***Highlights (for review)**

1. Putrescine was beneficial both as seed soaking and applied hydroponically under Cd stress.
2. Spermidine seed soaking was beneficial, but added hydroponically, enhanced Cd-induced stress.
3. High putrescine accumulation may be in relation with the negative effect of spermidine.
4. The putrescine content was correlated with the accumulation of Cd and salicylic acid.

Comparative study on the effects of putrescine and spermidine pre-treatment on cadmium stress in wheat

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Abstract

In several cases a correlation was found between polyamines and abiotic stress tolerance. However, the individual polyamines may have different effects, which also vary depending on the type of treatment. When applied as seed soaking or added hydroponically 0.5 mM putrescine and spermidine, different changes were induced during 50 µM cadmium stress in wheat plants. Seed-soaked plants were exposed to cadmium immediately after germination for 5 days, while plants pre-treated with polyamines hydroponically were stressed at age of 14 days for 7 days.

Putrescine pre-treatment was beneficial both as seed soaking and applied hydroponically, while spermidine only had a protective effect in the case of seed soaking, enhancing the Cd-induced oxidative stress when were pre-treated hydroponically. The differences observed were related to the polyamine metabolism. The accumulation of endogenous putrescine beyond a certain amount may be in relation with the negative effect of hydroponic spermidine pre-treatment during Cd stress. The increased putrescine content was also correlated with the highest accumulation of Cd, salicylic acid and proline contents in plants treated with a combination of spermidine and Cd. However, the expression level of the gene encoding phytochelatin synthase was only influenced by hydroponically applied spermidine, which decreased it under cadmium stress. Changes in the activities of antioxidant enzymes, diamine and polyamine oxidases were also discussed.

Keywords: cadmium; phytochelatin synthase; putrescine; salicylic acid; spermidine; wheat

Abbreviations: ADC: arginine decarboxylase; APX: ascorbate peroxidase; DAO: diamine oxidase; CAT: catalase; GR: glutathione reductase; G-POD: guaiacol peroxidase; GST: glutathione-S-transferase; ODC: ornithine decarboxylase; PCS: phytochelatin synthase; PA: polyamine; PAO: polyamine oxidase; PUT: putrescine; SA: salicylic acid; SPD: spermidine; SPM: Spermine.

1. Introduction

Investigations on compounds capable of reducing the stress sensitivity of plants are of great importance in the ever changing environment, where the accumulation of heavy or other toxic metals further inhibits the growth and development of plant organisms. Polyamines (PAs) could be promising compounds for the reduction of abiotic stress sensitivity in plants.

The most abundant PAs, namely putrescine (PUT), spermidine (SPD) and spermine (SPM), their biosynthetic pathways and the key enzymes of their metabolism are well documented. Putrescine is synthesized by the decarboxylation of ornithine, catalysed by ornithine decarboxylase (ODC), or indirectly by the decarboxylation of arginine by arginine decarboxylase (ADC), via agmatine. Higher polyamines (SPD and SPM) are produced by the sequential addition of aminopropyl moieties to the PUT skeleton through enzymatic reactions catalysed by the spermidine and spermine synthases (SPDS and SPMS). S-adenosylmethionine decarboxylase is responsible for the synthesis of decarboxylated S-adenosylmethionine which is used for the addition of the aminopropyl moiety. PUT is catabolized by diamine oxidases (DAO) and SPD and SPM by polyamine oxidases (PAOs) (Moschou et al., 2012; Liu et al., 2015).

Although in several cases a correlation was found between the endogenous PA content and stress tolerance, this relationship cannot be generalized (Minocha et al., 2014; Pál et al., 2015). PAs should no longer be considered simply as direct protective molecules, but rather as compounds that are involved in a complex signalling system and have a key role in the regulation of stress tolerance. PA metabolism and signalling are involved in interactions with other metabolic pathways and hormonal cross-talk. Furthermore, individual PAs can be converted into each other in the PA cycle (Pál et al., 2015). Thus, the precise mechanism of how PAs control plant responses is largely unknown. There are still gaps in our information

78 on the regulation of PA biosynthesis under control and stress conditions, on their role in gene
79 expression and on their interaction with plant hormones.
80 Greater attention should be paid to cadmium (Cd), not only due to its high penetration
81 ability, but also because it causes subsequent damage to animals and humans when entering
82 the food chain (di Toppi and Gabrielli, 1999; Pál et al., 2006). Cd induces a number of
83 physiological changes, from visual symptoms, such as growth inhibition and chlorosis, to the
84 inhibition of photosynthesis, the formation of free radicals and the induced synthesis of
85 protective compounds. Among the potential defence mechanisms, chelation, followed by
86 compartmentalisation to the vacuoles, can be performed by compounds of thiol origin such as
87 phytochelatins (PCs). The early activation of PC synthesis was reported in several plant
88 species during cadmium stress (Stolt et al., 2003; Pál et al., 2005; Kovács et al., 2014a;
89 López-Climent et al., 2014; Gondor et al., 2016). Cd stress has been reported to increase the
90 PUT content and to induce the enzymes involved in its synthesis in the leaves of wheat plants,
91 while Cd-induced oxidative stress was alleviated by PA treatment in the same plant species
92 (Groppa et al., 2007). However only a few controversial studies have been published on the
93 effect PA treatment on PC synthesis under heavy metal stress, especially on the gene
94 expression level (Groppa et al., 2007; Hsu et al., 2007; Nahar et al., 2016; Pál et al., 2017).
95 The content of the plant hormone salicylic acid (SA) has also been reported to exhibit
96 a concentration-dependent increase after treatment with Cd (Pál et al., 2005). Although other
97 authors found no increase in the endogenous SA content (Landberg and Greger, 2002), or no
98 direct relationship between the SA content and the level of Cd tolerance in wheat, it has been
99 suggested that SA has some role in heavy metal tolerance (Kovács et al., 2014a). A
100 relationship between SA and PAs was suggested by several studies performed under control
101 and stress conditions (Németh et al., 2002; Pál et al., 2013; Szalai et al., 2017).

Although PAs are usually considered as a family of similar molecules, different PAs may have different effects, and the protective effect may vary as a function of the type of treatment. In order to show the differences between the various PA treatments and to gain a better understanding of the role of PAs under heavy metal conditions, the present work investigated the background of the potential effect of PA treatment (PUT and SPD applied as seed soaking or hydroponic pre-treatment) on wheat plants, in relation to changes in the PA pool. A possible relationship with SA was also studied under Cd stress conditions, as well as changes in the gene expression level of phytochelatin synthase, one of the key enzymes in heavy metal detoxification.

2. Materials and methods

2.1. Plant material and growth conditions

The wheat (*Triticum aestivum* L.) variety Emese was selected for the present experiment based on a previous study on Cd stress in wheat (Kovács et al., 2014b), while the PUT and SPD concentration applied as a pre-treatment was chosen on the basis of a hydroponic study performed also on wheat (Szalai et al., 2017). In order to compare differences between the different PA application modes, the same concentration of PUT or SPD was used for seed pre-treatment.

In the first experiment, the seeds were divided into three groups, one of which was primed in distilled water, while the others were treated with 0.5 mM PUT or SPD. After 16 h soaking the seeds were moved to filter paper and allowed to germinate for 3 days, after which half of each group was grown under control conditions (control), while the others were treated hydroponically with 50 µM $Cd(NO_3)_2$ for 5 days (Cd, PUTss+Cd and SPDss+Cd) in modified Hoagland solution including 0.3125 mM KNO_3, 0.45 mM $Ca(NO_3)_2$, 0.0625 mM KH_2PO_4, 0.125 mM $MgSO_4x7H_2O$, 11.92 µM HBO_3, 4.57 µM $MnCl_2x4H_2O$, 0.191 µM $ZnSO_4x7H_2O$,

0.08 µM $CuSO_4x5H_2O$, 0.024 µM $(NH_4)_6Mo_7O_{24}x4H_2O$, 15.02 µM $FeSO_4x7H_2O$, and 23.04 µM $Na_2EDTAx5H_2O$ (Pál et al., 2005) at 20/18°C with 16/8-h light/dark periodicity and 75% relative humidity in a Conviron PGR-36 plant growth chamber (Controlled Environments Ltd, Winnipeg, Canada). The photosynthetic photon flux density was 250 µmol m^{-2} s^{-1}. Glass beakers were used, with 12 plants/beaker. Plants were grown on stainless steel net.

In the second experiment the wheat seeds were germinated and grown under control conditions for a week, after which three groups were formed, one control and two PA treatments (0.5 mM PUThyd or SPDhyd), all of which were moved to plastic pots for 7 days in order to avoid the interaction between PAs and glass. After that the roots were washed and the plants were either grown under control growth conditions, for a recovery period (control) or treated with 50 µM $Cd(NO_3)_2$ for 7 days (Cd, PUThyd+Cd and SPDhyd+Cd), again in glass beakers without PAs.

