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**DEFINING THE ROLE OF POLYAMINES AND ITS LIGHT-  
RELATED MODULATION IN THE DEVELOPMENT AND  
STRESS TOLERANCE OF CROP PLANTS**

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**Altafur Rahman**

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**The Doctoral School of Horticultural Sciences**

**Name: Altafur Rahman**

**Discipline:** Plant Physiology

**Leader of the school:** Prof. Dr. Zámборiné Németh Éva, DSc

Head of Horticultural Sciences Doctoral School

Hungarian University of Agriculture and Life Sciences, Department of Medicinal and Aromatic Plants

**Supervisors:** Prof. Magda Pál (DSc) and Prof. Papp István (DSc)

Centre for Agricultural Research, Hungarian Research Network, Department of Plant Physiology and Metabolomics, Agricultural Institute

Hungarian University of Agriculture and Life Sciences, Institute of Agronomy, Department of Plant Physiology and Plant Ecology

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Approval of the PhD School leader

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Approval of the Supervisor

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Approval of the Co-supervisor

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## 1. INTRODUCTION

Polyamines (PAs) are aliphatic nitrogenous bases with a low molecular weight that comprise two or more amino groups. They are present in nearly all cells and are produced by organisms during metabolism and classified as a novel type of plant biostimulant due to their significant contributions to a variety of plant growth and developmental processes, as well as their responses to environmental duress. They are responsible for maintaining cellular structure, gene expression, and signaling (Tiburcio et al., 2014; Chen et al., 2019). A sequence of enzymatic steps is employed to synthesize PAs in plants: spermidine (SPD) and spermine (SPM) are synthesized by adding aminopropyl groups to PUT (Putrescine) skeleton, while PUT is synthesized from ornithine and/or arginine through decarboxylation as in *Arabidopsis*, only arginine decarboxylase (ADC) pathway exists. *ADC2* is an important component of the ADC pathway, as it promotes PUT biosynthesis during stress (Hummel et al., 2004). The ADC pathway is encoded by the *ADC1* and *ADC2* gene. The activity of spermidine synthase (SPDS) and spermine synthase (SPMS) is responsible for the synthesis of higher PAs, SPD and SPM, respectively, using decarboxylated S-adenosylmethionine (dcSAM) as the aminopropyl donor. The dynamic equilibrium between PA synthesis and degradation, which is essential for cellular homeostasis, is maintained by the regulation of these pathways, which includes the activity of SAMDC and the back-conversion enzymes such as polyamine oxidases (PAOs) (Kolesnikov et al 2024; Pál et al., 2021). This biosynthesis is maintained in balance by a catabolism that involves the enzymes diamine oxidase (DAO) and polyamine oxidase (PAO). Consequently, the actual levels of PAs in the plant's tissues are well-regulated (Shao et al., 2022; Jiménez-Bremont et al., 2014). It is interesting that PAO, which is primarily located in the apoplast, is responsible for the degradation of PAs, while the peroxisomal form, pxPAO, is responsible for back-conversion of higher PAs (SPD and SPM), but both processes result in the formation of hydrogen peroxide and thus involved in redox homeostasis/ROS regulation (Yu et al., 2019). The so-called PA cycle is crucial for plants, as PAs are molecular stabilizers that help plants with managing in different kinds of stresses (Chen et al., 2019).

In adverse environmental conditions including drought, salinity, and exposure to heavy metals, the biosynthesis of PA is increased to allow the plant to cope up with these stress conditions. The increased PA levels can provide enhanced antioxidant activity and membrane stability and help in regulating osmotic pressure thus reducing the effects of stresses such as

drought, salinity and freezing (Tyagi et al., 2022; González-Hernández et al., 2022; Alcázar et al., 2010). Additionally, PAs may also mitigate oxidative stress by eliminating ROS to maintain the integrity of the cells. For example, PAs can alleviate the toxicity of heavy metals like cadmium (Cd) that inhibits nutrient absorption and induce oxidative stress by removing ROS, partly by the induction of the antioxidant defense systems, and by their metal chelator function (Stewart et al., 2018; Hasanuzzaman et al., 2019). However, balance can be disrupted by an excessive amount of PAs, particularly PUT, which underscores the fact that the relationship between PA concentration and stress tolerance is complicated rather than straightforward (Shu et al., 2012).

Light is one of the most critical qualitative factors in the environment that regulates photosynthesis, growth, secondary metabolism, and even PA metabolism, and can also influence the stress responses of plants (Lerin et al., 2019; Landi et al., 2020; Oliveira et al., 2020; Roeber et al., 2021). As noted by Gondor et al. (2021), light intensity affected PA metabolism and influenced the effects of PA treatments on wheat plants. PA synthesis and catabolism have also been reported to be regulated by certain wavelengths of light including blue and red light as reviewed by Pál et al. (2021). Phytochromes (Phys), such as PhyA, are fundamental positive regulators of light-dependent metabolic readjustments, including PA metabolism. In addition, PhyA mediates red light response and the expression of genes and enhances the synthesis of the higher PAs, such as SPD and SPM, under stress conditions. A *phyA* mutation causes a distinct shift in the expression of PA-related genes across different light conditions, indicating that light quality is crucial in metabolic reprogramming during stress (Rahman et al., 2023; Ibarra et al., 2013). Consequently, these factors may influence responses to abiotic stresses. The relationship between light and PAs is also apparent in secondary metabolism, as PAs and their metabolism linked to the formation of other protective compounds, such as certain amino acids, flavonoids, thiols and phytochelatin (PCs), and plant hormones that are involved in the stress response (Napieraj et al., 2023; Xu et al., 2021; Pál et al., 2018; Hu dec et al., 2006, Pál et al., 2021; Demetriou et al., 2007). Thus, the potential for light-induced PA regulation is quite notable, although more studies are needed to determine its effectiveness under a wide number of stresses, including heavy metals.

Cadmium (Cd) toxicity severely impairs plants by disrupting photosynthetic regulation and inhibiting normal growth and development. Studies have shown that blue light can alleviate Cd stress by stimulating the antioxidant defense mechanism (Sebastian and Prasad, 2014; Wang et al., 2023). PCs, which are short cysteine-rich peptides synthesized by the enzyme, phytochelatin

synthase (PCS) from glutathione, are also involved in reducing Cd toxicity. These are then sequestered into vacuoles as stable complexes with Cd ions, thus lowering the amount of free Cd in the cytosol and reducing the extent of oxidative damage. The relationship between PAs and PCs, both of them use precursors like cysteine, reveals a common defense mechanism that is vital for plant tolerance towards heavy metal stress (Faizan et al., 2024; Pál et al., 2017).

Although the light-PA relationship has been investigated, there is still a lack of comprehension regarding the influence of specific wavelengths on PA metabolism in the context of heavy metal stress. Blue light has the potential to be beneficial in the alteration of plant metabolism, which has an interesting implication in the improvement of PA function during heavy metal stress. This could assist in the identification of strategies that can be implemented to improve the well-being of plants, particularly those that are cultivated in suboptimal conditions.

The majority of the previous research has focused on tolerance and the very limited knowledge of the effects of certain light conditions on the synthesis and degradation of PAs in plants. The need to gain insight on how different light qualities affect PA biosynthesis, degradation and their interconnection with the stress-related metabolites led to these experiments. These experiments address the knowledge gaps by investigating the impact of varying light quality on the regulation of PAs and stress response, with Cd stress also being taken into account. This study enhances comprehension of the regulation of PAs in plants as a function of light by examining the impact of light spectra on the amount of PAs also at the gene expression level. The information presented in this research has the potential to be applied in the agricultural sector, enabling the use of light and PA interventions to improve crop resistance to stress, thus enhancing the understanding of plant stress physiology and improving agricultural practices.

## 2. OBJECTIVES TO ACHIEVE

The main objective of the present studies was to determine the function of PAs in promoting growth and/or improving the physiological condition of plants in crop species and the model plant *Arabidopsis*. In a series of experiments, we changed the light conditions or the light spectra and observed the effects of different PA treatments to determine their potential as biostimulants to improve crop performance. The following are the specific objectives of each experiment:

1. The response of wheat, maize and rice crops to exogenous PUT treatment, with emphasis on the variability of PA metabolism in these crops. The objective was to ascertain whether the level of the basal PA affects the outcome of the supplementation with PUT, depending on the species.
2. How the daily uptake pattern of exogenous PUT differs across three wheat genotypes and how this relates to early changes in PA metabolism-related gene expression. This experiment aimed to reveal genotype-specific variations in PA absorption and how these genotypes regulate PA metabolism in response to external PUT.
3. The effect of different light qualities (blue, red and far red) on the PA metabolism of wheat plants. The emphasis was on the understanding of the light quality regimes and their effects on the levels of PUT, SPD and SPM and the consequent modulation of gene expression associated with PA metabolism.
4. The role of PhyA in regulating PA metabolism under different light conditions using *Arabidopsis* wild type and the *phyA* mutant. The objective was to evaluate the effect of light perception by PhyA on PA synthesis and catabolism and the changes in the gene expression profile associated with PA metabolism.
5. The experiment was conducted to examine the protective role of PUT on wheat plants exposed to Cd stress and the role of blue light in this process. It was designed to establish the effects of light spectra on PUT potential to modify Cd uptake, antioxidant activity, and the expression of genes involved in PA and PC synthesis.

### 3. RESULTS AND DISCUSSION

#### 3.1. Differential responses of wheat, maize, and rice plants to putrescine treatment

This study involved three cereal species—wheat, maize, and rice—exposed to a 0.3 mM hydroponic PUT. Specifically, we chose domestically bred genotypes for each species: Béres (winter wheat variety from Martonvásár), Mv 350 (maize hybrid from Martonvásár), and Janka (japonica rice variety from Szarvas). Healthy seedlings were cultivated under controlled conditions using a modified Hoagland solution, with established temperature and light parameters in the growth chamber for each species. In the case of rice, the conditions set were 28/26 °C, while for wheat and maize, the temperature was set at 22/20 °C, respectively with 75% humidity and a 16/8 hours light/dark cycle. After one week, maize, wheat, and rice plants were randomly divided into control (C) and PUT-treated with 0.3 mM concentration in the nutrient solution for seven days. After this one week of PUT treatment, leaf and root samples were collected from both C and PUT treated plants.

Photosynthetic parameters, including gas exchange and chlorophyll fluorescence, were measured using a portable photosynthesis system and PAM fluorometer. Stress markers like malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were quantified spectrophotometrically. The measurement of nitric oxide (NO) was conducted utilizing the Griss reagent method. PA levels and metabolism-related gene expression were analyzed after pre-column derivatization with reverse phase chromatography using a high-performance liquid chromatograph (HPLC) equipped with a fluorescent detector (FLD), and quantitative reverse transcription PCR (qRT-PCR). Statistical evaluations on data from three biological replicates were performed using ANOVA and Duncan's post hoc test to assess significance.

**(Refer to the result figures for visual representation and the detailed materials and methods in [Rahman et al., 2024a], included in Appendix A.)**

##### 3.1.1. Changes in photosynthesis-related parameters

The treatment with 0.3 mM PUT induced a significant increase in leaf chlorophyll content in wheat, maize and rice (Fig. 1). The maximum quantum yield of PSII (F<sub>v</sub>/F<sub>m</sub>) was not influenced by PUT treatment in all the species examined (Fig. 1b). Nevertheless, it had a substantial impact on the PS II activity, as evidenced by the significantly reduced electron transport rate (ETR) (Fig. 1d) and the measured parameters of PS II Y(II) in maize (Fig. 1c). Table 4 illustrates that the gas

exchange parameters of all plants were not significantly different, exception of the rice plant, which exhibited a significant increase in transpiration rate (E).

### **3.1.2. Effects of putrescine treatment on certain stress markers**

#### *3.1.2.1. Lipid peroxidation and H<sub>2</sub>O<sub>2</sub> content*

PUT did not induce any changes in the wheat or maize leaves and roots when the levels of lipid peroxidation marked by MDA were evaluated. However, it enhanced the levels of MDA in the leaves and roots of rice suggesting that this species was under stress (Fig. 2a). It is evident there were no changes in the leaves of all the species with respect to H<sub>2</sub>O<sub>2</sub>. However, there was an increase in the H<sub>2</sub>O<sub>2</sub> levels in the roots of all the plants with the highest levels being seen in the rice roots (Fig. 2b).

#### *3.1.2.2. Proline and NO contents*

The PUT treatment enhanced proline content in the leaves of wheat and rice under the given conditions but to a lesser extent, while proline content increased dramatically in the roots of rice (Fig. 3a). However, the proline levels were not changed in either the leaves or the roots of maize. The level of NO was not affected by PUT treatment in wheat and maize, while it was significantly increased in the leaves and roots of rice (Fig. 3b). High increases of these stress markers indicated stress conditions in the rice plants.

#### *3.1.2.3. Antioxidant enzyme activities*

The activities of antioxidant enzymes were significantly changed by the PUT treatment, which was particularly evident in rice (Fig. 4). The glutathione reductase (GR) enzyme activity was increased in both the leaves and the roots of rice plants (Fig. 4a). Furthermore, Figure 4b illustrates that the activity of glutathione-S-transferase (GST) was also induced by PUT treatment in the rice leaves and roots. The figures below demonstrate that the levels of ascorbate peroxidase (APX) and glutathione peroxidase (GPX) in rice were also influenced by PUT treatment. APX activity increased in leaves, but it decreased in roots, whereas GPX activity increased in the roots (Fig. 4c, d).

### **3.1.3. The impact of putrescine treatments on the metabolism of polyamines**

#### *3.1.3.1. Changes in polyamine content*

The initial PA composition varied between the species, with rice showing the highest total PA content in both leaves and roots, followed by wheat and maize. In rice, the order of SPD > PUT ≥ SPM was seen in the leaves, whereas in the roots, the order was PUT > SPD > SPM (Fig. 5a-c). When treated with PUT, there was an increase in PUT content in the rice leaves, roots and wheat leaves while there was no change in the maize (Fig. 5a). Accumulation of SPD also enhanced in wheat and rice leaves (Fig. 5b). The levels of SPM in rice showed the same trends as those of SPD (Fig. 5c). 1,3-diamino propane (DAP), the end product of SPD and SPM terminal catabolism, did not change (Fig. 5d).

### 3.1.3.2. Gene expression of polyamine metabolism-related genes

Genes associated with PA metabolism exhibited distinct expression patterns as a consequence of PUT treatment (Fig. 6a-f). *ADC* gene expression was significantly elevated in rice leaves and roots (Fig. 6a-b), whereas *SPDS* was upregulated only in rice and maize roots (Fig. 6d, f). The *pxPAO* gene exhibited an increased expression in wheat and maize leaves, as well as in wheat roots (Fig. 6a-c). The expression of apoplasmic *PAO* decreased in wheat leaves, while it increased in maize roots (Fig. 6a, c). Interestingly, the expression of the *pxPAO* (*OsPAO5* and *OsPAO3*) genes in rice were not pronouncedly affected by PUT treatment (Fig. 6e-f). The *PUT1* and *PUT2* genes did not exhibit any significant changes in the leaves of any crop. However, *PUT2* expression was induced in maize roots, while it was downregulated in wheat roots (Fig. 6b, d, f).

**The responses to PUT treatment varied markedly between wheat, maize, and rice, indicating a species-specific dynamic in PA metabolism. In wheat, PUT enhanced chlorophyll content and photosynthetic efficiency without triggering oxidative stress, thus suggesting balanced PA metabolism. In maize, PUT induced chlorophyll content while photosynthetic performance remained normal and root hydrogen peroxide levels increased, suggesting that PA oxidase activity was upregulated as an oxidative stress response. On the other hand, rice showed disturbed PA homeostasis; PUT accumulated, higher PAs decreased, and oxidative stress markers like MDA and H<sub>2</sub>O<sub>2</sub> were increased. These responses bring out very clearly how the effectiveness of exogenous treatments depends vastly on the basal levels of PAs and metabolic capacity in different species. Hence, species-specific optimization of PA application is required to improve stress tolerance and metabolic balance.**

### **3.2 The dynamics of polyamine uptake and metabolism are partly genotype-dependent but were not affected by the absence of light in wheat**

Two winter wheat varieties from Martonvásár (Mv Béres and Mv Emese, genotypes with different agrotechnical needs and different protein and gluten contents) and one Thatcher-based near-isogenic line (TC33) were used in two experimental setups to study the regulation of PA uptake and metabolism under different light conditions. For the detection of genotype-dependent differences, plants were grown in a controlled environment with a light/dark cycle (16h light/8h dark) and on the last day treated with 0.5 mM PUT. To assess how light or darkness influences PA dynamics (light-dependent differences) plants were grown as above, but on the last day, they were treated either with 0.5 mM PUT or SPD both under continuous light and continuous dark conditions (see Suppl. Fig 1 for detailed growth conditions).

