT and/or Cd treatments) induced several different mechanisms to control endogenous PA levels, such as the conversion of PUT to higher PAs, and the activation of the catabolic enzymes was also detected. The amount of DAP, the product of the catabolism of higher PAs, significantly increased in the PUTpre+Cd treatment, while declining in DFMO- or DFMO+Cd-treated plants, indicating differences in the PA metabolism in response to PUT and DFMO treatments. In the same way, DFMO alone was found to have no influence on the endogenous PUT, SPD or SPM contents in wheat, but in DFMO+Cd-treated plants the root PUT content was lower than in those treated with Cd alone [8].

In order to reveal why DFMO application was more beneficial than excess PUT under Cd stress, the contents of metal-chelating agents, such as thiols and PCs, were then determined. Phytochelatins are synthesised from GSH and able to chelate heavy metals due to their thiol groups, after which the metal–phytochelatin complexes are sequestered into the vacuoles [41]. It has also been found that the genotypic differences in the tolerance of rice plants to Cd stress are related to their ability to enhance of GSH and PC contents [6]. In the present study, after 7 days of PUT treatment a significant decrease in the gene expression level of PCS was found in the leaves of 14-day-old rice plants. This was still manifested in the *in vitro* PCS activity of the leaves in 21-day-old plants (when PUT treatment was followed by a 7-day recovery period: PUTpre), while at this date the thiol and PC contents were not influenced significantly in either the leaves or roots of rice. However, when PUT pre-treatment was followed by Cd stress, both the total thiol and PC contents were depleted compared to treatment with Cd alone or DFMO+Cd. In a similar way, both the GSH and PC levels decreased in the roots of *Canavalia lineata* after SPM+Cd treatment; in addition, SPM+Cd-treated roots were found to contain an additional PC with lower affinity for Cd [11]. In SPDS-overexpressing transgenic European pear the GSH content was significantly depleted under heavy metal stress conditions compared to the wild type [37]. Interestingly, a comparative study on Cd treatment in poplar and willow

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plants revealed that Cd enhanced the phytochelatin content of poplar, especially in the leaves, but not in willow, whereas Cd increased PA contents in both the roots and leaves of willow but only that of putrescine in the roots of poplar [42]. SPM treatment was also found to provide protection against cadmium-induced oxidative damage in wheat, but failed to reverse the depletion of GSH content [8]. However, in rice treatment with SPD and SPM, but not PUT reduced cadmium toxicity, and prevented a cadmium-induced decrease in GSH [9]. This difference in the effect of the treatment with PUT or higher PAs (SPD and SPM) may be explained by the fact that the exogenous application of higher PAs does not induce further synthesis requiring dcSAM, while after PUT treatment, the PUT taken up is interconverted to higher PAs in the PA cycle [7], thereby using up large quantities of Cys. The relationship between PAs and thiols has not yet been considered from this aspect. In contrast, SPD applied in combination with Cr increased the GSH and PC content in radish, but did not lead to normal biomass [12]. As PCs are synthesized from GSH, the cells need to replace the GSH utilized. This, however, is an energy-demanding process, which may cause a delay in growth [3].

5. Conclusions

In certain cases PAs provide protection against heavy metals, even inhibiting Cd uptake or its entry into the cells, as exogenous PA is mainly allocated to the apoplast [8]. However, it should also be taken into consideration that the synthesis of higher PAs and PCs have an antagonistic relationship. This may explain why SPD and SPM, but not PUT, were found to reduce Cd toxicity in rice [9]. Nevertheless, the higher level of SPD and SPM may convert back to PUT or SPD in the PA cycle, leading to higher H_2O_2 accumulation and the inhibition of root elongation [36]. It is also possible that, as PAs are metal chelators, PA-Cd binding reduced the induction of the PC synthesis. However, it has not yet been demonstrated whether PA-Cd complexes are as efficiently compartmentalised to vacuoles as PC-Cd complexes.