2.2. Chlorophyll-a fluorescence induction measurements

Chlorophyll-*a* fluorescence was measured using a pulse amplitude modulated fluorometer (Imaging-PAM M-Series Fluorometer; Walz, Effeltrich, Germany). The maximum quantum yield of PSII photochemistry, Fv/Fm, was calculated as Fv/Fm = (Fm-F0)/Fm, where Fm is the maximal fluorescence induced by a saturating flash (8000 µmol m^{-2} s^{-1} PPFD for 0.8 s) in leaves dark-adapted for 20 min, and F0 is the minimum chlorophyll fluorescence yield in the dark (PPFD < 1 µmol m^{-2} s^{-1}). The effective PSII quantum yield (ΔF/Fm'), which represents the proportion of absorbed light energy consumed in photochemistry, was measured at a light intensity of 250 µmol m^{-2} s^{-1} and calculated as (Fm'-F)/Fm', where Fm' is the maximal fluorescence level induced by a saturating light pulse in the steady state light-adapted state, and F is the steady state chlorophyll fluorescence

151 immediately prior to the flash. Measurements were performed on the last fully expanded
152 leaves.
153
154 *2.3. Determination of growth biomarkers and Cd contents*
155 Measurements were made on the root and shoot length and the fresh weight of the root
156 and shoot. The Cd content in the leaves and roots was determined from air-dried
157 samples (approx. 0.5 g of each sample) using the inductively coupled plasma-atomic
158 emission spectrometry method (ICP-AES, Jobin-Yvon Ultima 2 sequential instrument)
159 after microwave Teflon bomb digestion with cc. HNO+HCl (Anton et al., 2012).
160
161 *2.4. Proline content*
162 The proline content was determined on the basis of its reaction with ninhydrin,
163 according to the Bates method (Bates et al., 1973).
164
165 *2.5. Enzyme assays*
166 For the analysis of antioxidant enzyme activity, 0.5 g tissue (fresh leaves or roots) was
167 homogenized in 2.5 mL ice-cold Tris-HCl buffer (0.5 M, pH 7.5) containing 3 mM $MgCl_2$
168 and 1 mM EDTA, and measurements were performed as described by Pál et al. (2005). The
169 ascorbate peroxidase (APX; EC 1.11.1.11.) activity of the extract was measured
170 spectrophotometrically by monitoring the decrease in absorbance at 290 nm, while the
171 catalase (CAT; EC 1.11.1.6.) activity was determined by the decrease in absorbance at 240
172 nm. The guaiacol peroxidase (G-POD; EC 1.11.1.7.) activity was determined at 470 nm and
173 the glutathione reductase (GR; EC 1.6.4.2.) activity at 412 nm. The glutathione-S-transferase
174 (GST; EC 2.5.1.18.) activity was measured by monitoring changes in the absorbance at 340
175 nm. Antioxidant enzyme activities were expressed in nkatal g^{-1} fresh weight (FW).

176

177 *2.6. PA analysis*

178 The analysis was carried out as described by Németh et al. (2002). The free fraction of
179 PAs, namely PUT, SPD and SPM were analysed as dansylated derivatives via HPLC using a
180 W2690 separation module on a reverse phase column (Kinetex C18, 5µ, 100 x 4.6 mm,
181 Phenomenex, Inc.) and a W474 scanning fluorescence detector with excitation at 340 nm and
182 emission at 515 nm (Waters, Milford, MA, USA).

183

184 *2.7. Diamine oxidase and polyamine oxidase enzyme activities*

185 The enzyme activities of diamine oxidase (DAO, EC 1.4.3.6.) and polyamine oxidase
186 (PAO, EC 1.5.3.3.) were estimated by the method of Takács et al. (2016) and expressed as
187 enzyme units per g fresh weight (U g^{-1} FW).

188

189 *2.8. SA extraction and analytical procedure*

190 SA extraction and analysis were performed according to Pál et al. (2005). After
191 separation on a reverse phase column (ABZ+, 150x4.5 mm, 5 µm, Supelco, Bellefonte, USA)
192 SA was quantified fluorimetrically (excitation: 305 nm; emission: 407 nm) using a W474
193 fluorescence detector (Waters, USA).

194

195 *2.9. Gene expression analysis*

196 For the gene expression analysis, fully developed leaf and root samples were collected in
197 three biological replicates. Each biological replicate was prepared with three technical repetitions.
198 Total RNA was extracted from samples using TRI Reagent®, after which the samples were treated
199 with DNase I, cleaned with a Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA)
200 according to the manufacturer's instructions. The purity and concentration of RNA was assessed by
201 using Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and the

samples were also run on agarose gel stained with ethidium bromide to check the integrity of total RNA. cDNA synthesis was made from 1 µg of total RNA using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA). Gene-specific primers (ADC, ODC and PCS) and housekeeping primers (Ta30797: Paolacci et al. 2009) (Suppl. Table 1), PCRBIO SyGreen Mix (PCR Biosystems, London, UK) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) were used for the quantitative real-time PCR reaction. The relative gene expression values were determined with the ΔCt method. Threshold cycle (Ct) values were normalized using the Ct values of housekeeping gene Ta30797, encoding phosphogluconate dehydrogenase.

2.10. Statistical analysis

The results were the means of at least fifty replicates for each treatment for the biomass parameters and of 5 replicates for the Cd content, enzyme activity and HPLC analysis. The data were statistically evaluated using the standard deviation and *t-test* methods.

3. Results

3.1. Biomass parameters, chlorophyll content, chlorophyll-a fluorescence parameters, cadmium and proline contents

In the first experiment (seed soaking) the biomass parameters showed that Cd treatment dramatically decreased the shoot and root growth and development of wheat seedlings (Table 1). The PUTss treatment did not influence the growth parameters and SPDss only induced a slight, but statistically significant increase in the shoot length. The combined treatment PUTss+Cd decreased the negative effect of Cd on shoot and root length and shoot FW, while SPDss only alleviated the Cd-induced decrease in shoot FW. Cd application significantly increased the Cd content in both of the leaves and roots of wheat plants, and similar values were also recorded after the PUTss+Cd and SPDss+Cd treatments (Table 1). Cd treatment decreased the chlorophyll content and the optimal (Fv/Fm) and effective

228 (ΔF/Fm') quantum yield of PSII, however, this was alleviated by PUTss and even more by
229 SPDss. The proline content increased in the roots of plants pre-treated with PUTss or SPDss.
230 Cd treatment also increased its level in the roots, but no additive effect of the combined
231 treatments (PUTss+Cd or SPSss+Cd) was found (Table 1). Neither PA pre-treatment nor Cd
232 treatment alone influenced the proline level in the leaves, while the PUTss+Cd and SPDss+Cd
233 treatments increased it.

234 In the second experiment, when 14-day-old plants were exposed to 7 days of Cd
235 stress, significantly decrease in the chlorophyll content, the Fv/Fm and ΔF/Fm' chlorophyll-*a*
236 fluorescence parameters in the leaves, while increase in the proline content in the roots were
237 detected. Hydroponically applied 0.5 mM PUT pre-treatment for 7 days induced greater shoot
238 and root development, manifested mainly in the shoot and root length parameters (Table 2).
239 The positive effect of PUT during Cd stress was proved by the shoot biomass parameters, as
240 the PUThyd+Cd plants had longer shoots than the Cd-treated plants and even than the control.
241 The chlorophyll content and ΔF/Fm' parameter were higher after PUThyd+Cd treatment than
242 after Cd alone. In comparison, when 0.5mM SPD was applied alone it had a negative effect
243 on root growth and chlorophyll content, and induced high proline accumulation in both the
244 leaves and roots; furthermore, SPDhyd pre-treatment enhanced the effect of Cd, as the lowest
245 biomass values and chlorophyll content and the highest proline accumulation were recorded
246 after SPDhyd+Cd treatment. The highest Cd accumulation was found in the leaves and roots
247 of SPDhyd+Cd-treated plants. However, the PUThyd+Cd treatment resulted in a root Cd
248 content similar to that of plants treated with Cd alone, while the leaf Cd content was higher
249 (Table 2).

250

251 ***3.2. Antioxidant enzyme activities***

252 Cd treatment usually induces oxidative stress in plants. In the seed soaking experiment
253 it resulted in increased GR, CAT, APX and G-POD activities, which were more pronounced
254 in the leaves, than in the roots in the case of GR, APX and G-POD. PAs applied alone as
255 seed soaking had no influence on these enzymes (Table 1), but after Cd treatment the positive
256 effects of PUT and SPD seed pre-treatments were manifested in the lower activity of CAT in
257 the leaves and roots, APX in the leaves, and GST in the roots, suggesting lower level of
258 damage. These results, together with the biomass and photosynthesis parameters, suggest that
259 pre-treatment of the seeds with PUT or SPD reduced the adverse effect of Cd to a certain
260 extent.

261 In the second experiment Cd stress also induced G-POD activity in the leaves and GR,
262 CAT and APX activity in the roots (Table 2). PA treatments alone applied hydroponically
263 influenced the antioxidant enzymes activities, as PUThyd pre-treatment decreased the leaf
264 CAT and APX activity, while SPDhyd pre-treatment tended to induce the antioxidant system,
265 leading to increased GR and GST activity in the roots, suggesting that SPD stressed the
266 plants. Similar antioxidant enzyme activities were observed in the PUThyd+Cd and Cd
267 treatments, while the SPDhyd+Cd treatment resulted in the highest GR, GST, APX and G-
268 POD activities in the roots, suggesting greater response to oxidative pressure as an additive
269 effect of SPD and Cd.