Techniques used included HPLC-FLC for the quantification of PAs and mass spectrometry for identifying phenolic compounds and conjugated PAs. Gene expression levels related to PA uptake, synthesis, and catabolism were determined through qRT-PCR. Statistical evaluations on data from three biological replicates were performed using ANOVA and Duncan's post hoc test to assess significance.

**(Refer to the result figures for visual representation and the detailed materials and methods in [Pál et al., 2024 under review], included in Appendix B.)**

#### **3.2.1. Genotype-dependent differences in polyamine metabolism in the presence of excess PUT**

##### *3.2.1.1 Changes in polyamine contents in the roots and leaves of three wheat genotypes*

Out of the most prominent PAs, PUT was found to be the most abundant PA in the roots, followed by SPD and SPM (Figure 1; Supplementary Figure 2-3). Basal levels were uniform among all genotypes. The PUT content in the roots of control plants remained largely unchanged by senescence or light over 73 hours (Fig. 1A, C, E). Although patterns differed, exogenous PUT caused considerable PUT accumulation in all genotypes. The Mv Béres showed a steady increase, peaking at 13 hours (Fig. 1A). Mv Emese had a significant increase at 5 hours before declining (Fig. 1C), while TC33 attained its maximum at 1 hour and maintained a constant level until a decrease occurred at 13 hours (Fig. 1E). PUT levels had nearly returned to baseline in all genotypes by 25, 49, and 73 hours.

The PUT content in the leaves of the three genotypes was similar under control conditions. After PUT treatment, the genotypes demonstrated differing patterns of PUT accumulation and decline, despite their initial similarity in PUT content (Fig. 1B, D, F). PUT accumulation was noted in Mv Béres and TC33 after one hour of PUT treatment, whereas Mv Emese had a significant increase at five hours. The accumulation of PUT was found to be highest in TC33, followed by Mv Béres, and lowest in Mv Emese. Following its maximum duration of 5 hours, PUT in Mv Béres reverted to the 1-hour level (Fig. 1B). The highest accumulation of Mv Emese occurred at 9 hours, and the levels remained elevated for 1 to 3 days (Fig. 1D). TC33 gradually increased PUT levels, reaching a peak at 13 hours, followed by a decrease after 1 day and subsequent increases after 2 and 3 days (Fig. 1F).

PUT treatment resulted in a comparable SPD peak in Mv Béres at 3-hours (Suppl. Fig. 2A). Additionally, SPD and SPM levels increased in the roots of both Mv Béres and Mv Emese under control conditions, observed 3 hours post-light shift (Suppl. Fig. 2A, C, E; Suppl. Fig. 3A, C, E). The peak was relocated to 5 hours in Mv Emese by PUT (Suppl Fig. 2C). However, PUT did not further affect SPD or SPM levels in these genotypes. Conversely, PUT resulted in a slight initial increase in SPD in TC33, which reverted to control levels after 5 hours (Suppl Fig. 2E). The SPM in TC33 roots displayed a similar pattern (Suppl Fig. 3E).

SPD was the most dominant PA in leaves, whereas a constant baseline was noticed in all the genotypes (Suppl. Fig. 2B, D, F). SPM levels were relatively constant in Mv Béres and TC33, but were more than twice as high in Mv Emese (Suppl. Fig. 3B, D, E). The levels of SPD or SPM in the leaves were not influenced significantly by the light or the PUT treatment (Suppl. Fig. 2, 3). DAP levels in the roots did not show any significant changes either (Suppl Fig. 4A, C, E). However, DAP levels in the leaves of Mv Emese were enhanced after 5 hours of PUT treatment and then reached to the maximum level at 9 hours and then returned to the initial level (Suppl Fig. 4D).

### *3.2.1.2 Changes in gene expression in the roots and leaves of three wheat genotypes*

We analyzed the expression of genes involved in PA metabolism in Mv Béres and Mv Emese. *TaADC2* expression in Mv Béres roots decreased after PUT treatment, reaching a minimum after 5 h before approaching control levels (Figure 2A). Similarly, *TaSPDS* and *TapxPAO* initially showed decreased transcript levels, which were then restored (Figure 2C-E). In contrast, *TaPUT1* expression increased from 5 h onwards (Fig 2G). PUT treatment inhibited

*TaADC2* expression in Mv Emese roots, with the effect still observable after 9 h (Fig. 3A). The initial increase in *TaSPDS* expression was ambiguously related to PUT treatment (Fig.3C). *TapxPAO* transcripts showed a steady decrease after PUT treatment (Fig.3E), while *TaPUT1* showed no significant change (Fig.3G).

Comparable patterns were observed in leaves. PUT treatment inhibited *TaADC2* expression, which returned to basal levels after 7 h (Fig.2B). However, *TaSPDS* did not show any significant change (Figure 2D). *TapxPAO* expression decreased in response to light; however, it was not affected by PUT (Figure 2F). A notable change was observed in *TaPUT1*, which showed a significant increase compared to 7 h after PUT treatment (Figure 2H). *TaADC2* expression in Mv Emese leaves was reduced under light conditions compared to dark conditions, independent of the presence of PUT (Fig.3C). Additionally, PUT decreased expression after 9 h. The expression levels of *TaSPDS* and *TapxPAO* showed no significant changes in response to light or PUT treatments (Figure 3D-F). *TaPUT1* levels increased after 5 h of PUT but decreased at 7 and 9 h (Figure 3H).

### **3.2.2 Light-dependent differences in polyamine metabolism in the presence of excess putrescine or spermidine**

#### *3.2.2.1 Changes in polyamine contents in Mv Emese after polyamine treatments under continuous dark or light*

This experiment investigated the daily PA dynamics of Mv Emese under continuous dark or light conditions, with and without PUT/SPD treatments. Leaf PUT levels showed minor fluctuations in dark conditions, whereas a slight increase was observed in light conditions, returning to baseline after one day (Suppl. Fig. 5A). Maximum PUT levels were recorded in PUT-treated plants under both conditions after 7 and 9 hours. PUT induced an initial rise in PUT levels in roots, demonstrating specific patterns under both light and dark conditions (Suppl. Fig. 5B). The PUT content remained unaffected by the SPD treatment.

The levels of SPD in leaves were stable, showing no response to light or PA interventions, yet they were higher in the presence of light compared to its absence (Fig. 4A). Exogenous SPD elevated SPD levels in roots under both light and dark conditions; however, these levels decreased after one hour, especially in illuminated plants (Fig. 4B).

SPM levels decreased under dark conditions but rose until 25 hours, regardless of PA treatment. In contrast, levels showed a slight increase throughout the day under light conditions

(Suppl. Fig. 6A). The SPM of the roots exhibited fluctuations that lacked a clear correlation with the treatment involving light or PA (Suppl. Fig. 6B). Irrespective of PA application, leaf DAP content exhibited an inverse relationship with SPM, declining throughout the day under both light and dark conditions (Suppl. Fig. 7A). The root DAP content was similar to SPD patterns, and SPD treatment led to an increase in levels under both light conditions (Suppl. Fig. 7B).

### *3.2.2.2 Changes in gene expression in Mv Emese after polyamine treatments under continuous dark or light*

*TaSPDS* transcript levels in the dark exhibited an initial increase followed by a decline, despite the fact that SPD levels in leaves remained unaffected by PA treatment (Fig. 5A). In both control and PA-treated plants, *TaSPDS* levels in roots fluctuated in the dark but significantly decreased under light (Fig. 5B). Under dark conditions, the expression of *TapxPAO* was reduced in both leaves and roots (Suppl. Fig. 8A-B). In leaves, it remained stable in the dark; however, PUT and SPD interventions resulted in an early rise followed by a decrease. *TapxPAO* expression was significantly elevated under light, particularly following SPD treatment. The expression of *TapxPAO* in the roots exhibited a daily periodicity, with a peak at the beginning of the day and a subsequent decline (Suppl. Fig. 8B).

**This study identified mechanisms regulating PA metabolism in wheat on a genotype basis and depending upon the light. Exogenous PUT resulted in differential PA homeostasis responses in different genotypes. Mv Béres showed a transient down-regulation of the genes involved in PA synthesis and back-conversion, reducing PUT accumulation. On the other hand, Mv Emese triggered terminal catabolism, which efficiently metabolizes excess PUT. The light condition and the dark condition both showed that leaf metabolism was predominantly controlled by circadian rhythms for leaf metabolism while root responses were mainly controlled by PA treatments. Overall, these results provide insight into the complex interaction between genotype and light in coordinating PA uptake, metabolism, and conjugation dynamics.**

### **3.3 Light spectral composition may modify the polyamine metabolism in young wheat plants**

In this experimental setup, four distinct light conditions were applied to wheat plants grown hydroponically under controlled conditions (22/20 °C day/night temperatures, 75% humidity, and a 16/8-hour light/dark cycle). Light regimes included a continuous, wide-spectrum LED source for

white light (W), a blue (B) light dominated by 82.55% blue light, a red + far-red (R) light with 66.28% red and 6.59% far-red, and a pink (P) light with an equal blue-red ratio. The specific spectral characteristics for each regimen are detailed in Table 3. PA treatments—PUT, SPD, and SPM—were applied at a 0.3 mM concentration exclusively under B, R, and P conditions for a week.

Chlorophyll fluorescence parameters, including maximum quantum yield and electron transport rate, were measured using a pulse amplitude modulated (PAM) fluorometer. PA levels and metabolic profiles were analyzed using HPLC as previously described, while proline content was measured via spectrophotometric method. Gene expression of enzymes involved in PA metabolism was analyzed using qRT-PCR.

**(Refer to the result figures for visual representation and the detailed materials and methods in [Pál et al., 2022], included in Appendix C.)**

### **3.3.1. Effects of different spectral conditions in combination with polyamine treatments on physiological parameters**

#### *3.3.1.1. Changes in fluorescence induction parameters*

The maximum quantum yield of PS II (Fv/Fm, Figure 1A) was not affected by light quality, as indicated by the fluorescence quenching analysis. However, the actual quantum yield [Y(II)] and the electron transport rate (ETR) were substantially influenced by light quality (Figures 1B and C). While the lowest Y(II) values were observed under R light, the highest was recorded under the P light regime, and the B light condition produced Y(II) values that were comparable to W light (Figure 1B). PA treatments under B, R, and P light conditions enhanced Y(II), with the most significant increases observed under SPM treatments, which demonstrated a 147% and 143% improvement under B and R lights, respectively, in comparison to the untreated controls. The lowest values were observed under R light, while the highest values were observed under P light, even in the absence of PA treatment. Similar patterns were observed for ETR. ETR was substantially increased by SPM under B and R light, whereas it remained high regardless of PA application under P light (Figure 1C).

#### *3.3.1.2. Differences in growth parameters*

Under W light, the plants showed the best response in terms of shoot and root elongation (Table 2). Blue light inhibited shoot and root lengths and thus resulted in a compact morphology;

however, the weight of the shoots and roots was similar to those grown under white light. The effect of blue was so dominant, that reduced elongation was also detected under P light conditions. The biomass trend patterns were the same for both B and R lights, with little variation being observed across the different PA treatments. SPM enhanced the shoot biomass, while the root biomass was not affected or slightly reduced by higher PAs including SPD and SPM.

#### *3.3.1.3. Changes in proline content*

Proline content in leaves and roots was not affected significantly by light quality (B, R, or P) compared to W light (Figure 2). Conversely, leaf proline levels were diminished by R light in comparison to B light. Under B light, all PA treatments resulted in an increase in leaf proline. However, under R and P conditions, only SPM significantly increased proline levels. The root proline content was increased by SPD and SPM in all light conditions (Figures 2A-B).

### **3.3.2. Effects of different spectral conditions in combination with polyamine treatments on polyamine metabolism**

#### *3.3.2.1. Changes in polyamine contents*

Compared to white light, B light did not influence PUT, SPD, or SPM levels; however, it did increase DAP in the leaves (Figure 5A-D). The P light exhibited negligible changes in PA levels, while the R light increased PUT and SPD. PUT levels were influenced by PA treatments under B light and SPM treatment under R light. SPD levels experienced a substantial increase following PA interventions under B light, whereas SPM levels increased under P light but decreased under R light. DAP levels increased following SPM treatment under R and P conditions, indicating that red light promotes PA catabolism.

Root PA levels were not influenced by light treatments only; however, exogenous PAs caused significant changes (Figure 6). The content of PUT, SPD, SPM, and DAP was also greatly influenced, and the variation was similar for all the light treatments, especially after the SPM treatment.

#### *3.3.2.2. Differences in the expression levels of genes involved in polyamine metabolism*

The leaves showed differential gene expression of the genes involved in PA metabolism as a result of the light conditions (Figure 7). In the non-PA treated plants, only R light enhanced the expression of *TaADC*, while the PUT content was also slightly higher under R light. Under the B light, all the PA treatments enhanced the expression of *TaADC* to near the control level under the

R light. On the other hand, under R light, SPM treatment downregulated the expression of *TaADC* while P light had no significant impact on it (Figure 7A).

The expression of *TaSPDS* was reduced under both B and P lights, with a more significant reduction noted under dominant blue light. *TaSPDS* expression exhibited a slight increase in response to R light (Figure 7B). The level of *TaSPDS* was further modulated by exogenous PA treatments with all PAs increasing expression under B light, although this level of expression was not as high as that seen under W light. The level of *TaSPDS* expression was also controlled by the SPD and SPM treatments, and expression of this gene was reduced under R light while under P light the expression of the gene was inhibited by both SPD and SPM in a similar manner.

The expression of *TaPAO*, which is involved in the terminal catabolism of SPD and SPM, was light-dependent. It was decreased by the B light, whereas it was increased by the R light. In comparison to W light, *TaPAO* expression was also reduced by P light (Figure 7C). *TaPAO* expression was restored to W light levels by PA treatments under B light, whereas it was reduced by SPD and SPM treatments under R light. After the PA treatments, no substantial alterations were observed under P light.

In the roots, the expression of PA metabolism-related genes showed a contrasting pattern to that observed in the leaves (Figure 8). B light increased the expression level of *TaADC*, *TaSPDS*, *TapxPAO*, *TaPUT1* and *TaPUT2*, while P light had similar effects on *TaADC* and *TapxPAO*, but its effect on *TaSPDS*, *TaPUT1*, and *TaPUT2* was similar to that of R light. *TaSPDS* transcription generally decreased under all light conditions, whereas PA treatment induced *TaADC* expression under R light. Under PA treatments, *TapxPAO* expression was markedly upregulated, with the highest levels of expression being seen under R and P lights. It is intriguing that B light significantly increased the expression of *TaPUT1* and *TaPUT2*, both PA transporters; however, PA interventions tended to reduce their expression (Figures 8D-E).

**This study revealed that the spectral composition of light reasonably alters physiological and metabolic activities in wheat. Blue light increased photosynthetic efficiency since all measured parameters—quantum yield of photosynthesis (Y[II]), electron transport rate, chlorophyll and carotenoid content- performed significantly better under this light. While blue light had positive impact on some photosynthetic characteristics such as chlorophyll content and electron transport rate, it did not affect the endogenous polyamine content as compared to white light. Blue light generally induced PA metabolism-related gene**

expression in the root but repressed it in the leaf. Red light appeared to have opposite influences on PA metabolism in that it stimulated certain PA synthesis in the leaves while suppressing gene expression in the roots. Furthermore, the effects of applied PA depended on the light quality and plant organs, and spermine treatment was most effective under both blue and red light conditions. This experiment highlights how the spectral composition of light can be used in combination with PA treatments as a principal factor for plant growth promotion and stress tolerance enhancement.

### **3.4 Influence of *phyA* mutation on polyamine metabolism in *Arabidopsis* under different light spectra**

In the presence or absence of exogenous SPM treatment, wild-type Col-0 and *phyA* mutant *Arabidopsis* were grown under a variety of light spectra (L1: "white light" from a continuous wide-spectrum LED; L2: "red light" with an elevated far-red component; L3: "blue light" dominated by blue light). Plants were grown under controlled light spectra to assess spectral-specific effects on PA metabolism.

Photosynthetic activity and chlorophyll fluorescence were evaluated using PAM fluorometry. Gene expression of enzymes involved in PA metabolism was quantified via qRT-PCR. Metabolite profiling, including PA content, was performed using HPLC.

**(Refer to the result figures for visual representation and the detailed materials and methods in [Rahman et al., 2023], included in Appendix D.)**

#### **3.4.1 Determination of the physiological status of plants under different light spectra**

##### *3.4.1.1. Differences in shoot weight*

The *phyA* mutation did not significantly affect shoot weight across all light conditions (Fig. 1A). Nevertheless, the shoot weight of both genotypes increased in comparison to L1, indicating that light quality has a greater impact on growth than the mutation for both L2 and L3. Shoot weight was not substantially affected by a one-day SPM treatment in any light condition (Fig. 1A).