At least three inhibition mechanisms may explain the changes in PCS activity in the leaves of rice: 1. PUT pre-treatment decreases the PCS gene expression level, 2. PUT pre-treatment also decreases PCS activity, probably due to the depletion of GSH content by the increased PA metabolism, and 3. in the PUTpre+Cd treatment, where the lowest PCS activity was found, PA-Cd binding may have reduced the amount of free Cd^{2+} to below the level required for the induction of PCS activity, also decreasing PCS gene expression.

Acknowledgements

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Table 1. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 µM Cd(NO$_3$)$_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 µM Cd(NO$_3$)$_2$ treatment on biomass parameters, cadmium and hydrogen peroxide contents in rice plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at P<0.05.

	Control	PUTpre	Cd	PUTpre + Cd	DFMO	DFMO + Cd
Shoot length (cm)	43.12±4.87 c	34.81±4.87 b	36.05±5.7 b	29.87±4.35 a	40.83±2.65 c	34.5±4.89 b
Shoot FW^a (g)	0.49±0.13 b	0.42±0.14 b	0.31±0.09 a	0.31±0.08 a	0.45±0.1 b	0.4±0.09 b
Root length (cm)	19.5±3.23 d	15±2.16 c	11.57±0.79 b	7.68±3.29 a	21.5±1.87 d	15.38±2.14 c
Root FW^a (g)	0.39±0.05 d	0.3±0.06 c	0.18±0.03 b	0.11±0.02 a	0.43±0.06 d	0.22±0.03 b
Leaf Cd (µg g^{-1} DW^b)	0.14±0.05 a	0.24±0.25 a	4.00±0.53 b	6.58±0.62 b	0.09±0.04 a	6.63±0.51 b
Root Cd (µg g^{-1} DW^b)	5.12±0.4 b	1.88±0.57 a	1446.85±222.27 d	1362.86±134.98 d	5.27±0.6 b	989.16±150.2 c
Leaf H$_2$O$_2$ (mM g^{-1} FW^a)	20.77±6.92 a	24.49±3.44 a	40.94±7.76 b	64.87±10.15 c	17.26±5.16 a	51.41±5.98 bc
Root H$_2$O$_2$ (mM g^{-1} FW^a)	11.71±2.21 a	14.44±4.7 a	11.71±1.41 a	10.26±0.36 a	13.16±3.25 a	9.23±5.08 a

a: fresh weight
b: dry weight

Table 2. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM Cd(NO$_3$)$_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM Cd(NO$_3$)$_2$ treatment on 1,3-diaminopropane (DAP), the enzyme activities of diamine oxidase (DAO) and polyamine oxidase (PAO) and the ratio of PUT/(SPD+SPM) in rice plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at P<0.05.

	DAP (nmol g^{-1} FW[a])		DAO (U g^{-1} FW[a])		PAO (U g^{-1} FW[a])		PUT/(SPD+SPM)	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	47.2±14.5 b	2730.7±847.6 b	10.7±2.8 a	11.1±2.6 a	14.6±2.4 a	12.4±0.4 a	0.685	1.016
PUTpre	67.4±1.7 bc	1428.6±254.9 ab	8.9±1.2 a	12±2.3 a	17.2±1.6 a	21.3±0.5 b	1.013	0.846
Cd	52.2±9.9 b	2086.6±231.5 b	10.9±1.2 a	11.7±1.3 a	24±2.2 b	22±1.8 b	1.048	1.888
PUTpre + Cd	78.23±12 c	3918.1±881.7 b	10.5±0.9 a	18.6±1.2 b	24.7±3.9 b	32.3±0.9 c	2.947	2.638
DFMO	27.4±6.3 a	693.7±211 a	10.7±0.4 a	13.9±1.6 a	13.7±0.6 a	21.4±1.2 b	0.375	0.762
DFMO + Cd	24.2±5.9 a	573.5±38.7 a	9.7±0.4 a	12.9±1.4 a	25.2±1.2 b	21.1±2.7 b	1.404	2.196

a: fresh weight

Table 3. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM Cd(NO$_3$)$_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM Cd(NO$_3$)$_2$ treatment on total thiol contents, namely cysteine (Cys), *gamma*-glutamyl-cysteine (*gamma*-Glu-Cys), hydroxylmethyl-glutathione (hmGSH) and glutathione (GSH) in rice plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at P<0.05.