270

271 ***3.3. Changes in PA content and catabolism***

272 Soaking seeds in PUT and SPD alone induced various changes in the PA contents.
273 The PUTss treatment did not significantly influence the PUT, SPD or SPM levels in the
274 leaves, but decreased the PUT level in the roots. In contrast, SPDss treatment increased the
275 amounts of PUT and SPM in the leaves, revealing the functioning of the PA cycle (Fig. 1A-
276 C). Cd stress caused a great accumulation of PUT in both the leaves and roots, but hardly

277 influenced the level of higher PAs (SPD or SPM), as only slight though statistically
278 significant decrease in root SPD and leaf SPM concentration were observed (Fig. 1A-C). Cd-
279 induced increases in the PUT level was also found in PUTss+Cd and SPDss+Cd-treated
280 plants. In the case of the leaves of PUTss+Cd-treated plants this increment was tendentiously
281 but not statistically significantly lower compared to the other Cd treatments (Cd or
282 SPDss+Cd)(Fig. 1A-C).

283 The DAO and PAO activities in the leaves were hardly influenced by the
284 treatments, except for PA seed soaking alone, where the DAO activity decreased in the PUTss
285 and increased in the SPDss treatment (Table 1). In the roots Cd treatment either alone or in
286 combination with PAs decreased the DAO activity. The lowest root DAO and PAO activities
287 were observed in the case of the PUTss+Cd treatment, while the SPDss pre-treatment only
288 slightly alleviated the decreasing effect of Cd stress (Table 1).

289 The PUThyd and SPDhyd treatments alone increased the endogenous PA content
290 (PUT, SPD and even SPM), especially in the roots (Fig. 1D-F), showing that the PUT or SPD
291 taken up by wheat plants is metabolised in the PA cycle. Among the PAs the most
292 pronounced changes were observed in the PUT content. Besides the PA pre-treatments, Cd
293 also increased the PUT level in the leaves and roots, and the combined treatments
294 (PUThyd+Cd or SPDhyd+Cd) were found to have an additive effect, with the highest
295 accumulation of PUT in the SPDhyd+Cd treatment.

296 As also found in the first experiment (seed soaking treatment), PUT pre-treatment
297 decreased but Cd did not change the DAO activity in the leaves statistically significantly
298 compared to the control plants (Table 2). However, leaf DAO activity was lower in
299 SPDhyd+Cd than in plants treated only with Cd, which was also manifested in the lower Cd-
300 induced increment of PUT in the leaves. None of the treatments influenced the DAO activity

301 in the roots or the PAO activity in the leaves, and only Cd, either alone or in combination with
302 SPD pre-treatment, increased the root PAO activity.
303
304 ***3.4. SA content***
305 PA pre-treatments applied as seed soaking did not influence the SA content. Cd
306 alone induced a dramatic accumulation of SA in both the free and bound forms in the leaves,
307 while it only increased the bound form in the roots (Fig. 2A-B). However, when PA pre-
308 treatment preceded the Cd stress, there was a less pronounced increase in the SA in the leaves,
309 with the lowest SA level in the case of the SPDss+Cd treatment.
310 PUThyd pre-treatment decreased the SA content, especially in the bound form both
311 in the leaves and roots, while SPDhyd did not influence it (Fig. 2D-E). Cd stress induced SA
312 accumulation in the leaves of wheat. When PUT pre-treatment preceded Cd stress lower leaf
313 SA content was detected, especially in the bound form, while in the case of the SPDhyd+Cd
314 treatment a higher root level of both free and bound SA was found compared to Cd stress
315 alone.
316
317 ***3.5. Gene expression***
318 The transcription levels of ADC and ODC genes, encoding the synthesis enzymes of
319 PUT, showed different pattern, ADC showed higher level of expression than ODC. Changes
320 in the gene expression level of them were depended on the type of the PA treatments. PUT or
321 SPD as seed soaking alone did not influence the expression of either ADC or ODC, while
322 when were applied hydroponically, these PAs significantly increased the expression level of
323 ADC, but not ODC (Fig. 3A-C). Cd stress also increased the level of ADC transcript in both
324 of the experiments (seed-soaking and hydroponic treatments) (Fig. 3A-B). However, in case
325 of the combined treatments, PA seed-soaking, especially SPDss+Cd increased the ADC and

ODC expression, while in the hydroponic treatment lower transcription levels were found in the leaves of PUThyd+Cd- and SPDhyd+Cd-treated plants than in the Cd-treated ones (Fig. 3B-D).

In the first experiment, only PUTss pre-treatment decreased the expression level of PCS gene in the leaves, while no significant changes were found after Cd treatments (Fig. 3E). In the second experiment, although PUThyd or SPDhyd pre-treatment and Cd stress had no influence on the expression of the PCS gene, SPD pre-treatment in combination with Cd significantly decreased it in the leaves of the 21-day-old wheat plants (Fig. 3F).

4. Discussion

Many studies have reported an increased level of PAs when plants are exposed to abiotic stress conditions; furthermore, enhanced abiotic tolerance has been reported after various PA treatments, mainly due to PAs acting as antioxidants or influencing the antioxidant system (Ghosh et al., 2012; Parvin et al., 2014). However, recent reviews have indicated that PAs are key compounds in signalling, interacting with endogenous plant hormones and influencing several defence mechanisms even at the gene expression level (Liu et al., 2015; Miller-Fleming et al., 2015; Pál et al. 2015).

PAs are usually considered to be similar molecules with roborant and protective roles. However, not only may different PAs have different effects, but the effect may also vary as a function of the type of treatment. In the present experiment the protective effect of PUT and SPD used in the same concentration largely depended on the type of treatment. Both PUT and SPD were effective against Cd stress in wheat when used for seed soaking, but only PUT was beneficial in the case of hydroponic pre-treatment. The positive effect of the PUTss treatment was manifested mainly as an increase in biomass parameters, that of SPDss as improved photosynthesis-related parameters, and hydroponic PUT pre-treatment increased shoot

biomass production, chlorophyll-*a* fluorescence parameters and chlorophyll content under cadmium stress conditions. In contrast, spraying with SPD and SPM were found to reduce Cd stress in detached rice leaves, while PUT did not (Hsu et al., 2007).

Several studies have demonstrated the protective role of PAs against various stress factors through the regulation of cation concentration, antioxidants, photosynthesis, phytohormones and gene expression (Minocha et al., 2014; Pál et al., 2015). It is evident that individual PAs (PUT, SPD or SPM) have different effects, mainly due to differences in the PA metabolism induced in the plant by PA treatment. When applied hydroponically, PUT did not induce any negative changes in either wheat or maize plants, while the same concentration of higher PAs (SPD and SPM) induced growth inhibition and oxidative stress, especially in maize, which could be explained by the high accumulation of PUT in the PA cycle (Szalai et al., 2017). In the present experiment we focused on the comparison of the effects of different PA treatments on wheat plants under Cd stress involving changes in the PA metabolism. In the first experiment only SPDss increased the SPM content in the leaves, while in the second experiment PUThyd or SPDhyd increased the PUT and SPD contents especially in the roots, and the SPM level only rose in the roots, suggesting that PUT or SPD uptake resulted in further synthesis to SPD or SPM. As also reported by other authors (Groppa et al., 2007; 2008), the greatest increase in PA content induced by Cd was observed for PUT in the leaves and roots of wheat plants, in both the experiments, which can be explained by the increased gene expression level of ADC. Although the combined treatments, PUTss+Cd, SPDss+Cd and even PUThyd+Cd resulted in similar PA patterns, SPDhyd+Cd caused higher leaf PUT accumulation than the corresponding Cd treatment. The PA pool is dynamic, changing over time as PAs undergo rapid interconversion in the polyamine cycle (Pál et al., 2015). The most abundant PAs are PUT, SPD and SPM in the order of the synthesis pathway, but usually SPD is the most dominant PA in wheat. PA pool is influenced by the age of the plants, as higher

PA level can be found in young seedlings, compared the older one; the contents of PA also change during the day; furthermore differences may occurs between different organs (root, stem, leaf or seed), upper and lower leaves, or between basal and apical leaf segments (Paschalidis and Roubelakis-Angelakis, 2005). Plants try to maintain an optimum PA pool and PA ratio, as the accumulation of PUT in the present experiment resulted from the uptake of PUT (PUThyd+Cd) or the back-conversion of the SPD taken up to PUT (SPDhyd+Cd), leading to a high PUT/(SPD+SPM) ratio, has been reported to result in plant injury under stress conditions (Shu et al., 2012). That may also be the reason why the inhibition of the expression of ODC was found in PUThyd+Cd- and SPDhyd+Cd-treated plants (Suppl. Fig.1.). The resulting high PUT level in SPDhyd+Cd-treated plants may have negative effects, as shown by the decrease in biomass parameters and chlorophyll content and the highest Cd accumulation in both the leaves and roots. Although increased PA content was found to be responsible for the Cd-induced damage, the inhibition of PUT synthesis could not restore normal root growth in wheat plants (Groppa et al., 2008). The uncontrollable increase in the PUT level may also explain the negative effect of higher PAs (SPD and SPM) observed in maize (Szalai et al., 2017). However, SPD has been reported to stimulate root Cd uptake, but not translocation into the leaves of Brassica juncea (Aoun et al., 2009). SPD and SPM treatments decreased the Cd content in detached rice leaves (Hsu and Kao, 2007). In the present experiments, Cd treatments in combination with SPD resulted in the highest Cd accumulation in the roots; however, the translocation of Cd into the leaves in SPDhyd+Cd-treated plants was not as intensive as in the case of PUThyd+Cd treatment. PAs were also found to have a metal-chelator function (Groppa et al., 2007; Wen et al., 2010), indicating a relationship between metal toxicity and the pattern of PA accumulation, as recently reported in the case of rice (Pál et al., 2017) but it has not been demonstrated clearly yet whether PA-Cd complexes are as efficiently compartmentalised to vacuoles as PC-Cd complexes.