##### *3.4.1.2. Changes in chlorophyll-a fluorescence induction parameters*

The Fv/Fm values (~0.8) were unaffected by genotype or treatment, suggesting that the growth conditions were optimal (Fig.1B). There were no differences observed between the genotypes under L1 and L2, but L3 slightly decreased Y(II) in *phyA* mutants in comparison to Col-0 (Suppl. Fig. 1b). The SPM treatment had a greater impact on Y(II) in wild-type plants under L1

and L3. Still, it had a minimal effect on the mutant (Suppl. Fig. 1a, b). L2 treatment with SPM slightly increased Y(II) in *phyA* mutants, though these changes were minor overall.

### 3.4.2 Investigation of polyamine metabolism modulated by light spectra

#### 3.4.2.1. Polyamine content and gene expression modulation by light spectra

The *AtADC2* gene was primarily investigated because the activity of its promoter region is affected by light and stress factors. In Col-0, L2 and L3 downregulated *AtADC2* expression (Fig. 2A). The *phyA* mutants demonstrated diminished expression in L1, while exhibiting comparable expression levels in L2 and L3. SPM treatment resulted in the expression of *AtADC2* in both genotypes, particularly in mutants under L1 and L3 (Fig. 2A), suggesting that SPM causes de novo PUT synthesis.

In the absence of SPM, the transcription levels of *AtSPDS2*, a hormone-inducible gene, were comparable between genotypes under L1 and L3, but marginally higher in *phyA* mutants under L2 (Fig. 2B). In both genotypes, SPM reduced *AtSPDS2* expression at L1 and L2, while no significant change was observed at L3 (Fig. 2B). The expression level of *AtSPMS* was downregulated in the *phyA* mutant compared to the wild type under L1 and L2 light treatments; however, under L3 light conditions, the expression level was comparable to that of the wild type and initially lower (Fig. 2C). Under L3, SPM produced the most substantial increase in *AtSPMS* in Col-0, exhibiting moderate effects under L1 and L2.

The PUT content for both genotypes remained consistent under all light conditions (Fig. 3A) and exhibited a significant increase after SPM treatment. PUT levels increased consistently across genotypes, independent of light conditions, although *AtADC2* expression exhibited variation. SPD was the predominant PA in *Arabidopsis* leaves (Fig. 3B), whereas PUT and SPM were significantly less abundant. The SPM treatment led to a slight decrease in SPD, especially under white and far-red light conditions. SPM content was consistent across genotypes under all light conditions, although *AtSPMS* expression exhibited variation (Fig. 3C). Exogenous SPM elevated SPM levels in Col-0 and *phyA* comparably under L1 and L2; however, Col-0 exhibited greater SPM accumulation under L3.

Current findings indicate that (Copper Amine Oxidase) *AtCuAO1* expression is unaffected by light conditions or the *phyA* mutation. In mutants, the expression was elevated during L1 and L3 in the presence of excess SPM (Fig. 4a). The expression of *AtCuAO3* was reduced in Col-0 under L3; however, its levels showed a slight increase in both genotypes under L1 and in Col-0

under L3 following SPM application (Fig. 4b). SPM treatment elevated the expression of *AtPAO2* across all light conditions, regardless of genotype (Fig. 4c). The expression of *AtPAO5* exhibited greater variability, with the minimum value recorded in Col-0 under L3 (Fig. 4d). SPM increased *AtPAO5* expression, especially under L2, while *phyA* mutants showed elevated levels under L3, even without SPM.

#### 3.4.2.2. Correlation and principal components analysis (PCA)

Table 1 shows that the correlation analysis found a positive correlation between the levels of PUT and SPM and the expression levels of *AtADC2*, *AtSPMS*, and *AtPAO2*. The level of SPD and the expression of *AtSPDS2* were negatively correlated with the PUT/SPM levels. Furthermore, SPD content also showed a strong, negative correlation with *AtPAO2* and *AtPAO5* expression levels.

The PCA expression analysis (Fig. 5) showed that the genetic and biochemical traits of the treatment groups were separated. SPM was a major treatment discriminant, while L3 had a specific effect on PA metabolism. Three variable groups, *SPD—SPDS2*, *ADC2—CuAO1—SPMS*, and *PAO2—PUT—SPM*, could explain the differences observed between the twelve treated groups. SPM treatment under L1 and L3 differentiated *phyA* mutants while L2 differentiated SPM-treated Col-0 from the mutant.

**The *phyA* mutation influenced PA metabolism in a light-quality-dependent manner. Under blue light, *phyA* mutants showed reduced photosynthetic efficiency, while far-red light enhanced PA catabolism and back-conversion. Exogenous SPM amplified these effects, notably increasing PUT synthesis under blue light. The findings reveal spectral-specific pathways in *phyA*-regulated PA metabolism and emphasize the role of phytochrome signaling in plant adaptation to diverse light conditions**

### 3.5 Adaptation to cadmium stress under white and blue light influenced by putrescine pre-treatment in wheat

PA metabolism is significantly affected by light quality (Pál et al., 2021); however, its significance under stress conditions is still unexplored. This study is the first to investigate the potential protective function of PUT pre-treatment during Cd stress in wheat and the impact of light quantity on PA metabolism.

The experiment employed techniques including inductively coupled plasma optical emission spectrometry for quantifying cadmium content, high-performance liquid chromatography for the analysis of PCs, and spectrophotometric assays to measure oxidative stress markers like lipid peroxidation and hydrogen peroxide. Antioxidant enzyme activities were determined through enzymatic assays, while gene expression levels related to PA metabolism and stress responses were quantified using real-time quantitative PCR. Statistical analysis was performed on data from three independent biological replicates using ANOVA and Duncan's post hoc test to determine significance.

**(Refer to the result figures for visual representation and the detailed materials and methods in [Rahman et al., 2024b], included in Appendix E.)**

### **3.5.1 Cadmium-induced stress responses and protective mechanisms under white and blue light conditions after putrescine pre-treatment**

#### *3.5.1.1 Cadmium content, lipid peroxidation, hydrogen peroxide content, and antioxidant enzyme activities*

Cd was mainly stored in the roots during Cd stress, with only a small amount of Cd being transported to the leaves. The amount of Cd in the leaves was not significantly affected by the light conditions or the application of PUT pre-treatment, as similar levels of Cd were detected in both cases (see Table 1). However, the amount of Cd uptake was higher when the plants were subjected to W light as compared to B light. What is important to note is the fact that the uptake of Cd was reduced when the plants were pre-treated with PUT pre-treatment in both light conditions. The roots of Put+Cd-treated plants contained the least amount of Cd under B light (Table 1).

The lower levels of lipid peroxidation in the leaves and reduced H<sub>2</sub>O<sub>2</sub> accumulation in the roots of Cd-treated plants grown under B light, as compared to those grown under W light (Table 1), may be attributed to the reduced Cd uptake under B light. These results indicate that the combination of PUT pre-treatment and Cd exposure reduces Cd-induced stress under W light, as evidenced by lower leaf MDA and root H<sub>2</sub>O<sub>2</sub> levels. However, this protective effect was not observed under B light in comparison to Cd treatment alone (Table 1). Furthermore, under B light, higher initial antioxidant enzyme activities were observed, with GR in both leaves and roots and APX in roots being notably prominent (Table 1). The GST and APX activities in the leaves were diminished by Cd treatment under W light, whereas it had no effect under B light (Table 1). In contrast, the PUT pre-treatment resulted in an increase in GR and GST activities under W light

and an enhancement in GR and APX activities under B light (Table 1). Under both light conditions, an additive effect of Put+Cd treatment was observed in GR activity (Table 1).

The response to Cd stress in the roots was more pronounced, as GR activity increased under W light and GST and APX activities increased under both light conditions (Table 1). The combined Put+Cd treatment under B light resulted in the highest GR and GST activities, as well as the lowest APX activity across all treatments (Table 1), despite the fact that PUT pre-treatment alone caused minimal changes—slightly increasing GST and reducing APX activities under B light (Table 1).

#### 3.5.1.2. *Phytochelatin synthesis*

The root PC composition underwent significant changes depending on the Cd and Put+Cd treatments when exposed to W and B light (Table 2). The treatment with Cd led to the synthesis of PC2, PC3 and PC5 when exposed to W light while only PC3 and PC5 were produced when exposed to B light. The level of PC was about three times higher when the plants were grown in W light than in B light after Cd treatment. PUT pre-treatment did not affect the PC synthesis significantly; however, the combination of Put+Cd affected the PC profile as compared to the Cd treatment only. In Put+Cd-treated roots under W light, PC2 was not detected while the levels of PC3 and PC4 were low and there was an increase in the level of the longer chain of PC6 (Table 2). In B light, Put+Cd treatment led to the induction of the PC2 synthesis, reduced PC3 accumulation and promoted PC6 synthesis without affecting PC5 levels. The results of this study showed that the Put pre-treatment resulted in the formation of longer PC compounds (PC6) in both light conditions. The total PC synthesis showed a decrease under W light and an increase under B light (Table 2).

The treatments had little effect on *in vitro* PCS activity in leaves (Table 2). However, the activity of PCS was evident in roots for Cd-treated plants under both light conditions although the impact was not as pronounced in Put+Cd treated plants (Table 2). Moreover, the expression of *TaPCS* gene in leaves was down-regulated by Cd treatment in both light conditions. Although PUT pre-treatment enhanced the *TaPCS* expression slightly in W light; however, it failed to alleviate the Cd-induced decrease in expression in Put+Cd-treated plants across both light conditions (Table 2). On the other hand, Cd enhanced the gene expression of *TaPCS* under W light while it down-regulated the gene expression under B light. The Put+Cd treatment enhanced the gene expression of *TaPCS* under B light while no further effect was seen under W light (Table 2).

### **3.5.2 Changes in polyamine metabolism under white and blue light conditions after putrescine pre-treatment**

#### *3.5.2.1. Polyamine content*

The catabolite DAP, which is a byproduct of SPD and SPM, was also quantified together with the levels of PUT, SPD and SPM (Fig. 2A-H). Out of all the PAs, SPD was found to be present in the highest amount in both the leaves and the roots. The initial PA levels were almost similar between W and B light with the only exception of the SPM content in leaves which was higher and the PUT levels in roots which was lower (Fig. 2D, F). In leaves PUT biosynthesis was upregulated by Cd stress in both light conditions while SPD levels were higher only under B light and SPM levels were downregulated in both conditions. Also, DAP concentration was enhanced in response to Cd stress in both light conditions (Fig. 2A-D). The levels of PAs were not affected to a great extent by the application of PUT pre-treatment alone but it did affect the changes in the PA pool that were induced by Cd. The combination of Put+Cd was more effective in increasing the levels of PUT and SPD than the Cd-only treatments under W light conditions. However, this synergistic effect was not observed under B light (Fig. 2B, C). The Cd stress caused the decrease of SPM was a dominant factor while the effect of PUT pre-treatment was negligible (Fig. 2D). In both light conditions, the levels of DAP were lower when the Put+Cd treatment was given than when Cd alone (Fig. 2).

The PUT content in the roots was similar to that of the leaves. However, the Put+Cd treatment led to the highest PUT content in both light conditions (Fig. 2F). The Put+Cd treatment caused the highest SPD accumulation when plants were grown under W light while the lowest level of SPD was seen under B light (Fig. 2G). The SPD content showed a pattern similar to that of leaves. Under W light, only the roots treated with Put+Cd had a significant increase in SPM (Fig. 2H) while the levels of DAP in the roots did not change significantly (Fig. 2E).

#### *3.5.2.2 Gene expression of polyamine metabolism-related genes*

We also analyzed the expression of key genes involved in PA metabolism, including *TaPUT*, *TaSPDS*, and *TapxPAO*. The transcript levels of *TaPUT* in leaves showed minimal fluctuations and were not significantly affected by any treatment. This indicates that PA transport proteins were not the main contributors to PUT accumulation after PUT pre-treatment (Fig. 3A). This suggests that PA transport proteins were not the major players in the accumulation of PUT after the PUT pre-treatment (Fig. 3A). When supplied with Put+Cd and under W light the

expression of *TaSPDS* increased to the highest level as compared to the other treatments. This suggests that SPD synthesis is higher where there is excess exogenous PUT (Fig. 3B). The expression of *TapxPAO* was upregulated by Put pre-treatment and Put+Cd treatment in both light conditions; however, the effect was more pronounced in the W light condition. This suggests that PA back-conversion plays a more significant role in response to the excess external PUT (Fig. 3C). In the roots, compared to the control, under W light the *TaPUT* expression slightly increased after all the treatments (Fig. 3D). The levels of SPD in the roots were found to be correlated with the levels of *TaSPDS*, which were higher when expressed under Cd and Put+Cd treatment in W light while it was unchanged in B light (Fig. 3E). The expression of *TapxPAO* was enhanced by Cd and Put+Cd in white light while it was down-regulated in blue light (Fig. 3F); this shows that the regulation of PA back-conversion is also dependent on the light conditions.

**These results highlighted that antioxidant enzyme activities were key determinants in mitigating cadmium stress. A notable technical finding was the reduction in cadmium uptake, which correlated with increased PC synthesis and PA conjugation. Gene expression analyses revealed that exogenous PUT pre-treatment modulated stress-related pathways differently under blue and white light, suggesting a synergistic interaction between light spectrum and PA metabolism. These findings emphasize the importance of integrating metabolite and transcript-level analyses for a comprehensive understanding of plant stress responses.**

#### 4. CONCLUSION

In the presented experiments, the steps of PA metabolism, classified as uptake, synthesis, back-conversion, and degradation, were investigated in cereal and *Arabidopsis* plants, with an emphasis on the species- and genotype-dependent differences and on the interactions with light quality. Each experiment builds on the results of the previous one and together contributes to a systematic understanding of how PAs can be effectively used to improve plant growth, development and stress tolerance.

The first experiment focused on the responses of wheat, maize, and rice plants to the application of exogenous PUT. The results pointed out that wheat has a better capability of maintaining PA homeostasis when exposed to excess PUT, thus wheat could be considered as a good plant model for further studies. On the other hand, rice was suffering from stress after PUT treatment, mainly because of the initially higher PUT content and less effective regulatory mechanism of PA metabolic system compared to the other two species. This preliminary study provided a basis for further studies on genotype-dependent as well as environment-dependent interactions in the following experiments in wheat.

A more detailed analysis was then performed in order to examine the PA uptake and metabolism in three different genotypes of wheat, namely Mv Béres, Mv Emese (winter wheat genotypes), and TC33 (spring wheat genotype), partly under dark conditions. All the genotypes showed a differential accumulation of PUT, with continuously increasing accumulation in Mv Béres, while more rapid accumulation at the initial stage in TC33. The differences in the biosynthesis and the PA back conversion pathways were seen more clearly at the gene expression level. This genotype-specific study revealed the genetic factors that are involved in regulating PA homeostasis under the conditions described and showed the variation of PA metabolism in three wheat genotypes. This realization instigated more studies to establish the possible factors that could alter these responses.

The third experiment was to subject wheat plants to different light compositions, such as dominant blue or red and their combination, to reveal how light quality affects the plant's PA metabolism. The results showed that light quality is one of the most influential factors that determine photosynthetic performance as well as PA levels. More specifically, the main finding of this study was that blue light enhances the photosynthetic performance with the positive effects of SPM treatment. Therefore, this study establishes that light spectral composition influences the

PA metabolic pathways differently, and thus it can be used to support the findings that light quality can be used together with PA interventions to enhance plant growth. This led to more specific questions on how light works in conjunction with the genetic factors of different organisms.

The study extended the investigation to *Arabidopsis* where wild-type and *phyA* mutant plants were exposed to different light regimes. The study also showed that the *phyA* mutation had a very significant impact on the PA metabolism especially when the plants were grown under blue and far-red light. This experiment also supported the notion that light signalling pathways including PhyA can be important regulators of PA levels in plants. This view added another level of understanding of the role of PAs under different light regimes and genetic backgrounds.

A comprehensive investigation was carried out on wheat to understand the role of PAs in alleviating Cd stress under white and blue light exposure. The combination of blue light and PA treatment increased photosynthetic efficiency while reducing the levels of oxidative stress markers. Thus, this experiment demonstrated that PA can improve the resistance of plants to heavy metal stress, especially when exposed to blue light, thereby establishing the notion that they can act as essential modulators of stress tolerance.

Collectively, these experiments offer a thorough comprehension of the dynamic role that PAs play in the physiology of plant growth, development and stress responses. These findings indicate that PA metabolism is not only species- and genotype-dependent but also highly responsive to external factors, such as light quality, which can be leveraged to enhance stress responses. These results underscore the significance of customizing PA interventions to the unique environmental contexts and genotypes of specific crops, thereby providing strategies for improving the resilience of crops in agriculture. Additionally, this research provides opportunities to investigate the molecular mechanisms that underlie PA metabolism. By incorporating insights from genotype-dependent differences, light-regulated PA pathways, and stress mitigation strategies, this corpus of work contributes to a growing understanding of plant adaptations to complex conditions. The potential of PA interventions to improve crop resilience in the face of environmental stressors and climate change can be further explored through the integration of agronomic practices in future research.