	Cys (nmol g^{-1} FW[a])		gamma-Glu-Cys (nmol g^{-1} FW[a])		hmGSH (nmol g^{-1} FW[a])		GSH (nmol g^{-1} FW[a])	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	2.06±0.16 a	2.55±0.33 b	0.83±0.42 a	0.19±0.06 a	25.4±1.18 b	29.6±1.72 bc	8.97±1.74 a	10.96±2.96 ab
PUTpre	2.25±0.42 a	2.26±0.4 ab	1.53±0.13 b	0.17±0.03 a	24.75±3.76 abc	28.48±0.05 b	9.86±0.73 a	9.4±1.34 a

Cd	2.45±0.76 a	2.55±0.46 b	2.17±0.33 c	0.64±0.2 c	28.14±1.91 c	28.97±1.38 b	11.67±0.97 b	10.76±0.47 ab
PUTpre + Cd	1.75±0.14 a	1.78±0.06 a	1.8±0.2 bc	0.24±0.1 ab	20.83±2.78 a	23.84±0.71 a	9.04±1.26 a	8.15±1.72 a
DFMO	2.17±0.08 a	2.955±0.13 b	0.97±0.21 a	0.16±0.02 a	24.95±0.63 b	30.54±0.37 c	9.83±0.65 a	11.61±0.13 b
DFMO + Cd	2.18±0.63 a	2.65±0.27 b	1.26±0.52 ab	0.48±0.18 bc	27.1±2.25 c	28.54±0.02 b	9.65±2.15 ab	10.2±1.21 a

a: fresh weight

Legends:

Figure 1. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM $Cd(NO_3)_2$ treatment on glutathione reductase and guaiacol peroxidase enzyme activities in the leaves (A, C) and roots (B, D) of rice plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at P<0.05.

Figure 2. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM $Cd(NO_3)_2$ treatment on free polyamine contents in the leaves (A) and roots (B) of rice plants. White bars: putrescine (PUT); grey bars: spermidine (SPD) and black bars: spermine (SPM). Data represent mean values ±SD, n=5. Different letters indicate significant differences between the treatments at P<0.05.

Figure 3. Effects of 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM $Cd(NO_3)_2$ treatment on free cadaverine (CAD) contents in the leaves and roots of rice plants. Data represent mean values ±SD, n=5. Different letters indicate significant differences between the treatments at P<0.05. nd: not detected.
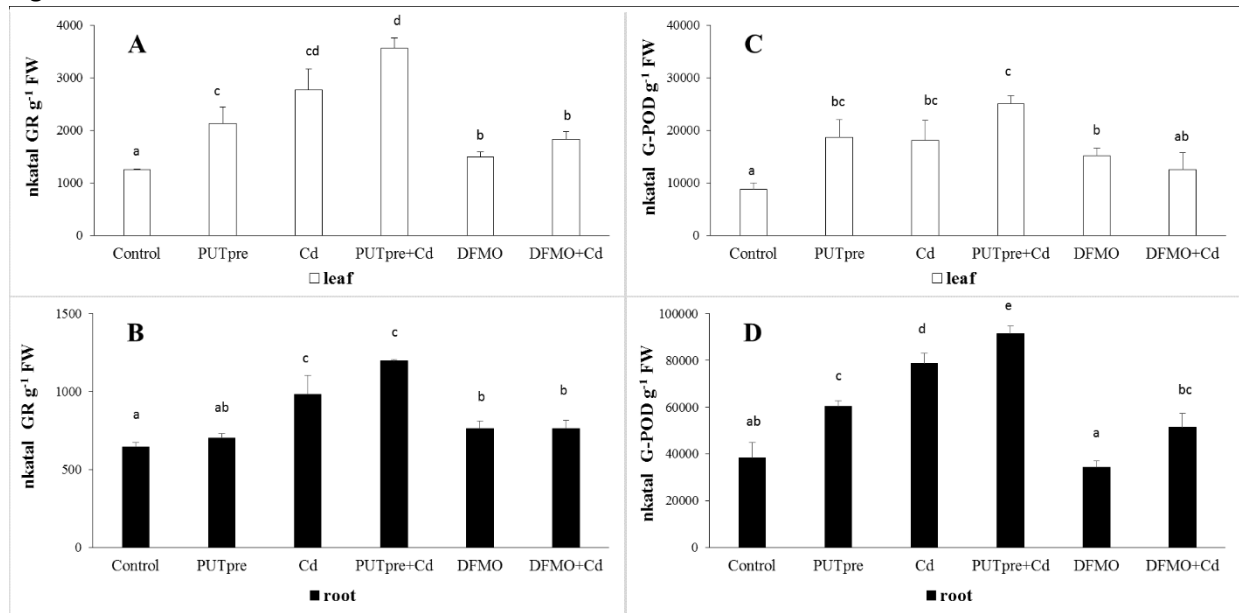
Figure 4. Changes in the phytochelatin, PC_2, PC_3 and PC_4 contents in the leaves (A) and roots (B) and phytochelatin synthase activity (C) of the leaves and roots of rice plants after 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM $Cd(NO_3)_2$. Data represent mean values ±SD, n=5. Different letters indicate significant differences between the treatments at P<0.05.