Cd and PA treatments may influence the expression level of ADC and ODC genes, as it was found in a previous study on rice (Pál et al., 2017). However, changes in PA content not always correlated with expression level of the genes, as post-transcriptional regulation may also occur (Kovács et al., 2010), and other mechanism may also be involved in the maintenance of the optimal PA pool. Apoplastic DAO activity did not change under the present conditions, but this DAO is responsible for the terminal catabolism of PUT, not for the back-conversion of SPD to PUT. According to these the already high apoplastic DAO/PAO activities are responsible for the catabolism of PA due to the controlled PA exodus into the apoplast (Cona et al., 2006). SPDss and SPDhyd treatments alone also increased the PUT content, in these cases the back-conversion of the uptaken SPD to PUT should be occurred, as suggested in wheat and maize plants (Szalai et al., 2017). In the present experiment, depending on the applied PA and on the type of treatment, different mechanisms may occur and result the observed changes in the PA pool. Possible mechanisms and changes were discussed on Suppl. Fig.1. comparing PUTss+Cd, PUThyd+Cd, SPDss+Cd and SPDhyd+Cd treaments.

Changes in endogenous SA content during abiotic stress and the protective effect of exogenously applied SA have also been demonstrated. As a plant hormone, SA is involved in general stress responses, in a complex relationship with other plant hormones, leading to the regulation of gene expression. There is evidence that not only may SA cause a rise in the quantity of ROS in the cell, but ROS may also lead to the accumulation of SA, suggesting the existence of a self-induced SA-H_2O_2 cycle (Pál et al., 2013). It has been reported that SA treatment influences the PA content and catabolism (Németh et al., 2002; Szepesi et al., 2011; Wang et al., 2012). Similarly, seed soaking with SPD or SPM enhanced the SA content (Iqbal et al., 2006). In wheat and maize plants hydroponic PA treatment induced SA accumulation, which was higher in the case of SDP and even higher after SPM, compared to PUT treatments

426 (Szalai et al., 2017). Based on these results a relationship is thought to exist between
427 endogenous SA and PA contents. In both of the present experiments Cd treatment led to SA
428 accumulation especially in the leaves, as previously reported in wheat (Kovács et al., 2014a).
429 It is possible that the initial basal and stress-induced levels of SA in the two experiments only
430 differed because in the first experiment the seedlings were immediately exposed to Cd after
431 germination and sampled on the 5th day, while in the second experiment 14-day-old plants
432 were treated with Cd and sampled on the 21st day. However, when PUTss or SPDss preceded
433 Cd treatment a less pronounced increase in SA content was found in the leaves compared to
434 plants treated with Cd alone, which may explain the observed protective effect. In contrast to
435 the seed soaking pre-treatment, only hydroponic pre-treatment with PUT was able to mitigate
436 the rise in SA content caused by Cd in the leaves, while the SPDhyd+Cd treatment had an
437 additive effect on SA content in the roots. SA is a well-known hormone involved in stress
438 signalling in plants, and exposure to Cd has been shown to stimulate SA accumulation in the
439 roots (DalCorso et al., 2010). In mutant lines of Arabidopsis SA deficiency decreased the
440 growth inhibition caused by Pb or Cd stress, and the consequent blockage of SA signalling
441 also had a slight protective effect (Tao et al., 2013). The highest SA and PUT accumulation
442 was found at whole plant level in SPDhyd+Cd-treated plants (Suppl. Fig. 1.). This is not
443 surprising, as a close correlation has recently been reported between the endogenous PA and
444 SA contents, which may be responsible for the negative effect of greater concentrations of
445 higher PAs (SPD and SPM) (Szalai et al., 2017). Compared to the corresponding Cd
446 treatments higher root GR, GST, APX and G-POD activities were also found for
447 SPDhyd+Cd.

448 Proline acts as a major reservoir of energy and nitrogen, which can be utilised to
449 resume growth under stress conditions, and it has been observed to accumulate in response to
450 heavy metals in several plant species, suggesting that it plays a role in heavy metal tolerance

451 (Szabados and Savoure, 2010). Proline is not only a direct ROS scavenger, but also helps to
452 stabilize antioxidant enzymes (Liang et al., 2013). Its accumulation was found to be SA- and
453 ROS-dependent in Arabidopsis (Fabro et al., 2004; Verslues et al., 2007). PUT treatment has
454 been reported to induce an increase in proline content, suggesting that the PA metabolism is
455 linked to proline synthesis (Pál et al., 2015; Szalai et al., 2017). In the leaves all the combined
456 treatments increased the proline content, with the most dramatic increase in the leaves of
457 SPDhyd+Cd-treated plants, where the highest Cd uptake was observed (Suppl. Fig. 1.). This
458 finding is in accordance with the suggestion that proline may function as a metal chelator
459 (Sharma et al. 1998).

460 When Cd enters the cytosol, the synthesis of metal-binding ligands is activated in
461 plants, resulting in the formation of complex-forming agents capable of compartmentalising
462 heavy metals to the vacuoles. PCs are some of the major heavy metal chelators in plants. The
463 enzyme responsible for the synthesis of PCs is γ-glutamyl cysteine dipeptidyl transpeptidase
464 (PCS), which is also expressed without heavy-metal stress, but is primarily activated by the
465 presence of heavy metals (Cobbet 2000; Pál et al., 2006).

466 In a previous study, it was demonstrated that PUT pre-treatment inhibited PC
467 synthesis at the molecular and gene expression levels in rice (Pál et al., 2017). Similarly,
468 SPM+Cd treatment decreased the PC levels in the roots of *Canavalia lineata* (Yun et al.,
469 1997). In contrast, the positive effect of PA treatments was found in some cases to result in
470 higher PC content (Hsu and Kao, 2007; Choudhary et al., 2012; Nahar et al., 2016), while
471 other authors reported that PAs had no effect on the PC level (Groppa et al., 2007). The PA
472 metabolism may influence the synthesis of PCs not only due to synthesis antagonism (Cys is
473 the common precursor of higher PAs and PCs), but also to the fact that PAs are metal
474 chelators, so PA-Cd binding may prevent Cd from stimulating the induction of PC synthesis.
475 Little information is available about the effect of PAs on PC synthesis at the gene expression

level. In the present work PA pre-treatments did not influence the expression level of the PCS gene in the leaves and roots of wheat plants when applied as seed soaking, but the hydroponic application of SPD reduced gene expression in the leaves during Cd stress. However, these changes alone are not responsible for the observed opposite effect of PUThyd and SPDhyd pre-treatments under Cd stress.

5. Conclusions

Although certain PA pre-treatments (PUTss, SPDss, or hydroponic PUT) provided protection against heavy metal stress in wheat, these beneficial effects were not related to decreased Cd uptake. PA pre-treatment in itself did not influence the PCS gene expression in the roots. However, PAs also have metal chelating ability, which could explain why greater Cd quantities were detected in plants treated hydroponically with SPD. The negative effect of hydroponic SPD was correlated with the highest accumulation of PUT, SA and proline. The results demonstrated that changes in the PA pool are important for the fine tuning of PA-induced stress tolerance and for the hormonal balance. However, a better understanding of the exact mechanisms exerting a protective effect under Cd stress conditions will still require further research.

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References

499 Anton, A., Rékási, M., Uzinger, N., Széplábi, G., Makó, A., 2012. Modelling the
500 potential effects of the Hungarian red mud disaster on soil properties. Water Air Soil
501 Pollut. 223, 5175-5188.
502 Bates L. S., Waldren R. P., Teare I. D., 1973. Rapid determination of free proline for water-
503 stress studies. Plant Soil, 39: 205-207.
504 Choudhary, S. P., Kanwar, M., Bhardwa,j R., Yu, J-Q, Tran L-S. P. 2012. Chromium stress
505 mitigation by polyamine-brassinosteroid application involves phytohormonal and
506 physiological strategies in *Raphanus sativus* L., PloS ONE 7, e33210.
507 Cobbett, C. S. 2000. Phytochelatins and their roles in heavy metal detoxification. Plant
508 Physiol. 123, 825-832.
509 DalCorso G, Farinati S, Furini A. 2010. Regulatory networks of cadmium stress in plants.
510 Plant Signal Behav. 5, 663-667.
511 di Toppi, L.S., Gabbrielli, R. 1999. Response to cadmium in higher plants. Environ. Exp.
512 Bot. 41, 105-130.
513 Fabro, G., Kovacs, I., Pavet, V., Szabados, L., Alvarez, M.E. 2004. Proline accumulation
514 and AtP5CS2 gene activation are induced by plant-pathogen incompatible interactions
515 in Arabidopsis. Mol. Plant Microbe Interact. 17, 343-350.
516 Gao, S., Yan, R., Cao, M., Yang, W., Wang, S., Chen, F. 2008. Effects of copper on growth,
517 antioxidant enzymes and phenylalanine ammonia-lyase activities in *Jatropha curcas* L.
518 Plant Soil Environ. 54, 117-122.
519 Ghosh, N., Das, S.P., Mandal, C., Gupta, S., Das, K., Dey, N., Adak, M.K. 2012. Variations
520 of antioxidative responses in two rice cultivars with polyamine treatment under salinity
521 stress, Physiol. Mol. Biol. Plants, 18, 301-313.