## 5. NEW SCIENTIFIC RESULTS

1. Putrescine (PUT) treatment induced oxidative stress in rice, with elevated H<sub>2</sub>O<sub>2</sub> levels, disrupted polyamine metabolism, and increased proline and NO contents. In maize, PUT enhanced root H<sub>2</sub>O<sub>2</sub> content and polyamine oxidase activity, indicating moderate stress. In maize roots, moderate stress was observed through enhanced H<sub>2</sub>O<sub>2</sub> content and increased polyamine oxidase activity. However, wheat had higher photosynthetic efficiency without any change in stress markers, which shows that the effect of exogenous putrescine depends on the different regulations of polyamine metabolism in different plant species.
2. It was also discovered that polyamine metabolism in wheat is also dependent on genotype and, to some extent, on light. Distinct variations in putrescine uptake patterns were found in Mv Béres and Mv Emese, which were accompanied by differential expression changes of genes involved in polyamine metabolism. These variations underscore genotype-dependent regulatory pathways for polyamine uptake and metabolism.
3. The metabolism of polyamines in wheat is influenced by light quality. Blue and red light had opposite effects on polyamine metabolism-related gene expression, and furthermore, leaves and roots exhibited differential responses. Polyamine treatments, especially spermine alleviated the blue light-induced inhibition of polyamine metabolism, and enhanced photosynthetic efficiency, thereby optimizing plant growth.
4. The *phyA* mutation in *Arabidopsis* was found to differentially affect polyamine metabolism under distinct light spectra. Under blue and red light, significant changes were observed in polyamine content and associated gene expression, emphasizing the regulatory role of light perception mechanisms in modulating polyamine metabolic pathways.
5. Under cadmium stress, putrescine pre-treatment combined with blue light reduced cadmium uptake and oxidative damage by promoting phytochelatin synthesis, increasing glutathione reductase activity, and stabilizing polyamine homeostasis. This highlights the synergistic role of putrescine and blue light in stress mitigation.

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## 7. LIST OF PUBLICATIONS (MTMT Identifier: 10089046)

### 7.1 Publications used in the thesis

- Pál, M., **Rahman, A.**, Hamow, K. Á., Nagy, K., Janda, T., Dernovics, M., & Szalai, G. (under review). Light and genotype affect the uptake of exogenous polyamines and their metabolism in wheat plants. *Plant Physiology and Biochemistry*
- Rahman, A.**, Nagy, K., Hamow, K. Á., Pál, M., Janda, T., Dernovics, M., Szóke, C., & Szalai, G. (2024b). Cadmium stress responses under white or blue light are influenced by putrescine pre-treatment in wheat. *Environmental and Experimental Botany*, 222, 105746. <https://doi.org/10.1016/j.envexpbot.2024.105746>
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### 7.2 Other Publications

- Saleem, K., Asghar, M. A., Javed, H. H., Raza, A., Seleiman, M. F., Ullah, A., **Rahman, A.**, Iqbal, S., Hanif, A., Imran, S., Nadeem, S. M., Du, J., Kocsy, G., Riaz, A., & Yong, J. W. H. (2023). Alleviation of arsenic toxicity-induced oxidative stress in lemon grass by methyl jasmonate. *South African Journal of Botany*, 160, 547-559. <https://doi.org/10.1016/j.sajb.2023.07.034>
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### **7.3 Conference Presentations and Posters**

**1. FIBOK2022 conference**, (April 11-12, 2022, Hungarian University of Agriculture and Life Sciences, Godollo, Hungary), Effect of different light spectral composition on polyamine metabolism in wheat - Poster Presentation. (ISBN 978-963-269-999-8).

**2. Vienna Bio Center PhD Program Symposium ‘Pushing Boundaries’** (November 3–4, 2022, Vienna, Austria), Effect of different light spectral composition on polyamine metabolism in wheat – (Poster presentation)

**3. 2nd Hungarian Polyamine Research Workshop**, (December 2, 2022, Szeged University) Influence of phyA mutation on polyamine metabolism in Arabidopsis in the presence of spermine excess under different light conditions - (Oral presentation).

**4. Plant Biology Conference**, (August 5-9, Savannah, Georgia, United States) Cadmium-induced changes under different light-quality conditions in wheat- Poster presentation

**5. 12th Congress of the Hungarian Free Radical Society**, (August 24-25, 2023, Hungarian Research Network, ATK, Martonvásár), Different effects of putrescine treatment in wheat, maize, and rice plants - Poster presentation

**6. 3rd Plant Polyamine Research Workshop**, (October 12-13, 2023, Hungarian Research Network, ATK Budapest and Martonvásár, Hungary)- Different adaptations to cadmium stress under white and blue light are partly further influenced by putrescine pre-treatment in wheat - Oral presentation

**7. 7th Conference on Cereal Biotechnology and Breeding**, (7–9 November 2023, Wernigerode, Germany), Different Responses of Wheat to Cadmium Stress under White and Blue Light, Modulated by Pre-Treatment with Putrescine- Poster presentation

**8. International Ceplas-IPK Summer School 2024**, (September 22-26, 2024, Drubeck, Germany), Methylation profile of wheat leaves under white and blue light after putrescine treatment using MeDIP-seq - Poster presentation

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## **9. APPENDICES**

# **Appendix A**



# Different reactions of wheat, maize, and rice plants to putrescine treatment

Altafur Rahman<sup>1,2</sup> · Eszter Kulik<sup>3</sup> · Imre Majláth<sup>1</sup> · Imran Khan<sup>2</sup> · Tibor Janda<sup>1</sup> · Magda Pál<sup>1</sup>

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## Abstract

Polyamines play an important role in growth and differentiation by regulating numerous physiological and biochemical processes at the cellular level. In addition to their roborative effect, their essential role in plant stress responses has been also reported. However, the positive effect may depend on the fine-tuning of polyamine metabolism, which influences the production of free radicals and/or signalling molecules. In the present study, 0.3 mM hydroponic putrescine treatment was tested in wheat, maize, and rice in order to reveal differences in their answers and highlight the relation of these with polyamine metabolism. In the case of wheat, the chlorophyll content and the actual quantum yield increased after putrescine treatment, and no remarkable changes were detected in the stress markers, polyamine contents, or polyamine metabolism-related gene expression. Although, in maize, the actual quantum yield decreased, and the root hydrogen peroxide content increased, no other negative effect was observed after putrescine treatment due to activation of polyamine oxidases at enzyme and gene expression levels. The results also demonstrated that after putrescine treatment, rice with a higher initial polyamine content, the balance of polyamine metabolism was disrupted and a significant amount of putrescine was accumulated, accompanied by a detrimental decrease in the level of higher polyamines. These initial differences and the putrescine-induced shift in polyamine metabolism together with the terminal catabolism or back-conversion-induced release of a substantial quantity of hydrogen peroxide could contribute to oxidative stress observed in rice.

**Keywords** Polyamine metabolism · Oxidative stress · Reactive species · Stress markers

✉ Magda Pál  
pal.magda@atk.hun-ren.hu

Altafur Rahman  
altafur.rahman@atk.hun-ren.hu

Eszter Kulik  
eszter.kulik@syngenta.com

Imre Majláth  
majlath.imre@atk.hun-ren.hu

Imran Khan  
imrankhanbtb@gmail.com

Tibor Janda  
janda.tibor@atk.hun-ren.hu

<sup>1</sup> Department of Plant Physiology and Metabolomics, Agricultural Institute, Centre for Agricultural Research, Hungarian Research Network, Brunszvik 2, Martonvásár 2462, Hungary

<sup>2</sup> Department of Plant Physiology and Plant Ecology, Institute of Agronomy, Hungarian University of Agriculture and Life Sciences, Budapest 1118, Hungary

<sup>3</sup> Syngenta Ltd., Alíz 2, Budapest 1117, Hungary

## Introduction

Polyamines (PAs) are a group of small, aliphatic organic molecules that are found widely throughout various living organisms, including plants (Tiburcio et al. 2014; Chen et al. 2019). The process of putrescine (PUT) synthesis takes place either through the decarboxylation of ornithine or indirectly via the decarboxylation of arginine (this latter reaction is catalysed by arginine decarboxylase (ADC)). The synthesis of higher PAs, namely spermidine (SPD) and spermine (SPM), involves the stepwise addition of aminopropyl moieties to the PUT structure through enzymatic reactions, catalysed by SPD synthase (SPDS) and SPM synthase. The process of catabolism in PAs is regulated by enzymes such as diamine oxidase (DAO) and PA oxidases (PAOs). It is worth mentioning that two different types of PAOs exist, members of the first one play a crucial role in the final breakdown of SPD or SPM, whereas the others are involved in the partial or complete conversion of SPM to SPD and SPD to PUT. PA oxidases (PAOs) in rice have been extensively characterized

regarding their subcellular distribution and temporal expression dynamics across growth stages. Notably, apoplastic PAO expression is markedly reduced during the initial two weeks following seed germination, whereas peroxisomal PAOs are prominently expressed during this pivotal developmental phase, as reported by Ono et al (2011). As a result, the PA pool demonstrates temporal variations, which are marked by swift interconversions that form the "PA cycle" (Pál et al. 2015). The collection of these polycationic compounds demonstrates a high level of precision in regulating important cellular processes, including DNA stabilization, RNA processing, maintenance of membrane integrity, and protein synthesis (Tiburcio et al. 2014). In addition to their fundamental role in normal plant development, PAs have become essential regulators in the field of plant stress adaptation. They function as molecular orchestrators that coordinate responses to various types of stress, including both abiotic and biotic factors (Pál et al. 2021; Alcázar et al. 2010; Hussain et al. 2011).

Stress tolerance in plants has been closely associated with their ability to enhance PA synthesis when exposed to stresses, often achieved through the overexpression of PA biosynthetic genes (Liu et al. 2015; Jia et al. 2021; Alcázar et al. 2020). The potential of PAs to enhance stress tolerance in crops has been suggested due to their capacity to mitigate oxidative damage resulting from environmental stressors (Takahashi and Kakehi 2009). Nevertheless, it is crucial to acknowledge that excessive accumulation of PUT during stressful circumstances, resulting in an elevated PUT/(SPD + SPM) ratio, can have adverse consequences on plant physiology (Shu et al. 2012a, b). PAs demonstrate a dual function, serving as scavengers of free radicals and as generators of free radicals (Takahashi and Kakehi 2009; Pottosin and Shabala 2014). Moreover, it should be noted that PAs also serve as signalling molecules, thereby adding complexity to their various functions (Mattoo and Sobieszczuk-Nowicka 2019). While existing research has primarily emphasized the positive effects of PAs on stress resistance, the influence of exogenous PAs on the PA pool's balance remains a critical aspect that has not been extensively discussed (Do et al. 2013; Liu et al. 2015; Shu et al. 2012a, b). Additionally, the intricate interaction between the treatment of PUT and the metabolism of PAs in various plant species continues to be a topic of research. The potential outcomes of this interaction may vary depending on the plant's initial PA levels and capacity to maintain PA balance after the supplementation of PUT. It is crucial to investigate whether the advantageous impacts of PUT treatment observed in specific crop species are universally applicable or limited to specific species.

The objective of this study is to evaluate the effect of PUT treatment on three economically important plant species, namely wheat (*Triticum aestivum*), maize (*Zea mays*), and

rice (*Oryza sativa*). The hypothesis suggested that there may be variations in the response to PUT treatment among these species, which can be attributed to differences in their basal PA levels and their ability to regulate PA homeostasis. The primary objectives of the present study encompass the comparison of PA metabolism in these plants, addressing the following research inquiries: (1) Does the applied 0.3 mM PUT treatment administered over a one-week duration induce stress in rice plants, and what physiological changes (photosynthesis-related parameters are some stress markers) are evident? (2) What initial disparities in PA content exist among these plant species, and what discernible variations emerge in the PA metabolism of plants following PUT treatment, with a particular focus on significant alterations at gene expression level? (3) Are changes in PA pool predominantly influenced by the mechanism of PA uptake or the PA metabolism, and consequently responsible for the observed changes in PA pool, and in turn for the negative effects? Through systematic investigation of these three crop species' responses to PUT treatment, we aim to unravel the intricate relationship between PUT supplementation and PA metabolism.

## Material and methods

### Plant material, growth conditions, and treatment

We conducted experiments on three cereal species: wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.). Specifically, we chose domestically bred genotypes for each species: Béres (winter wheat variety from Martonvásár), Mv 350 (maize hybrid from Martonvásár), and Janka (japonica rice variety from Szarvas).

Rice plants have higher temperature requirements than wheat and maize, so they were grown separately. Wheat and maize were germinated between moistened filter papers at 26 °C for 3 days, with daily monitoring of germination progress, while rice seeds were placed between soaked filter papers and germinated at 37 °C for one day and then at 27 °C for 5 days, in dark.

Healthy seedlings (15 plants per beaker for wheat and rice, 6 for maize) were grown on stainless-steel nets with modified Hoagland nutrient solution (Pál et al. 2005) in a Conviron PGV-36 plant growth chamber at 22/20 °C for wheat and maize, while at 28/26 °C for rice (16/8-h light–dark cycle, 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, 75% relative humidity). After one week, maize, wheat, and rice plants were randomly divided into control (C) and PUT-treated (PUT) groups. PUT treatment was applied at 0.3 mM concentration into the nutrition solution for 7 days. This concentration and duration were chosen based on previous results on wheat, maize and rice (Szalai et al. 2017; Pál et al. 2017).

**Table 2** Primer sequences for RT-qPCR analysis of reference and gene of interest genes in maize plants

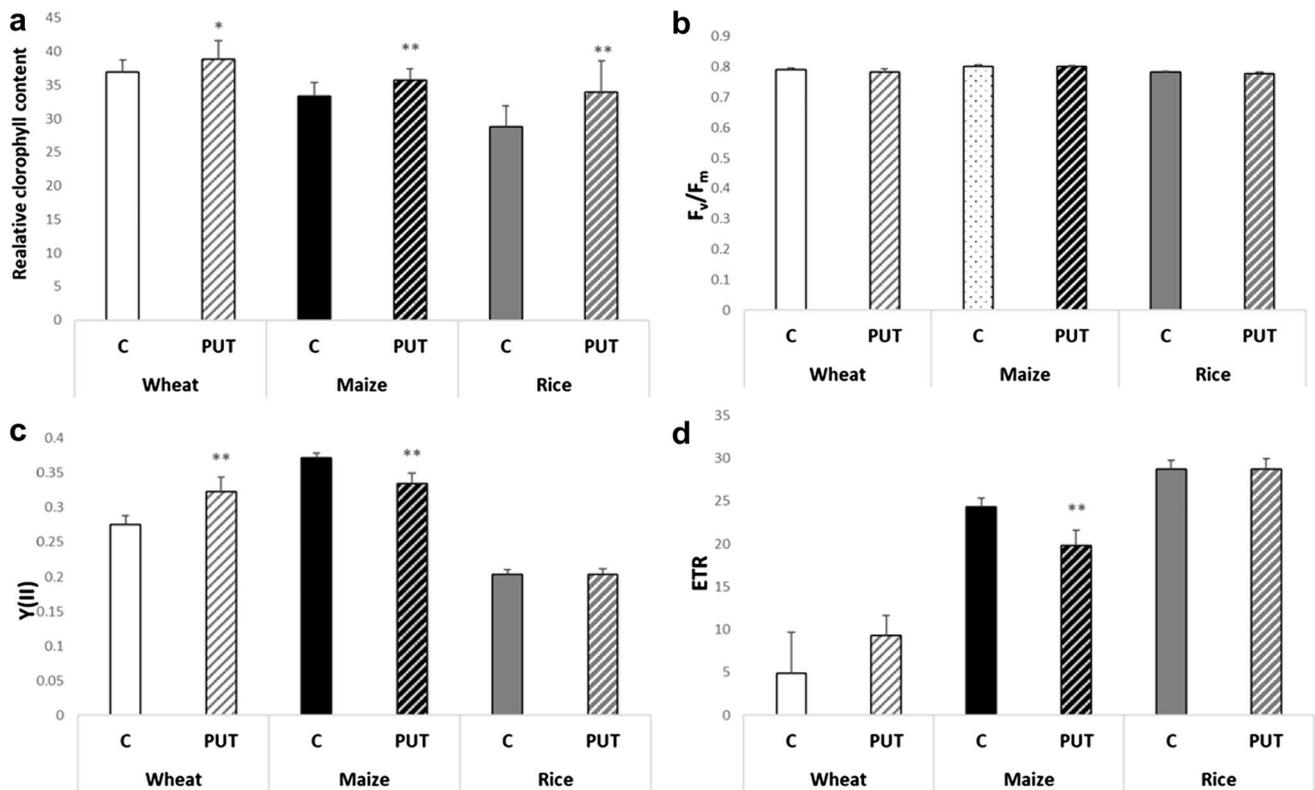
<i>ZmβTUB</i>	Forward	CTACCTCACGGCATCTGCTAT	139 bp	NM_001112218.1
	Reverse	AGGAAGGATGGAGAACACCC		
<i>ZmADC</i>	Forward	CTAATATGCCCGTATCCACC	167 bp	NM_001365614.1
	Reverse	GGCAATCATCATAAGTCGCAC		
<i>ZmSPDS</i>	Forward	CGAAAGAATCAGTGTGAGAACC	152 bp	AY730048.1
	Reverse	GTGCGGTGTGAGCAAAAAGC		
<i>ZmapoPA0</i>	Forward	GCAAGTACCATGTCCAGGG	148 bp	NM_001111636.2
	Reverse	CGAGGGAACATGGCTGTCA		
<i>ZmpxPA0</i>	Forward	TCCTACTCGTGCACCTG	142 bp	NM_001176693.2
	Reverse	CGATGCCTGACGAGTAAGC		
<i>ZmPUT1</i>	Forward	CATCGACAATGCCCTGTACC	190 bp	XM_035959254.1
	Reverse	AGGAAGGATGGAGAACACCC		
<i>ZmPUT2</i>	Forward	GGAACACGGCAATAACACGA	105 bp	BT035190.1
	Reverse	GCCCTCCCTTATGCTCTTCA		