Figure 5. Changes in gene expression of the ornithine decarboxylase: ODC (A), arginine decarboxylase: ADC (B), spermidine/spermine synthase: SPDS/SPMS (C) and phytochelatin
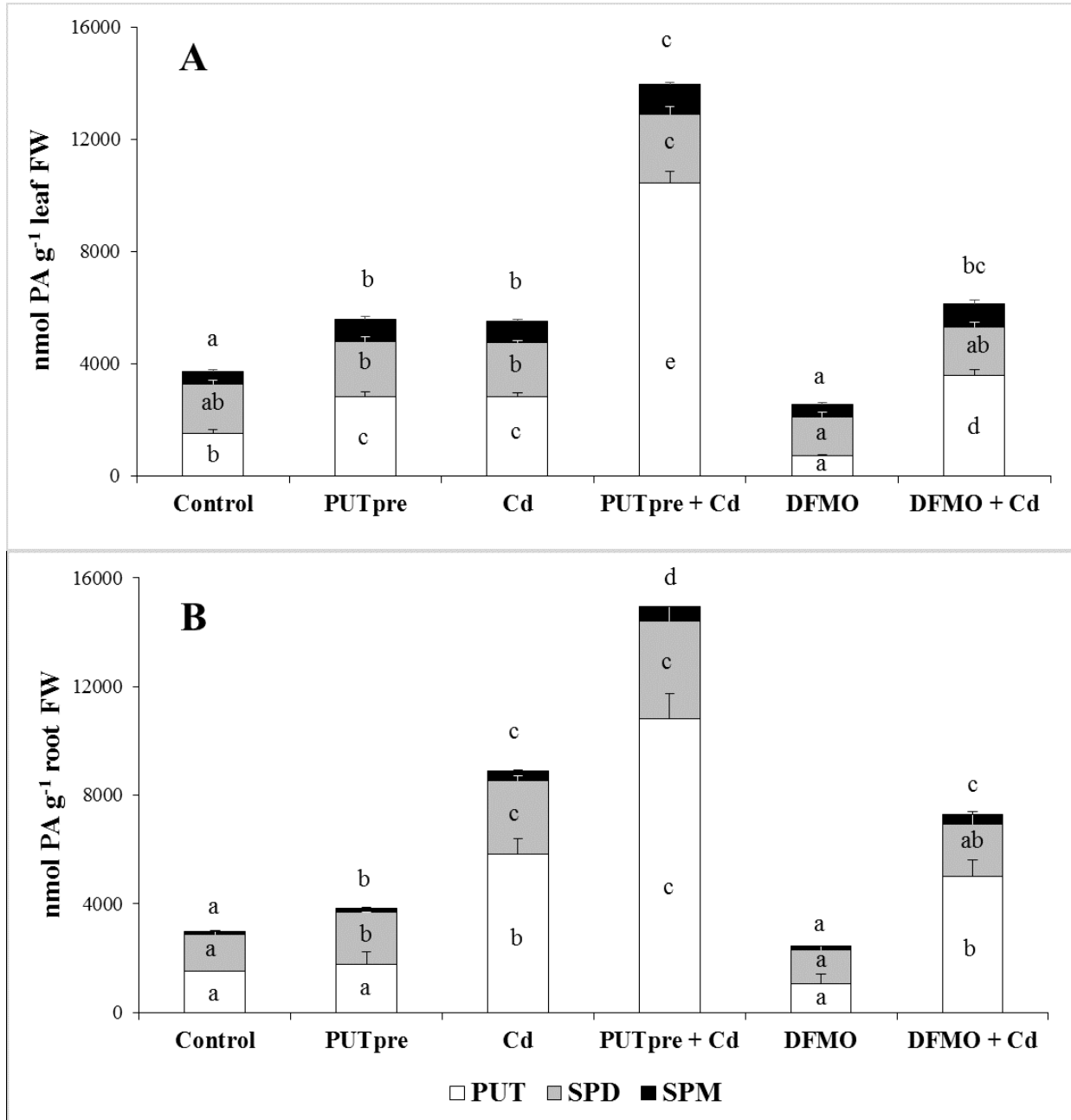
synthase: PCS (D) genes in the leaves of rice plants after 0.5 mM putrescine pre-treatment (PUTpre) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd), and 2-(difluoromethyl)ornithine (DFMO) with or without 50 μM $Cd(NO_3)_2$. Data represent mean values ±SD, n=3. *, ** and ***: significant differences between fold changes at the $P<0.05$, 0.01 and 0.001 level, respectively.