522 Gondor, O.K., Pál, M., Darkó, É., Janda, T., Szalai, G. 2016. Salicylic acid and sodium
523 salicylate alleviate cadmium toxicity to different extents in maize (*Zea mays* L.). PLoS
524 ONE. 11, e0160157.
525 Groppa, M. D., Tomaro, M. L., Benavides M. P. 2007. Polyamines and heavy metal stress:
526 the antioxidant behavior of spermine in cadmium- and copper-treated wheat leaves,
527 Biometals. 20, 185-195.
528 Groppa, M.D., Zawoznik, M.S., Tomaro, M.L., Benavides, MP. 2008. Inhibition of root
529 growth and polyamine metabolism in sunflower (*Helianthus annuus*) seedlings under
530 cadmium and copper stress. Biol. Trace Elem. Res. 126, 246-256.
531 Hsu, Y. T., Kao, C. H. 2007. Cadmium-induced oxidative damage in rice leaves is reduced
532 by polyamines, Plant Soil 291, 27-37.
533 Iqbal, M., Ashraf, M., Jamil, A., Rehman, S. 2006. Does seed priming induce changes in the
534 levels of some endogenous plant hormones in hexaploid wheat plants under salt stress?
535 J. Int. Plant Biol. 48, 181-189.
536 Kovács, V., Gondor, O.K., Szalai, G., Darkó, E., Majláth, I., Janda, T., Pál M. 2014a.
537 Synthesis and role of salicylic acid in wheat varieties with different levels of cadmium
538 tolerance, J. Hazard. Mater. 280, 12-19.
539 Kovács, V., Gondor, O.K., Szalai, G., Majláth, I., Janda, T., Pál, M. 2014b. UV-B radiation
540 modifies the acclimation processes to drought or cadmium in wheat. Environ. Exp.
541 Bot. 100, 122– 131.
542 Kovács, Z., Simon-Sarkadi, L., Szucs, A., Kocsy, G. 2010. Differential effects of cold,
543 osmotic stress and abscisic acid on polyamine accumulation in wheat. Amino Acids.
544 38: 623-631.
545 Landberg, T., Greger, M. 2002. Differences in oxidative stress in heavy metal resistant and
546 sensitive clones of *Salix viminalis*. J. Plant Physiol. 159, 69-75.

547 Liang, X., Zhang, L., Natarajan, S.K., Becker, D.F. 2013. Proline mechanisms of stress
548 survival. Antioxid. Redox Signal. 19, 998-1011.
549 Liu, J-H., Wang, W., Wu, H., Gong, X., Moriguchi, T. 2015. Polyamines function in stress
550 tolerance: from synthesis to regulation. Front. Plant Sci. 6, 827.
551 López-Climent, M. F., Arbona, V., Pérez-Clemente, R. M., Zandalinas, S. I., Gómez-
552 Cadenas, A. 2014. Effect of cadmium and calcium treatments on phytochelatin and
553 glutathione levels in citrus plants. Plant Biol J. 16, 79-87.
554 Miller-Fleming, L., Olin-Sandoval, V., Campbell, K., Ralser, M. 2015. Remaining mysteries
555 of molecular biology: The role of polyamines in the cell. J. Mol. Biol. 427, 3389-3406.
556 Minocha, R., Majumdar, R., Minocha, S.C. 2014. Polyamines and abiotic stress in plants: a
557 complex relationship. Front. Plant Sci. 5, 175.
558 Moschou, P.N., Wu, J., Cona, A., Tavladoraki, P., Angelini, R., Roubelakis-Angelakis K.A.,
559 2012. The polyamines and their catabolic products are significant players in the
560 turnover of nitrogenous molecules in plants. J. Exp. Bot. 63, 5003-5015.
561 Nahar, K., Hasanuzzaman, M., Alam, M.M., Rahman, A., Suzuki, T., Fujita, M. 2016.
562 Polyamine and nitric oxide crosstalk: Antagonistic effects on cadmium toxicity in
563 mung bean plants through upregulating the metal detoxification, antioxidant defense
564 and methylglyoxal detoxification systems. Ecotoxicol. Environ. Saf. 126, 245-255.
565 Németh, M., Janda, T., Horváth, E., Páldi, E., Szalai, G. 2002. Exogenous salicylic acid
566 increases polyamine content but may decrease drought tolerance in maize, Plant Sci.
567 162, 569-574.
568 Paschalidis, K.A., Roubelakis-Angelakis, K.A. 2005. Spatial and temporal distribution of
569 polyamine levels and polyamine anabolism in different organs/tissues of the tobacco
570 plant. Correlations with age, cell division/expansion, and differentiation. Plant Physiol.
571 138:142-152.

572 Pál, M., Csávás, G., Szalai, G., Oláh, T., Khalil, R., Yordanova, R., Gell, G., Birinyi, Zs.,
573 Németh, E., Janda, T. 2017. Polyamines may influence phytochelatin synthesis during
574 Cd stress in rice. J. Hazard. Mater. doi.org/10.1016/j.jhazmat.2017.07.016.
575 Pál, M., Horváth, E., Janda, T., Páldi, E., Szalai, G. 2005. Cadmium stimulates the
576 accumulation of salicylic acid and its putative precursors in maize (*Zea mays* L.)
577 plants, Physiol. Plant. 125, 356-364.
578 Pál, M., Horváth, E., Janda, T., Páldi, E., Szalai, G. 2006. Physiological changes and defence
579 mechanisms induced by cadmium stress in maize, J. Plant Nutr. Soil Sci. 169, 239-246.
580 Pál, M., Kovács, V., Vida, G., Szalai, G., Janda, T. 2013. Changes induced by powdery
581 mildew in the salicylic acid and polyamine contents and the antioxidant enzyme
582 activities of wheat lines, Eur. J. Plant Pathol. 135, 35-47.
583 Pál, M., Szalai, G., Janda, T. 2015. Speculation: Polyamines are important in abiotic stress
584 signalling. Plant Sci. 237, 16-23.
585 Parvin, S., Lee, O.R., Sathiyaraj, G., Khorolragchaa, A., Kim, Y.J., Yang, D.C. 2014.
586 Spermidine alleviates the growth of saline-stressed ginseng seedlings through
587 antioxidative defense system. Gene, 537, 70-78.
588 Paolacci, A.R., Tanzarella, O.A., Porceddu, E., Ciaffi, M. 2009. Identification and
589 validation of reference genes for quantitative RT-PCR normalization in wheat. BMC
590 Molecular Biology, 10, 11.
591 Sharma, S.S., Schat, H., Vooijs, R. 1998. *In vitro* alleviation of heavy metal-induced enzyme
592 inhibition by proline. Phytochemistry, 49, 1531–1535.
593 Shu, S., Yuan, L.Y., Guo, S.R., Sun, J., Liu, C.J. 2012. Effects of exogenous spermidine on
594 photosynthesis, xanthophyll cycle and endogenous polyamines in cucumber seedling
595 exposed to salinity. Afr. J. Biotech. 11, 6064-6074.

596 Stolt, J.P., Sneller, F.E.C., Bryngelsson, T., Lundborg, T., Schat, H. 2003. Phytochelatin and
597 cadmium accumulation in wheat. Environ. Exp. Bot. 49, 21-28.
598 Szabados, L., Savouré, A. 2010. Proline: a multifunctional amino acid. Trends Plant Sci. 15,
599 89-97.
600 Szalai, G., Janda, K., Darkó, E., Janda, T., Peeva, V., Pál, M. 2017. Comparative analysis of
601 polyamine metabolism in wheat and maize plants. Plant Physiol. Biochem. 112, 239-
602 250.
603 Szepesi, Á., Gémes, K., Orosz, G., Pető, A., Takács, Z., Vorák, M., Tari, I. 2011. Interaction
604 between salicylic acid and polyamines and their possible roles in tomato hardening
605 processes. Acta Biol. Szeged. 55, 165-166.
606 Takács, Z., Poór, P., Tari, I. 2016. Comparison of polyamine metabolism in tomato plants
607 exposed to different concentrations of salicylic acid under light or dark conditions,
608 Plant Physiol. Biochem. 108, 266-278.
609 Tao, S., Sun, L., Ma, C., Li, L., Li, G., Hao, L. 2013. Reducing basal salicylic acid enhances
610 Arabidopsis tolerance to lead or cadmium. Plant Soil 372,: 309-318.
611 Verslues, P.E., Kim, Y.S., Zhu, J.K. 2007. Altered ABA, proline and hydrogen peroxide in
612 an Arabidopsis glutamate: glyoxylate aminotransferase mutant. Plant Mol. Biol. 64,
613 205-217.
614 Wang, X., Zhang, Y. 2012.Regulation of salicylic acid on polyamine synthesize under NaCl
615 stress in leaves of the yali pear. Res. J. Appl. Sci. Eng. Technol. 4, 3704-3708.
616 Wen, X.-P., Ban, Y., Inoue, H., Matsuda, N., Moriguchi, T. 2010. Spermidine levels are
617 implicated in heavy metal tolerance in a spermidine synthase overexpressing
618 transgenic European pear by exerting antioxidant activities, Transgenic Res. 19, 91-
619 103.

Yun, I.S., Hwang, I.D., Moon, B.Y., Kwon, Y.M. 1997. Effect of spermine on the phytochelatin concentration and composition in cadmium-treated roots of *Canavalia lineata* seedlings. J. Plant Biol. 40, 275-278.