**Table 1** Primer sequences for RT-qPCR analysis of reference and gene of interest genes in wheat plants

<i>Ta35497</i>	Forward	GTGTGTCCCGTGTGCTGTC	131 bp	(Paolacci et al. 2009)
	Reverse	TCCAGCAGCCCAAAGAGTCC		
<i>TaADC</i>	Forward	AGGAGGAGGAGCTCGACATT	137 bp	(Gardiner et al. 2010)
	Reverse	GCCGAACCTTGCCCTTCTC		
<i>TaSPDS</i>	Forward	AGGTATTCAAGGGTGGCGTG	137 bp	(Pál et al. 2018)
	Reverse	TGGGTTTACAGGAGTCAGGA		
<i>TaapoPA0</i>	Forward	CCAGCCTCCAGTCCGCAAC	113 bp	(Xiong et al. 2017)
	Reverse	GCCCAGCTCCTCCACCTCGTC		
<i>TapxPA0</i>	Forward	GCTCATAAATCAGCCCAATTCCA	125 bp	(Xiong et al. 2017)
	Reverse	TTCGCCATTTGTTGAGCTCT		
<i>TaPUT1</i>	Forward	GGTCTTCTCCCTCTTGCCCT	156 bp	XM_044548016.1
	Reverse	GTGCTGATCGAGTCCCAGTA		
<i>TaPUT2</i>	Forward	TTCATCGCCTTCATCAAGCTG	124 bp	XM_044523314.1
	Reverse	TCACCACGACGATCAGGATAG		

**Table 3** Primer sequences for RT-qPCR analysis of reference and gene of interest genes in rice plants

<i>OsEF1alpha</i>	Forward	TTTCACTCTTGGTGTGAAGCAGAT	103 bp	(Phule et al. 2018)
	Reverse	GACTTCCTTCAGGATTCATCGTA		
<i>OsADC</i>	Forward	ATCATCCCAATCCAGCGCCT	107 bp	XM_015787552.2
	Reverse	TGCCTCCCGCCGATGAAGT		
<i>OsSPDS</i>	Forward	AGAGCATGTGGTTGCATACGC	69 bp	(Do et al. 2013)
	Reverse	GTGCTGATCGAGTCCCAGTA		
<i>OspxPA03</i>	Forward	TTTCTATTGCGAAGGCCATTG	100 bp	(Ono et al. 2011)
	Reverse	ATGCGGCACAAATACCACTGA		
<i>OspxPA05</i>	Forward	CATCCAGAGGTACAACAAAATAT	118 bp	(Ono et al. 2011)
	Reverse	TTCAAACCTTGATGATATTTGCTTTAA		
<i>OsPUT1</i>	Forward	GGTGGATGAAGTGGTTGAGC	152 bp	XM_015772006.2
	Reverse	ATTCAGCAATGTCAGCACGG		
<i>OsPUT2</i>	Forward	ATTGGCATCATGTTCTCCGC	137 bp	XM_015776404.2
	Reverse	ACCACCCTCAGCTTGATGAA		



**Fig. 1** Effects of 7-day 0.3 mM PUT treatments on the relative chlorophyll content (a), and chlorophyll-*a* fluorescence induction parameters (b: maximum quantum yield of PSII ( $F_v/F_m$ ), c: actual quantum yield of PS II ( $Y(II)$ ), and d: the electron transport rate (ETR))

in wheat, maize, and rice plants. Data represent mean values  $\pm$  SD,  $n=10$ . The significant difference at the  $p \leq 0.05$  and  $p \leq 0.01$  level is indicated by \* and \*\*, respectively

**Table 4** Effect of 7-day 0.3 mM PUT treatment on gas exchange parameters (A: net photosynthetic activity, gs: stomatal conductance, and E: transpiration)

		Net photosynthetic activity (A) ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	Stomatal conductance (gs) ( $\mu\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	Transpiration (E) ( $\mu\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )
Wheat	C	12.5 $\pm$ 0.55	89.6 $\pm$ 3.78	1.40 $\pm$ 0.10
	PUT	13.76 $\pm$ 1.01	106 $\pm$ 17.43	1.20 $\pm$ 0.10
Maize	C	12.86 $\pm$ 1.21	52 $\pm$ 5.56	0.66 $\pm$ 0.06
	PUT	13.06 $\pm$ 1.27	48 $\pm$ 1.52	0.63 $\pm$ 0.06
Rice	C	12.52 $\pm$ 1.65	95 $\pm$ 7.76	1.05 $\pm$ 0.10
	PUT	11.62 $\pm$ 1.20	89 $\pm$ 7.72	0.88 $\pm$ 0.08*

The significant difference at the  $p \leq 0.05$  is indicated by \*, compared to the adequate control

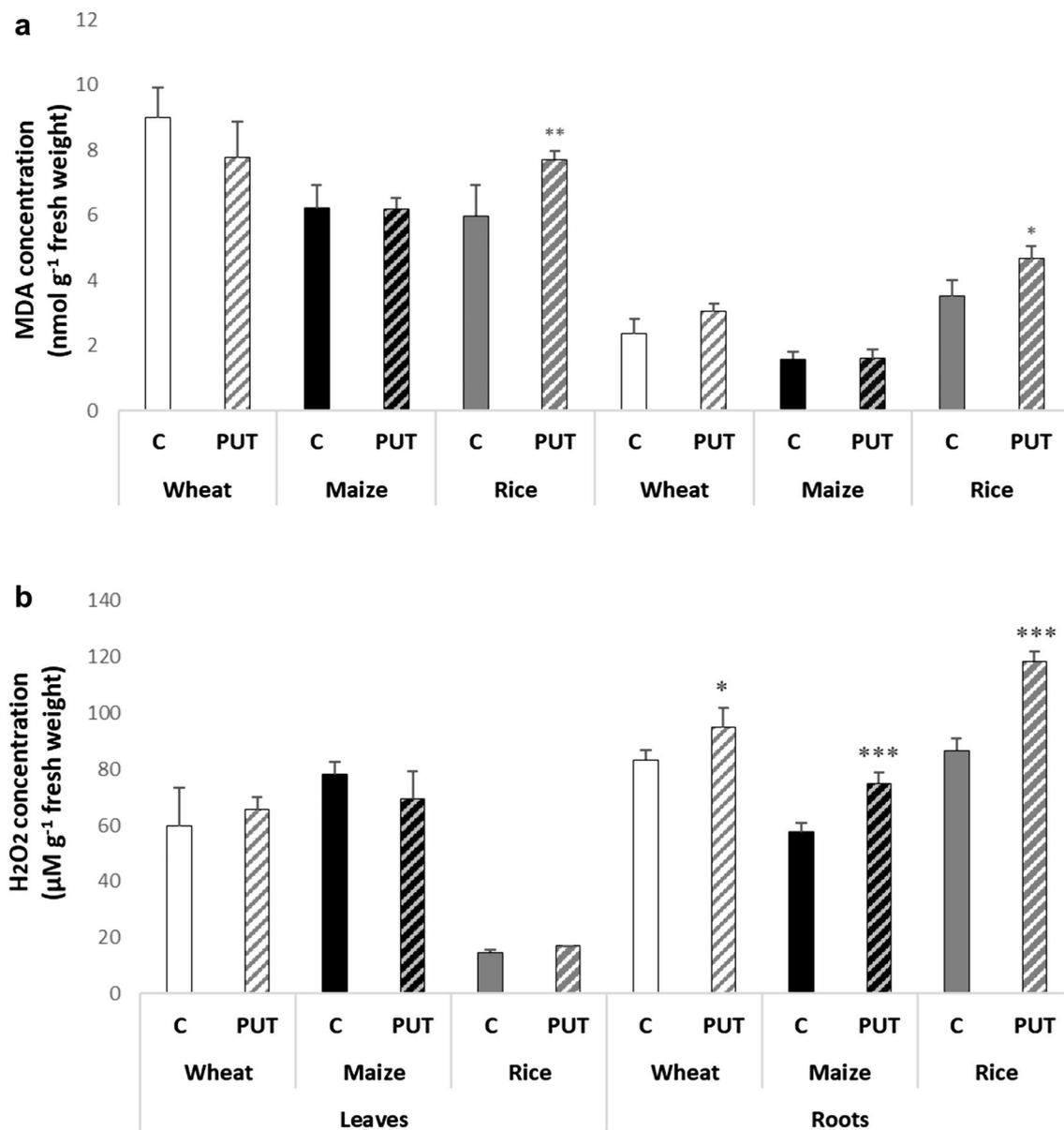
During the experiment, nutrient solutions were changed every two days, and pots were randomized. After one week of PUT treatment, leaf and root samples were collected from both C and PUT-treated plants.

### Chlorophyll content measurement and chlorophyll-*a* fluorescence induction analysis

The youngest, completely expanded leaves were used for the measurements. The chlorophyll content was measured

non-invasively with a portable SPAD-502 chlorophyll meter (Konica Minolta, Inc. Japan). The recorded values ranged from 0 to > 100.

The fluorescence imaging study was performed with a pulse amplitude modulated fluorometer (PAM) that was equipped with an Imaging-PAM MSeries from Walz (Effeltrich, Germany). The PAM was fitted with a blue LED-Array Illumination Unit IMAG-MAX/L, operating at a wavelength of 450 nm. Leaves had undergone 15 min of dark adaptation, in order to assure the activation of the acceptor



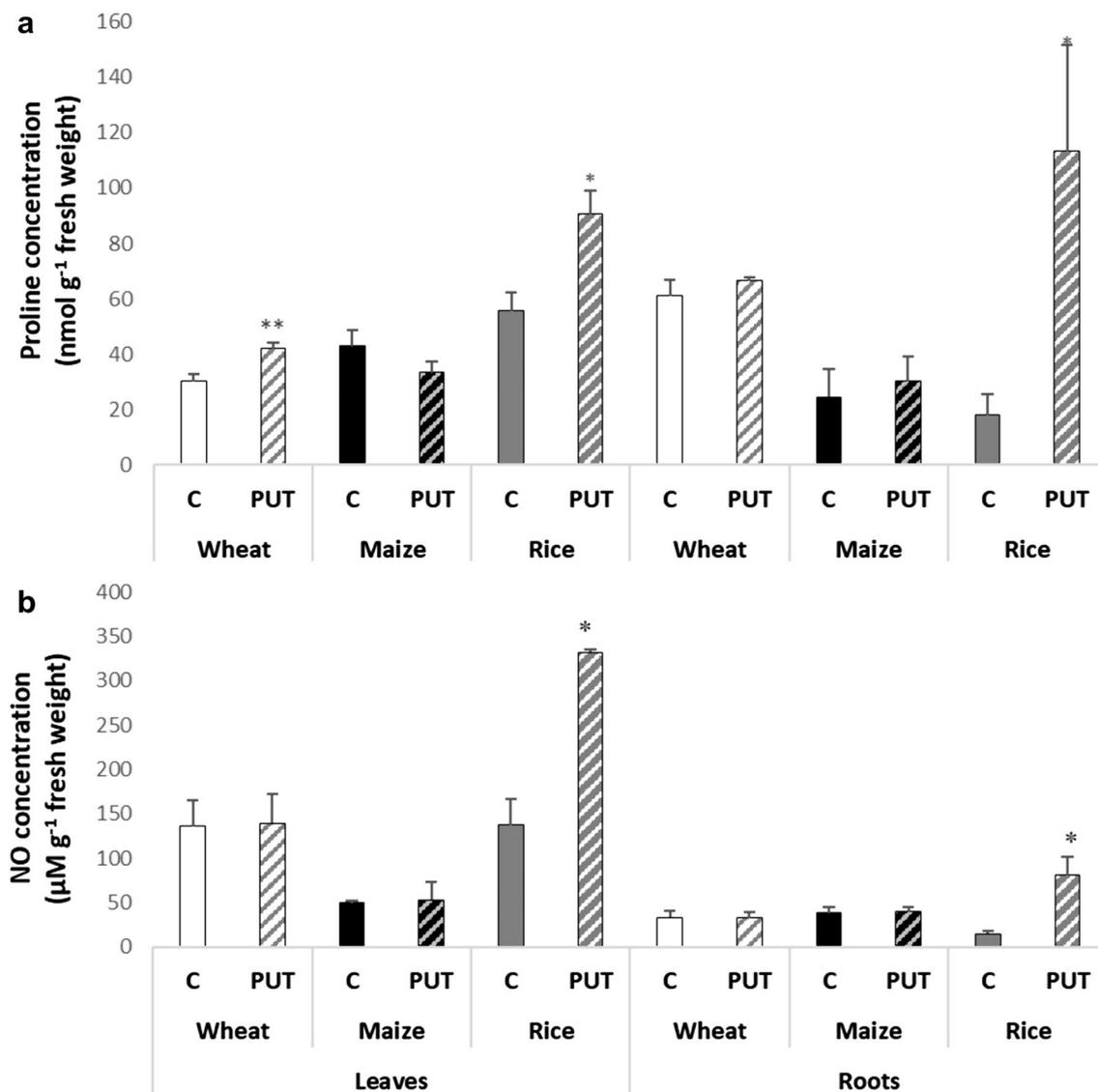
**Fig. 2** Effects of 7-day 0.3 mM PUT treatments on the MDA concentration (a) and H<sub>2</sub>O<sub>2</sub> concentration (b) in the leaves and roots of wheat, maize, and rice plants. Data represent mean values ±SD,

n = 10. The significant difference at the  $p \leq 0.05$  and  $p \leq 0.01$  level is indicated by \* and \*\*, respectively

side of the photosynthetic apparatus. The maximum quantum yield of photosystem II (PSII), represented as Fv/Fm, the actual quantum yield of PSII [Y(II)] were determined, and the linear electron transport rate (ETR) was calculated during the analysis. The investigation of chlorophyll-*a* fluorescence quenching was conducted in accordance with the methodologies outlined in the publication by Gondor et al. (2021).

### Gas exchange measurements

Gas exchange assessments were conducted after 7 days of PUT treatment, on the last fully developed leaves of the plants with a Ciras 2 Portable Photosynthesis System (Amesbury, USA) The reference CO<sub>2</sub> level was set at 380 μL L<sup>-1</sup>, with a light intensity of 250 μmol m<sup>-2</sup> s<sup>-1</sup>. These gas exchange analyses were conducted under ambient room temperature conditions, and air humidity was maintained at 50 ± 3% in both instances. Parameters such as net photosynthetic activity (A), stomatal conductance (gs), and



**Fig. 3** Effects of 7-day 0.3 mM PUT treatments on the proline concentration (a) and NO concentration (b) in the leaves and roots of wheat, maize, and rice plants. Data represent mean values  $\pm$  SD,

$n=10$ . The significant difference at the  $p \leq 0.05$  and  $p \leq 0.01$  level is indicated by \* and \*\*, respectively

transpiration (E) were measured during the steady-state phase of photosynthesis (Majláth et al. 2021).

### Determination of the level of lipid peroxidation and H<sub>2</sub>O<sub>2</sub> content

To assess lipid peroxidation, we followed the procedure outlined by Majláth et al. (2021), which involves the determination of MDA levels. The samples were analysed spectrophotometrically at 532 nm with Shimadzu UV-vis 160A (Shimadzu Corp. Kyoto, Japan), with the subtraction of non-specific absorption at 600 nm. The quantification

was carried out utilizing an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

For the determination of H<sub>2</sub>O<sub>2</sub> content in the samples, we employed the ferrous ammonium sulfate/xylenol orange (FOX-1) method, as described by Gay et al. (1999). This method involved spectrophotometric measurements at 560 nm (Shimadzu UV-vis 160A), utilizing an H<sub>2</sub>O<sub>2</sub> calibration curve for quantification.