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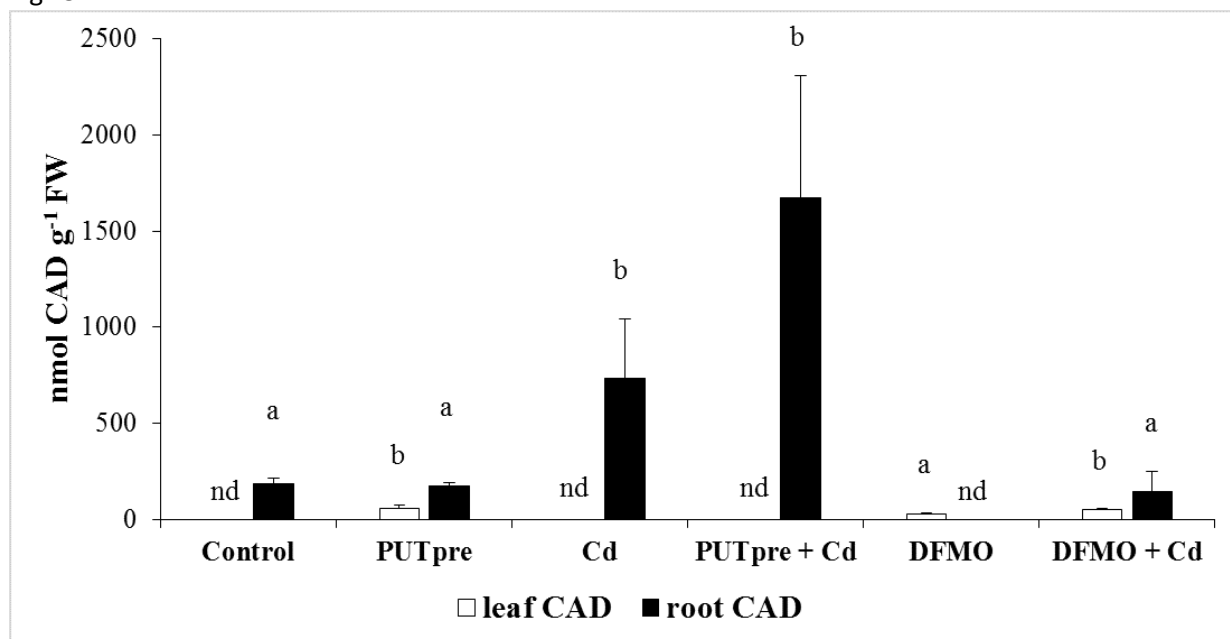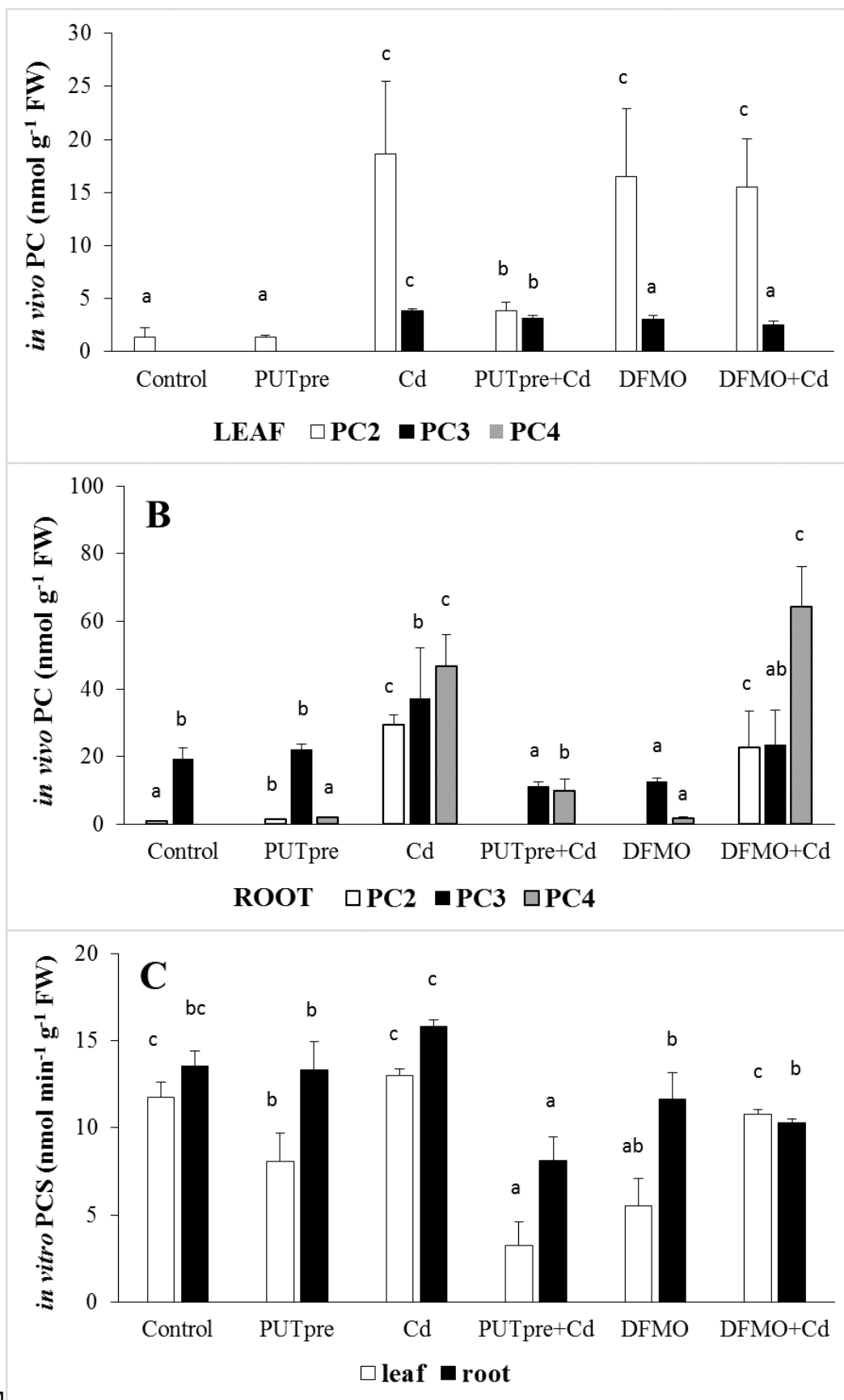