Legends:

Figure 1. Effects of 0.5 mM putrescine or spermidine pre-treatment as seed soaking (PUTss or SPDss) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUTss+Cd and SPDss+Cd); furthermore 0.5 mM putrescine or spermidine hydroponic pre-treatment (PUThyd or SPDhyd) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUThyd+Cd and SPDhyd+Cd) on the polyamine contents, namely putrescine (PUT, A and D), spermidine (SPD, B and E) and spermine (SPM, C and F) in the leaves and roots of wheat 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 or spermidine pre-treatment as seed soaking (PUTss or SPDss) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUTss+Cd and SPDss+Cd); furthermore

645 0.5 mM putrescine or spermidine hydroponic pre-treatment (PUThyd or SPDhyd) and/or 50
646 μM $Cd(NO_3)_2$ treatment (Cd, PUThyd+Cd and SPDhyd+Cd) on the salicylic acid (SA)
647 content in the free (A and C) or bound form (B and D) in the leaves and roots of wheat plants.
648 Data represent mean values ±SD. Different letters indicate significant differences between the
649 treatments at $P<0.05$.

650 **Figure 3.** Changes in the gene expression of the arginine decarboxylase (ADC) (A-B),
651 ornithine decarboxylase (C-D) and phytochelatin synthase (PCS) genes (E-F) in the leaves of
652 wheat plants after 0.5 mM putrescine or spermidine pre-treatment as seed soaking (PUTss or
653 SPDss) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUTss+Cd and SPDss+Cd); furthermore 0.5
654 mM putrescine or spermidine hydroponic pre-treatment (PUThyd or SPDhyd) and/or 50 μM
655 $Cd(NO_3)_2$ treatment (Cd, PUThyd+Cd and SPDhyd+Cd). Data represent mean values ±SD,
656 n=3. *, ** and ***: significant differences between fold changes at the $P<0.05$, 0.01 and
657 0.001 levels, respectively.

658 **Supplementary Figure 1.** Comparison of the effects of polyamine pre-treatments applied as
659 seed-soaking or hydroponically (putrescine: PUT or spermidine: SPD) under cadmium stress
660 on polyamine metabolism, contents of salicylic acid (SA) and proline (Pro), and on cadmium
661 uptake. The size of the letters either for polyamines (PUT, SPD and spermine: SPM) or for
662 SA, Pro and Cd indicated the level of accumulation. The thickness of the arrows indicate the
663 intensity of the effects: white arrows for changes in gene expression (stimulation or
664 inhibition), black arrows for the interconversion in the polyamine cycle, while black stripped
665 arrow for the polyamine uptake.

Table 1

Table 1. Effects of 0.5 mM putrescine or spermidine pre-treatment as seed soaking (PUTss or SPDss) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUTss+Cd and SPDss+Cd) on the biomass, fluorescence induction parameters and cadmium, chlorophyll and proline contents, the antioxidant enzyme activities, namely glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD), and on the diamine oxidase (DAO) and polyamine oxidase (PAO) activities in wheat plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at $P<0.05$.

	Control	Cd	PUTss	PUTss+Cd	SPDss	SPDss+Cd
Shoot length (cm)	11.81±0.97 c	7.07±0.88 a	12.19±1.18 cd	7.71±0.99 b	12.29±0.97 d	7.19±0.97 a
Shoot FW (g)	0.152±0.008 c	0.054±0.002 a	0.147±0.006 c	0.061±0.003 b	0.151±0.008 c	0.058±0.002 b
Root length (cm)	11.84±1.44 c	3.31±0.81 a	11.37±1.64 c	3.7±0.94 b	11.48±1.35 c	3.38±0.69 a
Root FW (g)	0.079±0.008 b	0.019±0.003 a	0.085±0.007 b	0.021±0.003 a	0.079±0.005 b	0.02±0.002 a
Leaf Cd (mg kg^{-1})	0.15±0.04 a	71.06±1.75 c	0.21±0.04 a	97.05±16.06 d	1.00±0.11 b	84.09±14.24 cd
Root Cd (mg kg^{-1})	0.73±0.03 a	2761.37±116.91 c	0.64±0.04 a	3068.46±112.5 d	1.33±0.05 b	3154.93±182.72 d
Chlorophyll content	38.78±4.74 c	27.59±8.16 a	38.56±3.89 c	26.52±6.89 a	36.57±4.13 c	32.74±3.68 b
Fv/Fm	0.775±0.003 c	0.73±0.023 a	0.774±0.005 c	0.767±0.01 b	0.782±0.003 c	0.769±0.015 b
ΔF/Fm'	0.35±0.027 d	0.181±0.026 a	0.336±0.029 d	0.26±0.037 b	0.313±0.01 c	0.237±0.024 b
Leaf proline (μg g^{-1} FW)	10.45±4.09 a	13.88±2.85 a	10.98±2.1 a	18.08±6.89 ab	9.22±1.04 a	28.43±5.66 b
Root proline (μg g^{-1} FW)	7.67±2.94 a	25.3±3.63 c	11.97±3.23 ab	23.27±3.62 c	14.89±3.23 b	23.35±3.6 c
GR leaf (nkatal g^{-1} FW)	43.93±1.08 b	39.51±2.5 a	36.92±1.43 ab	37.16±3.1 ab	37.4±2.99 ab	37.33±2.65 a
GR root (nkatal g^{-1} FW)	12.17±0.33 b	12.07±0.49 b	10.45±0.47 a	10.69±0.38 a	10.08±0.36 a	10.69±0.4 a
GST leaf (nkatal g-1 FW)	7.59±0.79 b	9.73±1.62 b	6.51±0.47 ab	4.62±1.1 a	7.48±0.56 b	7.28±0.4 b
GST root (nkatal g^{-1} FW)	11.77±0.83 ab	16.34±0.75 d	12.5±0.39 b	12.35±0.69 b	10.38±0.69 a	13.91±0.55 c
CAT leaf (nkatal g^{-1} FW)	3922.02±232.77 a	8199.54±391.93 c	4048.17±367.51 a	6383.8±854.52 b	4025.23±195 a	6903.67±950.6 b
CAT root (nkatal g^{-1} FW)	451.07±80.55 a	2431.19±105.6 d	340.21±56.57 a	1353.21±64.5 b	531.35±213.42 a	1823.4±120.4 c
APX leaf (nkatal g^{-1} FW)	132.52±6.29 a	320.84±9.32 c	121.13±11.88 a	282.48±23.79 bc	128.79±6.83 a	265.5±16.25 b
APX root (nkatal g^{-1} FW)	255.32±20.3 a	279.48±28.16 a	223.23±23.57 a	272.9±21.8 a	223.45±5.52 a	299.57±29.4 a
G-POD leaf (nkatal g^{-1} FW)	293.73±43.06 a	1281.5±70.4 b	224.24±67.29 a	1205.41±69.26 b	264.54±72.48 a	1178.2±153.17 b
G-POD root (nkatal g^{-1} FW)	1657.14±48.5 a	1891.67±107.5 a	1747.43±231.9 a	1525.17±87.2 a	1605.17±106.84 a	1574.62±71.8 a
DAO leaf (U g^{-1} FW)	343.02±21.08 bc	348.14±22.88 bc	265.37±28.58 a	328.02±14.56 b	410.5±1.79 d	380.53±11.75 c
DAO root (U g^{-1} FW)	248.44±49.9 c	129.93±33.93 ab	182.87±28.18 bc	79.05±31.82 a	207.89±17.95 c	157.04±14.31 b
PAO leaf (U g^{-1} FW)	386.96±20.7 abc	396.72±3.56 bc	345.15±30.29 a	419.95±26.07 c	402.23±6.38 b	372.89±11.04 a
PAO root (U g^{-1} FW)	176.23±17.61 bc	148.25±11.69 b	185.19±17.73 bc	90.6±1.41 a	193.22±10.67 c	177.29±4.49 c

Table 2

Table 2. Effects of 0.5 mM putrescine or spermidine hydroponic pre-treatment (PUThyd or SPDhyd) and/or 50 μM $Cd(NO_3)_2$ treatment (Cd, PUThyd+Cd and SPDhyd+Cd) on the biomass, fluorescence induction parameters and cadmium, chlorophyll and proline contents, on the antioxidant enzyme activities in wheat plants, namely glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (G-POD), and on the diamine oxidase (DAO) and polyamine oxidase (PAO) activities in wheat plants. Data represent mean values ±SD. Different letters indicate significant differences between the treatments at P<0.05.