### Enzyme assays

To analyse antioxidant enzyme activity, 0.5 g tissue was homogenized in 2.5 ml Tris-HCl buffer (0.5 M,

pH 7.5) containing 3 mM MgCl<sub>2</sub> and 1 mM EDTA. The measurements were conducted using spectrophotometry (Shimadzu UV–vis 160A), following the methodology described by Pál et al. (2005). The activity of glutathione reductase (GR) (EC 1.6.4.2.) activity was determined at 412 nm according to Smith et al. (1988). The reaction mixture contained 75 mM Na-phosphate buffer (pH 7.5), 0.15 mM diethylenetriamine-pentaacetic acid, 0.75 mM 5,5'-dithiobis (2-nitrobenzoic acid), 0.1 mM NADPH, 0.5 mM oxidized glutathione and 50 µl plant extract in a total volume of 1 ml. The increase in absorbance at 412 nm was monitored. The activity of glutathione-S-transferase (GST) (EC 2.5.1.18.) was measured by following changes in the absorbance at 340 nm in a mixture containing 72.7 mM Na-phosphate buffer (pH 6.5), 3.6 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene and enzyme extract (Mannervik and Guthenberg 1981). The activity of ascorbate peroxidase (APX) (EC 1.11.1.11.) activity was determined in the presence of 0.2 M Tris buffer (pH 7.8) and 5.625 mM ascorbic acid according to Janda et al. (1999). The reaction was started with 0.042% H<sub>2</sub>O<sub>2</sub>. The decrease in absorbance at 290 nm was monitored. The activities of antioxidant enzymes are expressed in units of nkatal (g<sup>-1</sup> FW).

### Proline and nitric oxide determination

The quantification of proline content was carried out using the Bates method (1973) with slight modifications, which relies on its reaction with ninhydrin. To summarize, 200 mg plant samples were homogenized in distilled water. After centrifugation at 15,000 rpm for 10 min at 4 °C, 0.5 ml supernatant was combined with 0.25 ml of glacial acetic acid and 0.25 ml of ninhydrin reagent. This mixture was incubated at 96 °C for 30 min, then the chromophore generated was subsequently extracted using 1 ml of toluene, and its absorbance at 518 nm was determined using a Shimadzu 160A.

The measurement of NO was conducted utilizing the Griss reagent method (Invitrogen™ Griess Reagent Kit, for nitrite quantitation, Catalog number: G7921) according to the manufacturer's instruction.

### Diamine oxidase and polyamine oxidase enzyme activities

The method employed by Takács et al. (2016) was used to estimate the enzyme activities of diamine oxidase (DAO, EC 1.4.3.6.) and polyamine oxidase (PAO, EC 1.5.3.3.). Enzyme activity was expressed in nmol Δ<sup>1</sup>-pyrroline min<sup>-1</sup> g<sup>-1</sup> FW using an extinction coefficient of 1.86 × 10<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup>.

### Polyamine analysis

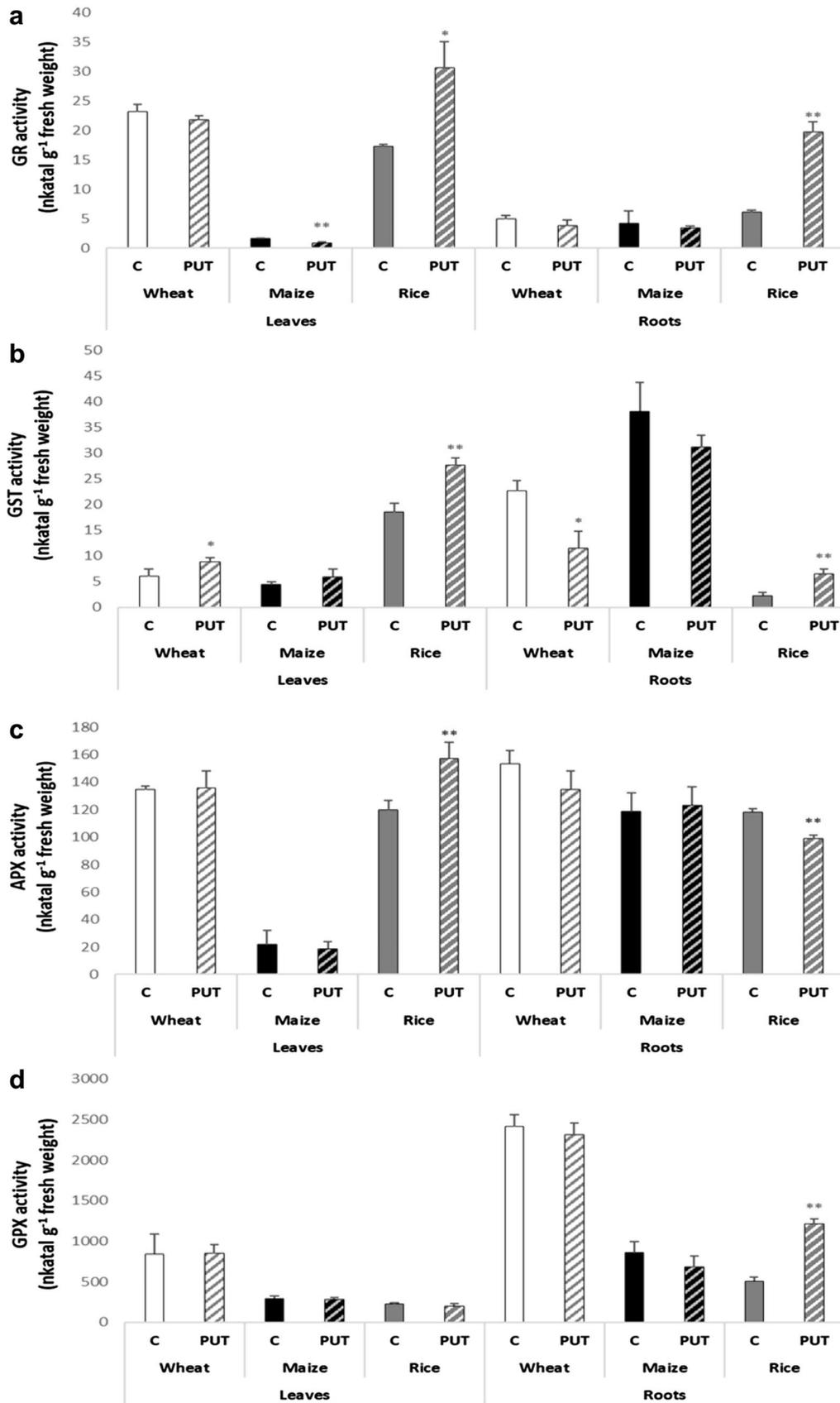
The leaf and root samples were subjected to homogenization in a 2 ml solution of 0.2 N HClO<sub>4</sub> and subsequently placed on ice for 30 min. The homogenates were centrifuged at 4 °C in a centrifuge for 10 min at 10,000 rpm. The supernatant was utilized for pre-column derivatization using dansyl chloride, as described by Németh et al. (2002). The compounds 1,3 diaminopropane (DAP), PUT, SPD, and SPM were subjected to analysis using a reverse phase Kinetex column (C18, 100 × 2.1 mm, 5 µm, Phenomenex, Torrance, CA, USA) by HPLC. The HPLC system employed for this analysis consisted of a W2690 separation module and a W474 scanning fluorescence detector, with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA).

### Gene expression analysis

To conduct gene expression studies, the third, fully matured leaves and roots of 14-day-old plants were collected and promptly preserved in liquid nitrogen. The procedures for total RNA extraction and cDNA synthesis were conducted in accordance with the methodology described by Tajti et al. (2021). The RT-qPCR measurements were conducted using a BioRad CFX96 Touch Real-Time Detection System. The experimental setup included the use of 1 µl of fourfold diluted cDNA, 200 nM forward and reverse primers (the primer sequences can be found in Table 1, 2, and 3), 2.5 µl of PCRBIO Mastermix (PCR Biosystem Ltd., London, United Kingdom), and 2.5 µl of molecular grade water. The 2<sup>-ΔΔCt</sup> method, as described by Livak and Schmittgen (2001), was employed to ascertain the relative transcript levels.

### Statistical analysis

The results were the means of at least ten replicates for each treatment for chlorophyll content, five repetitions for chlorophyll-*a* induction and gas exchange parameters, and three replicates for enzyme activity and HPLC analysis. All reactions for gene expression analyses were performed in triplicate using 3 biological and 3 technical repetitions. The data were statistically evaluated using the standard deviation and *t*-test methods. Significance levels were assessed based on the *p*-value, with a threshold of *p* < 0.05 denoted by a single asterisk (\*) in the figures. When the difference reached a significance level of *p* < 0.01 or lower, it was indicated by two asterisks (\*\*).



**Fig. 4** Effects of 7-day 0.3 mM PUT treatments on the **a** glutathione reductase (GR), **b** glutathione-S-transferase (GST), **c** ascorbate peroxidase (APX), and **d** guaiacol peroxidase (GPX) enzyme activity in the leaves and in the leaves and roots of wheat, maize and rice plants. Data represent mean values  $\pm$ SD,  $n=10$ . The significant difference at the  $p \leq 0.05$  and  $p \leq 0.01$  level is indicated by \* and \*\*, respectively

## Results

### Photosynthesis-related parameters

#### Chlorophyll content and chlorophyll-a fluorescence induction analysis

Application of a 0.3 mM PUT treatment elicited a noteworthy outcome across all three crop plant species, demonstrating a significant increase in leaf chlorophyll content (Fig. 1a).

Analysis of chlorophyll-*a* fluorescence quenching revealed that the PUT treatment did not have a significant impact on the maximum quantum yield of PS II (as indicated by the Fv/Fm parameter) in either examined plant genotypes (Fig. 1b). However, it did influence the photosynthetic activity of PS II, leading to notable differences in both the Y(II) (Fig. 1c) and the ETR (Fig. 1d). Nevertheless, the parameter Y(II) exhibited distinct responses in the three plant species (Fig. 3b). Specifically, in wheat plants, there was a significant increase in the Y(II) value, while it notably decreased in maize plants. No significant changes were observed in rice plants. Following PUT treatment, the ETR value exhibited an increase in wheat. However, no statistically significant alterations were observed in rice and maize (Fig. 1c).

#### Gas exchange measurements

The values of the gas exchange parameters after PUT treatment for the three plant species are shown in Table 4. Notably, there were no substantial alterations observed in any of the plant species as a direct outcome of PUT pre-treatment. The only parameters that displayed significant changes were the transpiration rate (E) in rice.

### Effects of PUT treatment on certain stress markers

#### Lipid peroxidation and H<sub>2</sub>O<sub>2</sub> content

The MDA concentration was used to examine lipid peroxidation. PUT treatment did not induce lipid peroxidation in the leaves and roots of the wheat and maize plants, but it did elicit a statistically significant effect in the accumulation

of MDA in the rice leaves and roots (Fig. 2a), indicating a condition of stress in the rice plants.

In the leaves of all three crop plants, PUT application had no significant effect on H<sub>2</sub>O<sub>2</sub> content, but a substantial increase in H<sub>2</sub>O<sub>2</sub> accumulation was observed in the roots of all three plant species, with the most pronounced effect found in the roots of rice (Fig. 2b).

#### Proline and NO contents

Under the present conditions, as a result of PUT treatment, the level of proline increased slightly, but statistically significantly in the leaves of wheat and rice, while in the roots of rice dramatic proline accumulation was detected compared to the control. Whereas proline levels did not change either in the leaves or in the roots of maize (Fig. 3a). The highest accumulation of proline in the root of the rice plant is also indicative of a stressed condition.

Treatment with 0.3 mM PUT for 7 days did not induce any changes in NO content in the leaves and roots of wheat and maize plants. However, exogenous PUT induced an increase in multiple folds of NO concentration in the leaves and roots of rice plants (Fig. 3b).

#### Antioxidant enzyme activities

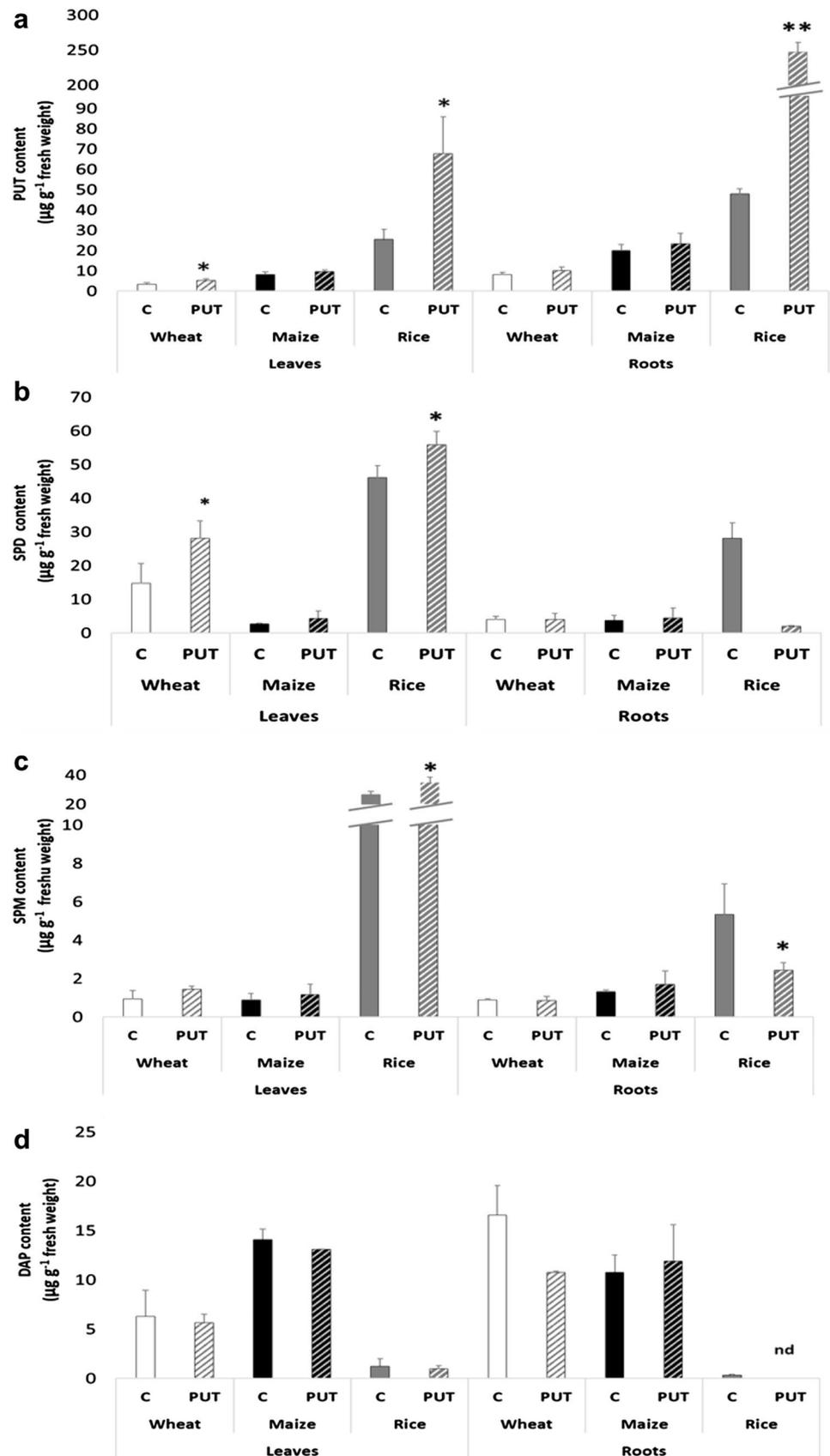
Figure 4 shows that the most remarkable changes were observed again in rice as a result of the PUT treatment. The activity of GR increased in both the leaf and the root of the rice plant. On the contrary, its activity in maize leaves slightly declined after PUT treatment (Fig. 4a). For GST, a significant increase in enzyme activity was also observed in rice plants following PUT treatment both in the leaves and roots (Fig. 4b). Interestingly in wheat plants, PUT treatment increased GST activity in the leaves, but decreased it in the roots (Fig. 4b). PUT treatment caused substantial changes in the activity of APX and GPX also in rice plants. The activity of APX increased in the leaves but decreased in the roots (Fig. 4c) whereas the activity of GPX increased in the roots compared to the control (Fig. 4d).