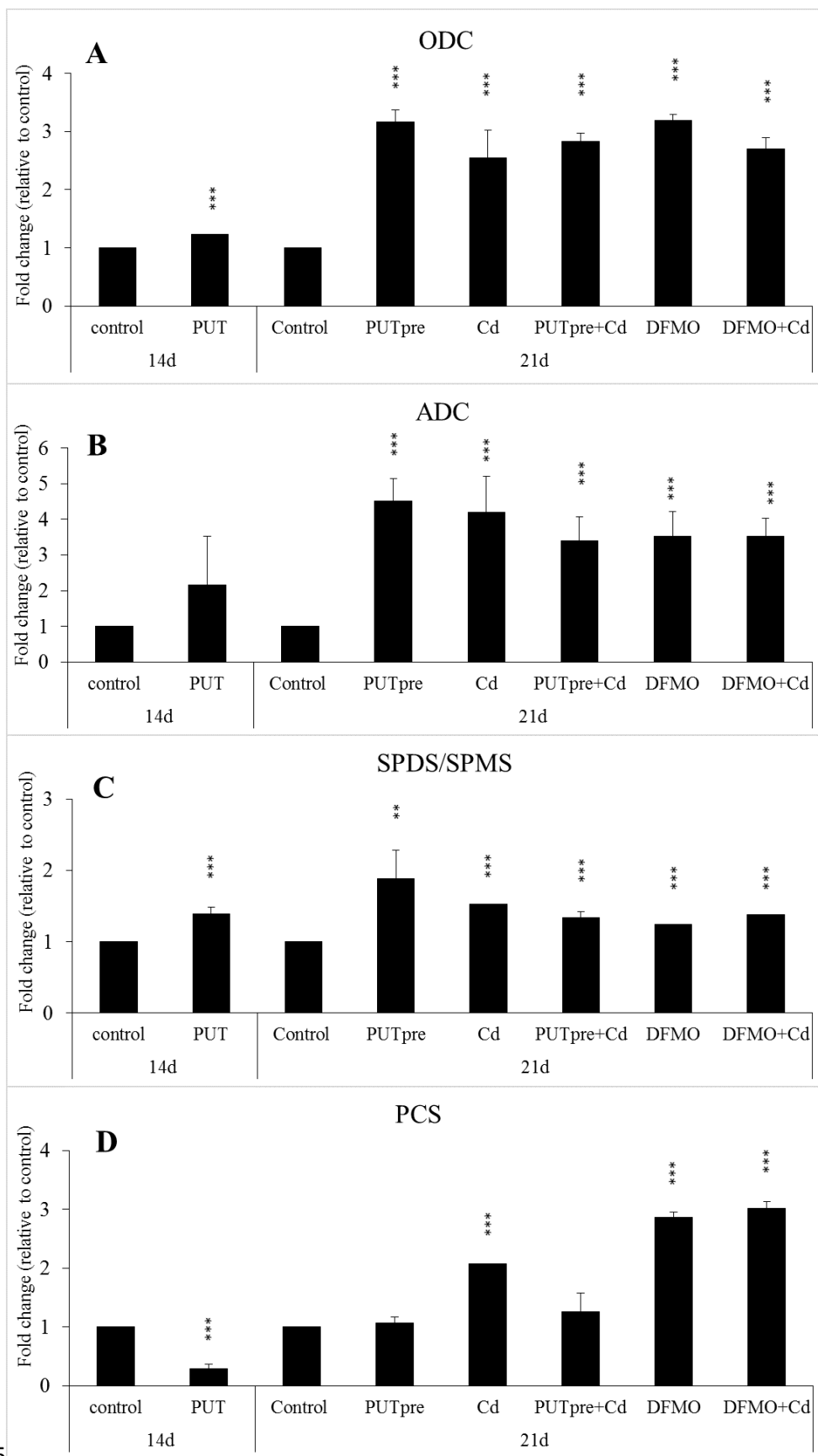
Figr-1

Figr-2

Figr-3

Figr-4

Figr-5