	Control	Cd	PUThyd	PUThyd+Cd	SPDhyd	SPDhyd+Cd
Shoot length (cm)	26.17±2.98 b	25.76±2.14 b	28.38±2.45 d	27.17±1.62 c	26.41±2.21 b	24.78±1.67 a
Shoot FW (g)	1.1±0.25 b	0.94±0.22 a	1.24±0.3 c	1.13±0.3 b	1.09±0.27 b	0.96±0.24 a
Root length (cm)	23.88±5.65 c	22.42±5.11 c	30.48±5.61 d	23.73±4.27 c	20.41±3.35 b	17.04±2.79 a
Root FW (g)	0.97±0.24 c	0.62±0.15 b	1.08±0.03 c	064±0.07 b	0.65±0.01 b	0.36±0.05 a
Leaf Cd (mg kg^{-1})	0.73±0.18 a	32.±5.22 b	0.62±0.24 a	82.16±16.5 c	0.51±0.11 a	127.6±10.22 d
Root Cd (mg kg^{-1})	2.07±0.62 a	1601.76±111.34 c	2.18±1.16 ab	1796.94±330.8 c	4.39±1.36 b	2365.53±123,34 d
Chlorophyll content	37.19±2.05 e	27.76±3.56 b	37.25±2.02 e	29.83±2.62 c	35.75±2.67 d	25.11±4.06 a
Fv/Fm	0.779±0.008 b	0.659±0.045 a	0.775±0.004 b	0.761±0.013 b	0.773±0.004 b	0.718±0.079 b
ΔF/Fm'	0.33±0.029 c	0.264±0.033 a	0.382±0.019 c	0.33±0.019 b	0.3±0.038 ab	0.292±0.046 abc
Leaf proline (μg g^{-1} FW)	12.56±1,21 a	16.32±4.88 ab	17.55±5.1 ab	21.64±6.46 b	24.27±8.1 b	47.67±8 c
Root proline (μg g^{-1} FW)	11.19±1.66 a	16±3.33 bc	15.09±3.69 ab	15.3±3.38 ab	15.82±3.69 bc	20.04±2.23 c
GR leaf (nkatal g^{-1} FW)	35.89±6.17 ab	31.16±0.94 a	34.58±5.56 a	35.15±3.38 a	41.9±3.48 b	34.87±4 a
GR root (nkatal g^{-1} FW)	7.96±0.47 a	10.77±1.67 b	11.47±2.32 b	11.63±1.56 b	12.17±1.38 b	18.08±1.05 c
GST leaf (nkatal g^{-1} FW)	14.86±1.77 c	9.63±1.09 b	10.41±0.47 b	10.08±1.1 b	16.36±2.42 c	7.64±0.46 a
GST root (nkatal g^{-1} FW)	32.11±3.64 a	31.51±3.55 a	31.93±3.26 a	29.95±4.43 a	42.23±4.59 b	45.51±1.17 b
CAT leaf (nkatal g^{-1} FW)	4782.11±997.31 b	3247.71±822.27 a	2977.06±171.37 a	3596.33±612.04 ab	4461.01±655.83 b	3368.69±231.52 a
CAT root (nkatal g^{-1} FW)	834.86±563.23 a	2112.96±342.42 b	977.06±238 a	1766.06±491.65 b	853.21±502.87 ab	1763.76±846.35 b
APX leaf (nkatal g^{-1} FW)	189.16±27.27 b	160.75±15.51 b	135.32±25.96 a	148.4±36.91 ab	177.94±26.08 b	155.31±13.95 ab
APX root (nkatal g^{-1} FW)	212.3±35.33 ab	275.72±18.65 c	199.38±7.5 a	259.71±34.87 bc	220.31±18.79 ab	308.09±19.93 d
G-POD leaf (nkatal g^{-1} FW)	368.01±16.9 a	494.166±93.85 b	367.26±14.55 a	452.55±39.88 b	384.11±46.1 a	500.64±51.6 b
G-POD root (nkatal g^{-1} FW)	1005.04±84.52 a	1023.05±81.66 a	1073.47±89.46 ab	1112.26±39.57 ab	1122.97±65.56 b	1369.79±133.65 c
DAO leaf (U g^{-1} FW)	169.12±35.45 bc	209.42±26.77 c	100.73±28.74 a	174.7±42.8 bc	147.05±37.44 ab	160.25±62.99 ab
DAO root (U g^{-1} FW)	25.9±2.72 a	25.67±2.87 a	23.52±6.37 a	23.08±3.22 a	24.83±5.04 a	28.3±5.14 a
PAO leaf (U g^{-1} FW)	238.37±40.22 a	213.8±39.01 a	223.96±57.06 a	245.54±40.63 a	244.07±23.67 a	178.99±53.33 a
PAO root (U g^{-1} FW)	24.02±2.98 a	30.53±5.32 b	19.67±6.22 a	24.73±9.39 ab	24±1.76 a	30.29±4.18 b

Figure 1
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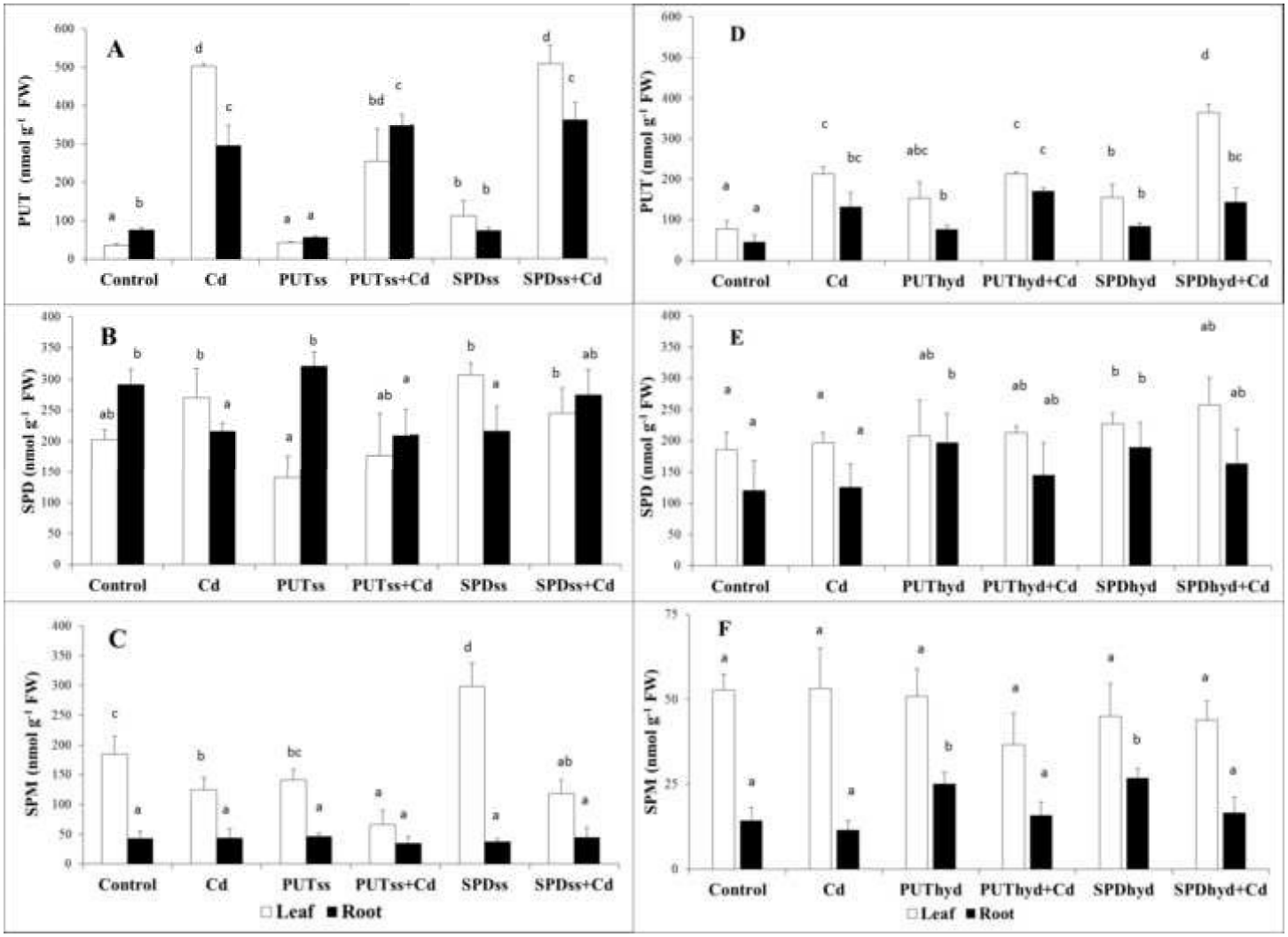

Figure 2
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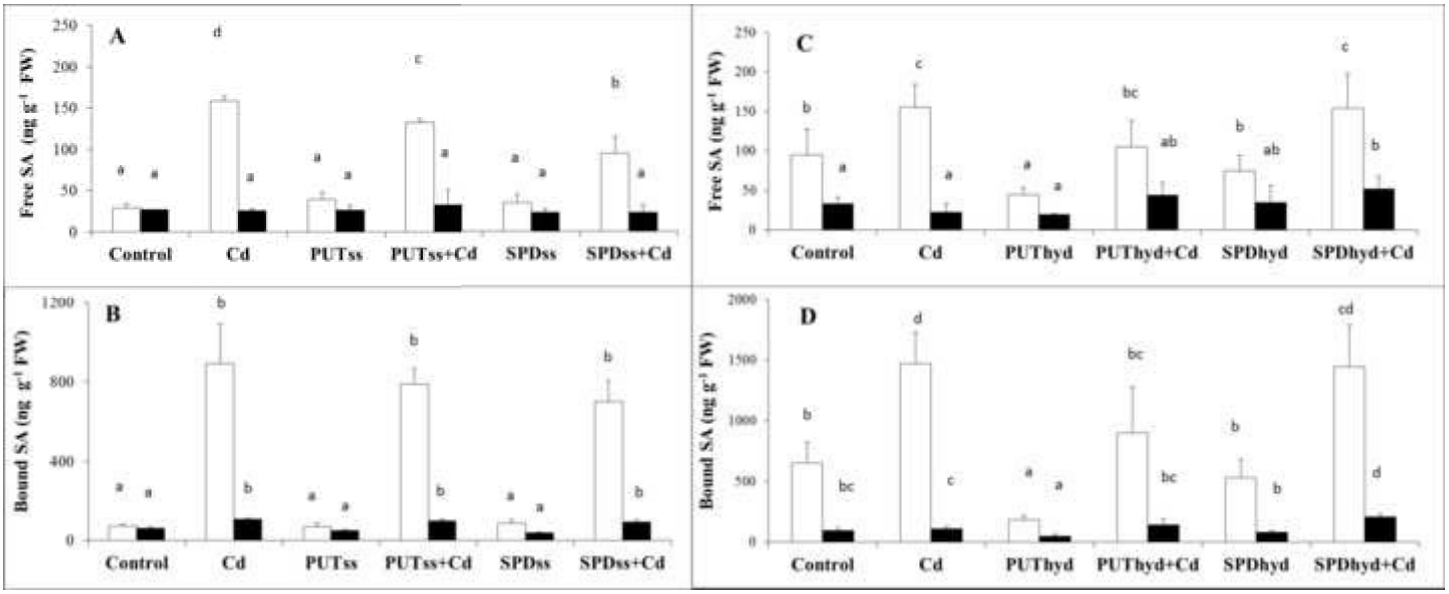