### Effects of the putrescine treatments on the polyamine metabolism

#### Changes in polyamine contents

Considerable variations were found in the initial PA composition among plant species. Rice plants displayed the highest total PA content in both leaves and roots, compared to wheat and maize. Wheat exhibited the highest levels of SPD, followed by PUT and SPM in leaves, while in the roots PUT content was higher than SPD. In maize leaves the highest

**Fig. 5** Effects of 7-day 0.3 mM PUT treatments on the PUT (a), SPD (b), SPM (c), and DAP (d) contents in the leaves and roots of wheat, Maize, and Rice plants. Data represent mean values  $\pm$  SD,  $n=3$ . The significant difference at the  $p \leq 0.05$  and  $p \leq 0.01$  level is indicated by \* and \*\*, respectively. nd means not detected



**Table 5** Effect of 0.3 mM PUT treatment on the diamine oxidase (DAO) and polyamine oxidase (PAO) enzyme activities after 7 days in wheat, maize, and rice plants

		PAO activity (nkatal g <sup>-1</sup> FW)		DAO activity (nkatal g <sup>-1</sup> FW)	
		Leaves	Root	Leaves	Root
Wheat	C	10.53 ± 3.17	13.55 ± 4.97	5.91 ± 1.32	9.44 ± 1.71
	PUT	11.67 ± 1.76	19.40 ± 2.97	6.55 ± 1.20	7.97 ± 0.39
Maize	C	50.42 ± 2.88	23.62 ± 3.02	7.58 ± 0.25	15.70 ± 0.46
	PUT	41.29 ± 6.45	31.30 ± 1.87*	9.09 ± 1.91	14.38 ± 1.19
Rice	C	12.35 ± 0.37	13.64 ± 0.84	8.35 ± 0.09	11.69 ± 1.53
	PUT	12.95 ± 1.75	17.96 ± 1.95*	7.89 ± 0.36	22.89 ± 5.85

Data represent mean values ± SD, n = 3. The significant difference at the  $p \leq 0.05$  level is indicated by \*, compared to the adequate control

PUT content was followed by SPD and SPM, mirroring the pattern observed in maize roots, too. In rice leaves, the PA distribution followed the sequence SPD > PUT ≥ SPM, whereas in roots, it was PUT > SPD > SPM (Fig. 5a–c). Notably, DAP content, which is the catabolite product of terminal oxidation of SPD and SPM, also showed remarkable differences between the plant species, and compared to wheat and maize, in rice, it was very low (Fig. 5d).

While maize plants did not display notable alterations in PUT, SPD, and SPM content following treatment, wheat and especially rice plants exhibited significant changes after PUT treatment (Fig. 5a–d). The content of PUT increased significantly in rice leaves and roots following treatment, in addition in wheat leaves, while no significant differences were observed in case of maize (Fig. 5a). SPD also increased by exogenous PUT in wheat leaves and rice leaves, but decreased in rice roots (Fig. 5b). The changes in SPM level in rice leaves and roots were similar to those described for SPD content (Fig. 5c). In contrast, DAP content remained relatively stable across the three plant species, except for a decrease below the detection limit in rice roots following PUT treatment (Fig. 5d).

#### Activity of PAO and DAO enzymes responsible for terminal catabolism of PAs

The activity of apoplastic PAO and DAO, which are responsible for terminal degradation of SPD/SPM and PUT, respectively. Notably, an increase in PAO activity was observed in the root samples of PUT-treated maize and rice plants (Table 5). While DAO did not exhibit significant changes following PUT treatment in either plant species, whether in leaves or root samples (Table 5).

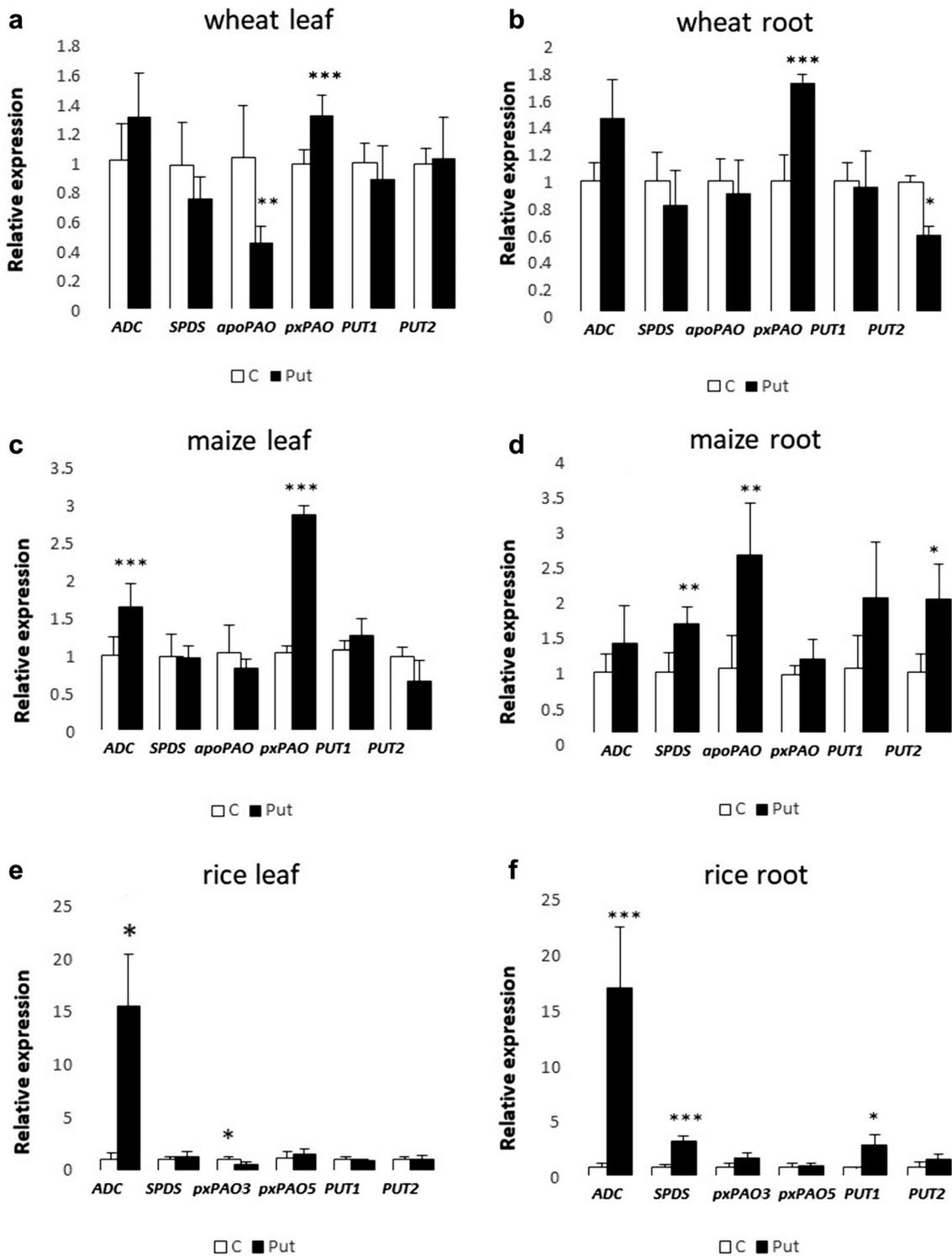
#### Expression level of certain polyamine metabolism-related genes

The application of PUT treatment resulted in statistically significant and distinct expression patterns of certain genes related to PA metabolism (Fig. 6a–f). Substantial increases were detected in the transcript levels of the *ADC* gene in the leaves and roots of rice plants after PUT application (Fig. 6a–b). Regarding *SPDS*, its transcript level is upregulated in maize and rice roots (Fig. 6d, f).

Interestingly, the expression level of the gene encoding the peroxisomal localised PAO (*pxPAO*), which is responsible for the back-conversion of higher PAs, exhibited a significant increase in the leaves of both wheat and maize plants, in addition in the roots of wheat (Fig. 6a–c). While the transcript level of *PAO*, which encodes the *apoPAO* catalysing the terminal oxidation of SPD/SPM, decreased in wheat leaves, but increased in maize roots (Fig. 6a, c). In contrast, the expression levels of *apoPAO* and *pxPAO3* genes showed a notable decrease in all cases, except in maize and rice roots. Notably, PUT treatment had no discernible impact on the expression level of the *pxPAO5* gene in either the leaves or roots of rice plants (Fig. 6e–f). In the leaves of all three crop plants, no substantial changes were observed in the gene expression levels of *PUT1* and *PUT2*. However, a significant increase in the expression levels of *PUT2* or *PUT1* genes was noted in maize and rice roots, respectively (Fig. 6d, f). While in wheat root, *PUT2* expression was inhibited by exogenous PUT (Fig. 6b, d, f).

#### Discussion

Although in several cases there is a positive correlation between PA levels and plant growth or stress tolerance, it has become apparent in recent years that not only PA depletion but also excessive PA accumulation may be detrimental (Iannone et al. 2013; Jiménez-Bremont et al. 2014; Szalai



**Fig. 6** Effects of 7-day 0.3 mM PUT treatments on the expression levels of PA synthesis-related genes, namely arginine decarboxylase (*ADC*) (a–f) spermidine synthase (*SPDS*) (a–f), PA metabolism-related genes, namely PA oxidases (a–f) *apoPAO* and, *pxPAO*, and

PA uptake transporter genes namely (a–f) *PUT1* and *PUT2* in the leaves and roots of wheat, Maize and Rice plants. Data represent mean values  $\pm$  SD,  $n=5$ . The significant difference at the  $P \leq 0.05$  and  $P \leq 0.01$  level is indicated by \* and \*\*, respectively

et al. 2017). Several authors have demonstrated the bio-stimulant effects of PA application during plant development (Chen et al. 2019) and the ameliorative function of PA treatments against diverse stress factors (Minocha et al. 2014; Li et al. 2015a, b). Nonetheless, the positive effect may vary depending on the investigated plant genotypes, the mode of application, or the concentration of the applied PAs (Szalai et al. 2017; Tajti et al. 2018; Pál et al. 2019). Thus, it remains a pertinent question: is more always better when it comes to PAs? Only a few investigations have focused on the negative effects of PAs up to the present. PA treatment has been reported to lead to root growth inhibition and alterations in plant morphology in *Arabidopsis* (Tassoni et al. 2000). In maize it induced programmed cell death (PCD) (Tisi et al. 2011), due to the cytotoxic by-products of PA metabolism (Moschou and Roubelakis-Angelakis 2014). Prior research also indicated a negative effect of 0.5 mM PUT treatment during cadmium stress, while the inhibition of PUT synthesis was favourable in rice (Pál et al. 2017). In maize 0.5 mM PUT pre-treatment did not result in a pronounced protective effect against osmotic stress as it was found in wheat due to the higher PA accumulation (Szalai et al. 2017). So, PAs seem to play important roles in normal cellular functions, plant development, or stress tolerance, but the balanced PA metabolism achieved by the regulation of biosynthesis, back-conversion, catabolism, and conjugation is the most important factor during the outcome of their effects (Handa et al. 2018). In the present study in the same vein, the potential effects of 0.3 mM PUT treatment on three economically important plant species, namely wheat, maize, and rice were tested, in order to reveal the changes in PA metabolism in the background, and their responsibility for the observed differences.

PAs can exert their effects on photosynthesis at several levels. PA treatments protected the chloroplast ultrastructure by preserving the thylakoid membrane structure, and could improve the photosynthetic capacity by increasing the level of the photochemical efficiency of PSII, interacting directly with thylakoid membranes, thus decreasing the loss of LHCII, increasing chlorophyll content, influenced stomatal opening, improved the leaf CO<sub>2</sub> assimilation rate (Shu et al. 2012a, b; Najafpour 2012; ElSayed et al. 2022; González-Hernández et al. 2022). Navakoudis et al. (2007) found that PUT can directly increase the size of the LHCII antenna complex, and bind to the PSI and PSII core proteins. Consequently, increased electron transport rate and photosynthetic activity can be attributed to PUT treatment. Our results demonstrate that treatment with 0.3 mM PUT significantly increased the chlorophyll content of the leaves of all three plant species, indicating a beneficial effect on photosynthetic processes to a certain extent. However, chlorophyll-*a* fluorescence quenching analyses revealed that PUT treatment increased the actual quantum yield (Y(II))

only in wheat, did not influence maize, and decreased it in rice plants. At the same time, these changes were not accompanied by changes in CO<sub>2</sub> exchange parameters. PUT treatment only induced a slight decrease in the transpiration rate in rice.

Although, under the given conditions 0.3 mM PUT treatment could not induce pronounced changes and differences in the photosynthesis-related parameters, the determination of certain stress markers proved that PUT application was not beneficial for all the three plant species. PUT treatment did not induce lipid peroxidation or H<sub>2</sub>O<sub>2</sub> accumulation in the leaves of wheat and maize. Nonetheless, a statistically significant increase was observed in the level of MDA and H<sub>2</sub>O<sub>2</sub> in the roots of all three crop plants, indicating that the roots were subjected to oxidative stress conditions. In addition, in the leaves of rice increased MDA content was detected revealing that rice is more sensitive to exogenous PUT. Results also suggested that not the decreased photosynthesis activity may be responsible for ROS production, the other processes. As both the terminal catabolism and the back-conversion of the excess PA produce H<sub>2</sub>O<sub>2</sub> PA metabolism can be implicated.

Further analysis of other stress markers, namely proline and NO contents, confirmed this hypothesis. Dramatic accumulation of both compounds was observed after PUT treatment in rice leaves and roots. Proline is an essential amino acid with multiple roles in plants. It functions as a nitrogen source, stress indicator, osmolyte, and antioxidant molecule in plants (Majumdar et al. 2016; Razavizadeh et al. 2017), thus the increase in proline content due to PA treatment indicated its essential protective role in rice roots under stress conditions. NO is a crucial gaseous free radical in plants, acting as an intra- and intercellular messenger to trigger processes including defence-related gene expression, programmed cell death, stomatal closure, seed germination, and root development (Neill et al. 2003; Lamotte et al. 2004). NO production can be mediated by H<sub>2</sub>O<sub>2</sub> resulting from the oxidation of PAs via DAO and PAO enzymes, or through other unidentified mechanisms associated with the PAs pathway (Wimalasekera et al. 2011). It is worth mentioning that proline and PA synthesis use a common precursor (glutamate), in addition, the catabolism of PAs may also be involved in proline production (Su and Bai 2008), furthermore, NO production is intricately linked to PA metabolism (Flores et al. 2008). Thus the increased level of these compounds can not only indicate stress condition in rice, but reflect on the imbalance in PA metabolism.

Along with these changes, induced activities of leaf and root GR, leaf and root GST, leaf APX, and root GPX were found in rice plants, indicating that the activated antioxidant system tried to maintain the redox balance. Species-specific role of PAs treatments in modulating the antioxidant defence system has been reported in various

cases (Shao et al. 2022). PAs can generally activate antioxidant enzymes and modulate ROS homeostasis and oxidative damage by inhibiting  $H_2O_2$  accumulation (Singh-Gill & Tuteja 2010). However, under the present conditions, the induction of the antioxidant system in rice plants indicated again the disturbance of PA homeostasis.

To highlight the role of the PA pool in the above-described stress conditions in rice, detailed analyses of PA metabolism were performed. Plants use a variety of mechanisms to control endogenous PA levels, such as the synthesis of PUT, its further synthesis to higher PAs, conjugation of them to small molecules, conversion of higher PAs back to PUT in the PA cycle, and terminal oxidation of them (Pál et al. 2015). Although upon exposure to exogenous PA, other mechanisms may be also involved, like modulation of PA uptake, and translocation from the roots to the shoots. PUT application induced a slight increase in PUT and SPM contents of wheat leaves, did not influence the DAP content and DAO or PAO activities, in addition, did not induce characteristic changes in the expression level of PA metabolism-related genes, except slight *pxPAO* induction both in the leaves and roots, and *PAO* inhibition in the leaves. These findings revealed that in wheat plants the applied PUT treatment did not affect the PA metabolism or the plants can re-adjust it properly, in order to maintain PA homeostasis. In maize, more changes were detected. Although the PA contents were not affected, in the roots the *PAO* activity increased, furthermore at the gene expression level, *PAO* was also induced in the roots. In contrast, in maize leaves the expression level of *pxPAO* increased after PUT treatment. These changes reflect that excess PUT induced the PA cycle and catabolism both at enzymatic and gene expression levels, which in turn helps the maize plants in acclimation to changed conditions. At the same time, PUT treatment caused pronounced PUT accumulation in the leaves and roots of rice, indicating not only the uptake of PUT but also its translocation. The application of PUT induced the accumulation of higher PAs (SPD or SPM) in the leaves of rice plants, indicating that PUT treatment resulted in additional synthesis of SPD or SPM. However, interestingly the *ADC* gene expression also increased both in the leaves and roots, which proved that *in vivo* PUT synthesis was also induced, and responsible for the dramatic PUT accumulation. In the roots of rice, the amount of SPD and SPM decreased after PUT treatment despite the increased transcript level of *SPDS*, but partly due to the increased *PAO* activity. In this instance, however, there was no detectable DAP accumulation following PUT treatment. Notably, even under controlled conditions, rice had the lowest DAP.

## Conclusion

PAs, including PUT, have been recognized for a long time to play essential roles in cellular growth, differentiation, and stress responses. While PUT had beneficial effects on certain aspects of plant physiology, its impact varied depending on the plant species and its inherent capacity to regulate PA homeostasis. This study challenges the simplistic notion that the higher PA level is always the better, emphasizing the context-dependent responses of plants to PA treatments. Wheat, maize, and rice were positively affected by PUT treatment in terms of chlorophyll content, but an investigation of various stress markers testified that rice plants experienced oxidative stress. As in rice, the initial PA content was much higher than in wheat or maize, disruption in PA metabolism after PUT application could be responsible for the observed negative effects. In conclusion, while PUT has the potential to improve plant growth, development, and stress tolerance, its negative effects vary across plant species, highlighting the importance of the dynamic nature of the PA metabolism. To fully understand the underlying mechanisms and maximize the potential use of PUT for crop improvement, additional research is required.