Figure 3.
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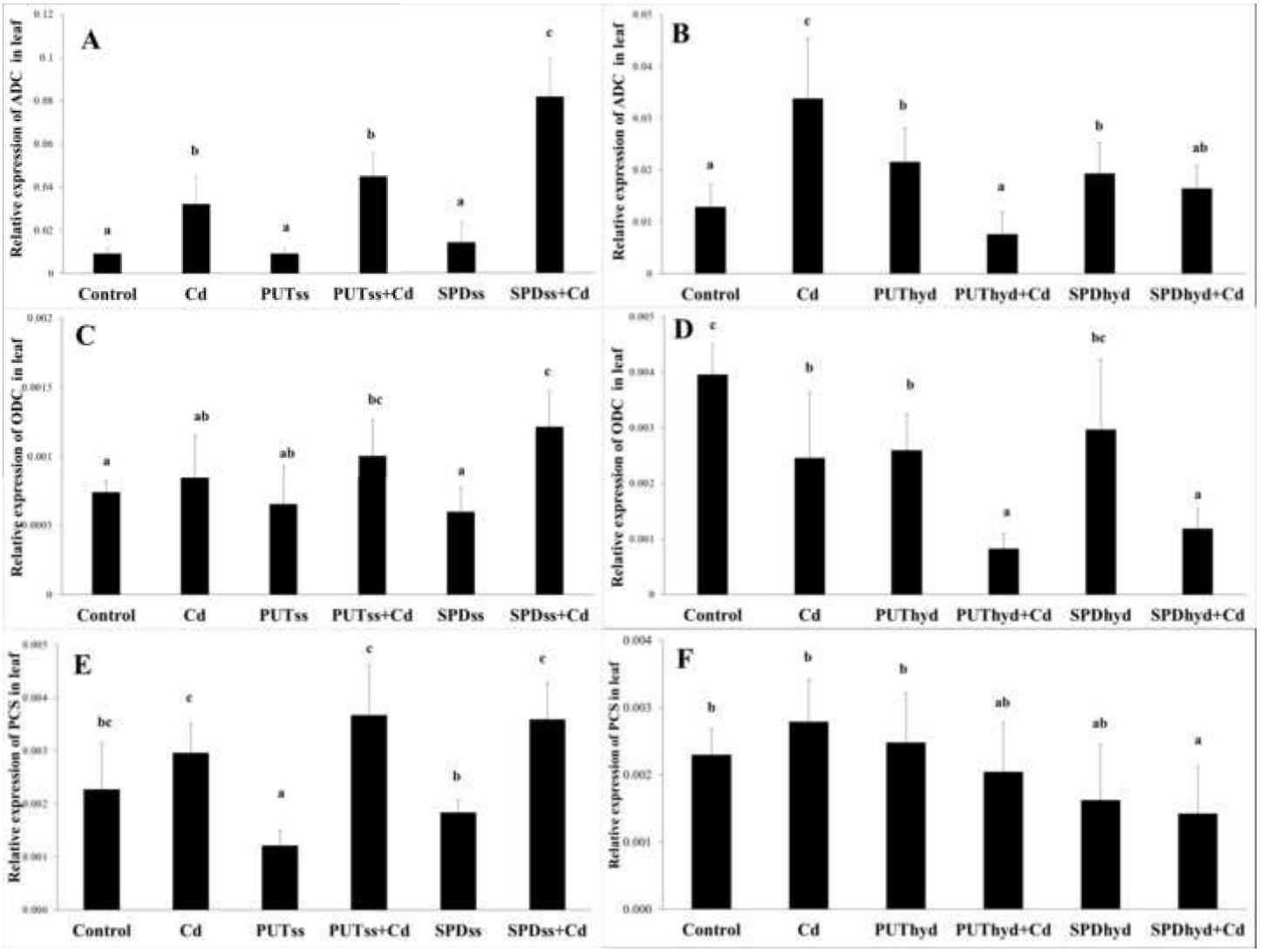

Supplementary Figure 1.
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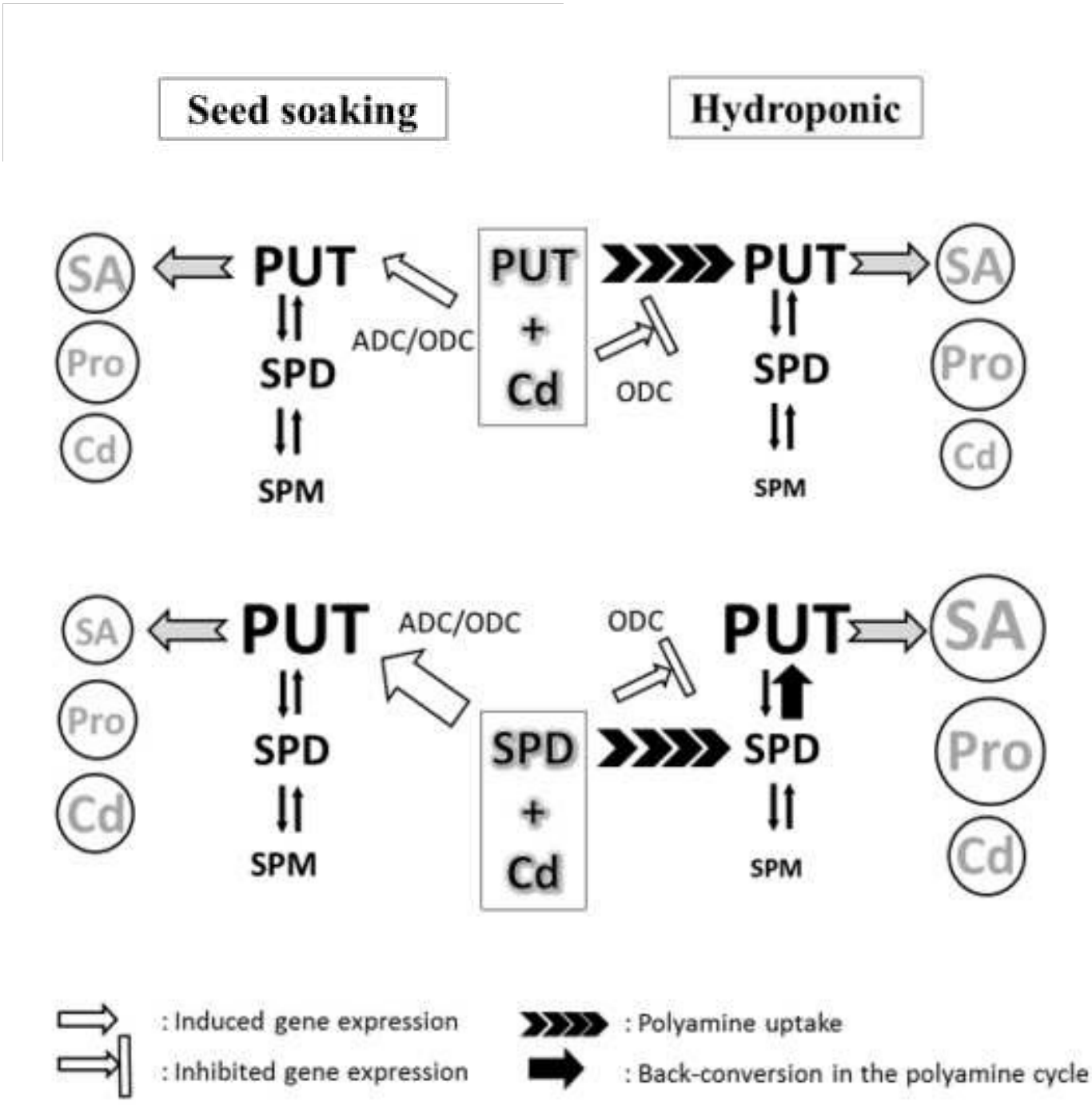

Suppl. Table 1. Reference gene and target gene investigated in wheat plants using qRT-PCR.

Gene name		Primer sequences (5' → 3')	Reference
Ta30797 *(Similar to phosphogluconate dehydrogenase)*	Forward	GCCGTGTCCATGCCAGTG	Paolacci *et al.*, 2009
	Reverse	TTAGCCTGAACCACCTGTGC	
TaADC	Forward	TCTACCCCGTCAAGTGCAAC	own designed
	Reverse	GACGAGGCAGCTCATGGT	
TaODC	Forward	CGTGCGTGGAGGTGATAGG	own designed
	Reverse	AGCTGAGGGTGCCGTAGA	
TaPCS1	Forward	CCTTCAAGCAGACTGGGACT	own designed
	Reverse	CCTTCAAGCAGACTGGGACT	