**Author contributions** A.R., E.K., I.K.: investigation, analyses, data evaluation, visualisation; A.R.: writing and editing; I.M.: methodology, data evaluation, T.J.: reviewing; M.P.: conceptualisation, analyses, data evaluation, visualisation, editing, reviewing.

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## Declarations

**Conflict of interest** The authors have stated that they do not have any conflicts of interest. The individuals or organizations providing funding for this study did not participate in the study's design, data collection, analysis, interpretation, manuscript writing, or the decision to publish the findings.

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## **Appendix B**

Submitted to *Plant Physiology and Biochemistry* Journal  
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# Plant Physiology and Biochemistry

## Light and genotype affect the uptake of exogenous polyamines and their metabolism in wheat plants --Manuscript Draft--

<b>Manuscript Number:</b>	
<b>Article Type:</b>	Research Paper
<b>Section/Category:</b>	Secondary metabolism
<b>Keywords:</b>	gene expression; light regulation; polyamine treatment; polyamine metabolism; Triticum aestivum L
<b>Corresponding Author:</b>	Magda Pál, Ph.D. HUN-REN Centre for Agricultural Research Martonvásár, HUNGARY
<b>First Author:</b>	Magda Pál, DSc
<b>Order of Authors:</b>	Magda Pál, DSc Altafur Rahman Kamirán Áron Hamow, Ph.D. Katalin Nagy Tibor Janda, DSc Mihály Dernovics, Ph.D. Gabriella Szalai, DSc
<b>Abstract:</b>	<p>Two experimental setups were conducted to better understand how light regulates polyamine uptake and metabolism. We aimed to characterise genotype-dependent differences in the pattern of daily uptake of exogenous putrescine in three wheat genotypes. Putrescine treatment induced accumulation of putrescine in all the genotypes; however, the pattern of changes were partly distinct indicating that different regulation strategies may exist. In Mv Béres, a decrease in expression of polyamine synthesis and back-conversion-related genes (arginine decarboxylase2, spermidine synthase and peroxisomal polyamine oxidase) was detected, which was partly efficient against the extreme putrescine accumulation. In Mv Emese, the putrescine synthesis (arginine decarboxylase2 gene expression) and absorption (polyamine uptake transport gene expression) decreased, the synthesis and back-conversion of higher polyamines (transcription of spermidine synthase and peroxisomal polyamine oxidase genes) were not, but the terminal catabolism was initiated, leading to successful faster metabolism of excess putrescine. The effects of putrescine or spermidine treatments on polyamine homeostasis were also monitored under continuous light or dark conditions in Mv Emese, with a special question on how polyamine conjugation is involved in the metabolism of polyamine excess. Results indicated that in the leaves, intrinsic circadian rhythm had a primary influence, at least in one day period, rather than polyamine treatments and the presence or absence of light. In contrast, in the roots, polyamine treatments had higher effects than the daily rhythm or light conditions. Here, we demonstrated that polyamine uptake and metabolism dynamics are partly genotype-dependent, but were not influenced by the absence of light in wheat.</p>
<b>Suggested Reviewers:</b>	Ewa Sobieszczuk-Nowicka ewa.sobieszczuk-nowicka@amu.edu.pl Cristiano Soares cristiano.soares@fc.up.pt Xiangnan Li lixiangnan@iga.ac.cn

Dear Editors-in-Chief,

We are sending our original study “**Light and genotype affect the uptake of exogenous polyamines and their metabolism in wheat plants**” by Magda Pál, Altafur Rahman, Kamirán Áron Hamow, Katalin Nagy, Tibor Janda, Mihály Dernovics, Gabriella Szalai

Homeostasis of polyamines is maintained by intricate multiple feedback mechanism at the levels of uptake, transport, biosynthesis, back-conversion, terminal catabolism and conjugation. Their levels fluctuate during the growth and development, depending on stress condition and light regime. In previous studies only one genotype of a plant species has been tested usually a few days later after the treatments, but it is important to monitor the genotype-dependent differences in the early period, too. Since polyamine metabolism may have light-dependent aspects, it is worth examining the effect of light, lighting hours or its absence on the uptake and metabolism of exogenous polyamine in plants.

Therefore, in order to better understand the light may regulate the maintenance of the polyamine homeostasis, in the present study first, we aimed to characterize differences in the pattern of daily uptake of putrescine in three wheat genotypes, and also to monitor especially the early changes in polyamine metabolism-related gene expression levels. After that, the results of putrescine and spermidine treatments were compared under continuous light or dark conditions at metabolite and gene expression levels, with a special question on how polyamine conjugation is involved in the metabolism of polyamine excess.

We confirm that neither the manuscript nor any parts of its content are currently under consideration or published in another journal. All authors have approved the manuscript and agree with its submission to Plant Physiology and Biochemistry.

Looking forward to receiving your reply,

Magda PÁL DSc  
[pal.magda@atk.hu](mailto:pal.magda@atk.hu)

Scientific consultant  
corresponding author

Department of Plant Physiology and Metabolomics  
Agricultural Institute  
Centre for Agricultural Research

pal.magda@atk.hu  
H-2462 Martonvásár, Brunszvik 2.  
Tel: +36-22-569-502  
Fax: +36-22-569-576

## Light and genotype affect the uptake of exogenous polyamines and their metabolism in wheat plants

Magda Pál\*, Altafur Rahman, Kamirán Áron Hamow, Katalin Nagy, Tibor Janda, Mihály Dernovics, Gabriella Szalai

<sup>1</sup>Hungarian Research Network, Centre for Agricultural Research, Agricultural Institute, H-2462 Hungary Martonvásár, Brunszvik 2.

[altafk013@gmail.com](mailto:altafk013@gmail.com); [hamow.kamiran@atk.hun-ren.hu](mailto:hamow.kamiran@atk.hun-ren.hu); [nagy.katalin@atk.hun-ren.hu](mailto:nagy.katalin@atk.hun-ren.hu);  
[janda.tibor@atk.hun-ren.hu](mailto:janda.tibor@atk.hun-ren.hu); [dernovics.mihaly@atk.hun-ren.hu](mailto:dernovics.mihaly@atk.hun-ren.hu); [szalai.gabriella@atk.hun-ren.hu](mailto:szalai.gabriella@atk.hun-ren.hu)

### \*Corresponding author:

[pal.magda@atk.hun-ren.hu](mailto:pal.magda@atk.hun-ren.hu)

Tel: +36-22-569-502

### Abstract

Two experimental setups were conducted to better understand how light regulates polyamine uptake and metabolism. We aimed to characterise genotype-dependent differences in the pattern of daily uptake of exogenous putrescine in three wheat genotypes. Putrescine treatment induced accumulation of putrescine in all the genotypes; however, the pattern of changes were partly distinct indicating that different regulation strategies may exist. In Mv Béres, a decrease in expression of polyamine synthesis and back-conversion-related genes (arginine decarboxylase2, spermidine synthase and peroxisomal polyamine oxidase) was detected, which was partly efficient against the extreme putrescine accumulation. In Mv Emese, the putrescine synthesis (arginine decarboxylase2 gene expression) and absorption (polyamine uptake transport gene expression) decreased, the synthesis and back-conversion of higher polyamines (transcription of spermidine synthase and peroxisomal polyamine oxidase genes) were not, but the terminal catabolism was initiated, leading to successful faster metabolism of excess putrescine. The effects of putrescine or spermidine treatments on polyamine homeostasis were also monitored under continuous light or dark conditions in Mv Emese, with a special question on how polyamine conjugation is involved in the metabolism of polyamine excess. Results indicated that in the leaves, intrinsic circadian rhythm had a primary influence, at least in one day period, rather than polyamine treatments and the presence or absence of light. In contrast, in the roots, polyamine treatments had higher effects than the daily rhythm or light conditions. Here, we demonstrated that polyamine uptake and metabolism dynamics are partly genotype-dependent, but were not influenced by the absence of light in wheat.

1 **Keywords:** gene expression, light regulation, polyamine treatment, polyamine metabolism,  
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3 *Triticum aestivum* L.  
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## 6 7 **1. Introduction**

8  
9 Polyamines (PAs) are small, naturally occurring compounds with several roles in  
10 fundamental cell functions and plant development (Kolesnikov et al., 2024). They are also  
11 involved in plants' stress responses as protective and signalling compounds (Alcázar et al.,  
12 2020). Applications of PA compounds in different ways (seed-soaking, hydroponic treatment  
13 or leaf spraying) at proper concentration, which can be specific depending on the PA compound  
14 and the plant species, have been proven to be positive and roborative under control conditions,  
15 or be beneficial against various stress factors in numerous plant species (Biondi et al., 2022;  
16 Chen et al., 2021; Marcińska et al., 2020). They can alleviate stress symptoms directly or  
17 indirectly and induce stress tolerance at physiological, metabolite and gene expression levels.  
18 The most reported and important elements of their action mechanism involve the enhancement  
19 of photosynthesis, induction of antioxidant system, metabolomic connection with the synthesis  
20 of other protective or signalling compounds, relationship with the synthesis of plant hormones  
21 and hormone signalling pathways, and regulation of gene expression (Bitrián et al., 2012; Chen  
22 et al., 2019; Napieraj et al., 2023; Navakoudis and Kotzabasis, 2022; Wuddineh et al., 2018).  
23 However, there are other roles, such as functioning as metal chelators and osmolytes, influence  
24 DNA methylation (Hasanuzzaman et al., 2019; Sengupta et al., 2016; Stolarska et al., 2023).  
25 Increasingly, it is clear that the positive effect is not evident in all cases. In a comparison study,  
26 it was found that even under stressless conditions, the same putrescine (PUT) concentration  
27 (0.3 mM) was beneficial for wheat, rather neutral for maize, but was harmful for rice plants  
28 (Rahman et al., 2024a). At higher concentrations, spermidine (SPD) and spermine (SPM) had  
29 negative effects, whereas at the same concentration, PUT was favourable in maize (Szalai et  
30 al., 2017). PUT pre-treatment (0.5 mM) had a higher protective effect against PEG-induced  
31 osmotic stress in wheat than in maize (Szalai et al., 2017), while the same pre-treatment further  
32 increased the Cd-induced oxidative stress in rice (Pál et al., 2017). The mode of application  
33 may also influence the outcomes of the treatments, depending on the applied PA compounds.  
34 For example, PUT as seed-soaking or applied as hydroponic treatment decreased the toxic  
35 effects of Cd stress, while spermidine (SPD) had positive effects only as seed-priming (Tajti et  
36 al., 2018).  
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1 The observed variations in the effects of the PA treatments may be attributed to the  
2 variability in PA metabolism across the plant species under investigation (Raziq et al., 2022;  
3 Roy et al., 2024; Szalai et al., 2020). The excessive accumulation of PAs may disturb their  
4 homeostasis, resulting in oxidative stress and an unfavourable alteration in the metabolism of  
5 additional compounds (Pál et al., 2017; Szalai et al., 2017; Szalai et al., 2020). To better assess  
6 the beneficial effects of a PA treatment, the absorption dynamics of excess PA during the  
7 treatment and the metabolism of the absorbed PAs should be studied more intensively. In most  
8 studies, only one genotype of a plant species has been tested usually a few days later after the  
9 treatments, but in terms of usability in agricultural practice, it is important to monitor the  
10 genotype-dependent differences in the early period, too.

11 Understanding the effect of light on plants holds enormous potential for sustainable  
12 agricultural production in the future. Since PA metabolism also has light intensity- and spectral-  
13 dependent aspects (Gondor et al., 2021; Pál et al., 2022), it is worth examining the effect of  
14 light or its absence on the uptake of exogenous PA, and their metabolism in plants.

15 According to these findings, in the present study, we were looking for answers to two  
16 questions: 1) Within the wheat species, to what extent can the response to putrescine treatment  
17 be generalised at the level of polyamine metabolism, and what genotype-dependent differences  
18 can be detected? 2) Is there any influence of the lack of light on polyamine uptake and  
19 metabolism in the early period of PA-treatments? Therefore, in the present study, we aimed to  
20 characterise differences in the pattern of daily uptake of an exogenously applied PA, namely  
21 PUT in three wheat genotypes, and also to monitor especially the early changes in PA  
22 metabolism-related gene expression levels. After that, to better understand the role of light in  
23 PA uptake and the maintenance of PA homeostasis, the results of PUT and SPD treatments  
24 were compared under continuous light or dark conditions at metabolite and gene expression  
25 levels, with a special question on how PA conjugation is involved in the metabolism of PA  
26 excess.

## 27 **2. Materials and Methods**

### 28 ***2.1. Plant material, growth conditions and treatments***

29 Two winter wheat varieties from Martonvásár ('Mv Béres' and 'Mv Emese', genotypes  
30 with different agrotechnical needs and different protein and gluten contents) and one Thatcher-  
31 based near-isogenic line (TC33=Thatcher\*6/P.I.58548, a less tolerant genotype against  
32 cadmium or powdery mildew stress (Pál et al., 2013; Kovács et al., 2014)) were selected based  
33 on previous experiments (Szalai et al., 2017; Tajti et al., 2018; Rahman et al., 2024a, b), where  
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PUT treatments had positive effects on these genotypes. After germination for 3 days at 26 °C in the dark, healthy, similarly developed seedlings were planted on stainless steel net into plastic pots (15 plants/plastic pot) and grown on modified Hoagland solution (Pál et al., 2005) in Conviron PGV-36 plant growth chamber (Controlled Environments Ltd., Winnipeg, Canada). Growing conditions were the same throughout the experiments: 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, 22/20°C day/night temperature with 16/8-h light/dark periodicity and 75% relative humidity. The nutrient solution was renewed every 2 days until the PUT or SPD treatments were started.

Two different experimental setups were conducted and to facilitate comprehension, supplementary figures provide specifics regarding the duration of treatments and the collection of samples (Suppl. Figs. 1A-B). In the first experimental setup, after growing under control conditions, at the end of the last 8-hour dark period, 10-day-old seedlings of all three genotypes were sampled in the dark (0h) as an absolute control. After this, one part of the plants was further grown in hydroponic solution without any treatment (control: C), while the other part of the plants was promptly treated with 0.5 mM PUT (PUT-treated: PUT) as the next light (day) period is started in the chamber. Leaves and roots of control and PUT-treated plants were collected at dedicated times, namely non-treated plants 3, 5, 13 and 73 hours, while PUT-treated ones 1, 3, 5, 13, 25, 49 and 73 hours after starting of the treatment period. During the treatment, the 16/8-h light/dark periodicity was continued, and the nutrient solution was not changed (Suppl. Fig. 1A).

In the second experiment, only Mv Emese was used. The growth conditions were the same, and the sample collection in the dark (samples of 0h) was similar to what was described for the first experiment. However, here after the last dark period, half of the plants were further grown under continuous dark conditions (DARK), while the other half of them were grown under continuous light conditions (LIGHT) for 25h, until the end of the experiment. Both under light or dark conditions, plants were divided into three different groups, namely control (C: without any treatment), PUT-treated (PUT: treated with 0.5 mM PUT) and SPD-treated ones (SPD: treated with 0.5 mM SPD) (Suppl. Fig. 1B.). Leaves and roots samples were collected from these six groups of plants 1, 3, 5, 7, 9 and 25 hours after the beginning of the treatments (Suppl. Fig. 1B).

## 2.2. Polyamine analysis

0.2 g of each sample was homogenised in liquid N<sub>2</sub> and extracted in 2.0 ml solution of 0.2 M HClO<sub>4</sub>. After centrifugation, the supernatant was utilised for pre-column derivatisation using dansyl chloride, as described by Németh et al. (2002). The following compounds were

1 separated using a reverse phase Kinetex column (C18, 100 × 2.1 mm, 5 μm, Phenomenex,  
2 Torrance, CA, USA) by HPLC: 1,3-diaminopropane (DAP), PUT, SPD, and SPM. The HPLC  
3 system consisted of a W2690 separation module and a W2475 scanning fluorescence detector  
4 (Waters; Milford, MA, USA). For detection, excitation at 340 nm and emission at 515 nm were  
5 applied.  
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### 10 **2.3. Analytical procedure for the quantitation of conjugated polyamines**

11 Quantitative estimation of certain polyamine derivatives (*N-p*-coumaroyl-*agmatine* and *N-*  
12 *p*-coumaroyl-*putrescine*) was performed using a Vion ion mobility quadrupole time-of-flight  
13 mass spectrometer (UPLC-ESI-MS-QTOF-MS) (Waters, Milford, MA, USA) as described in  
14 details by Szalai et al. (2022). The standards and identification of conjugated PAs can be found  
15 as supplementary information in our previous study (Rahman et al., 2024b).  
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### 23 **2.4. Analytical determination of phenolic compounds**

24 Measurement of phenolic compounds was carried out according to Pál et al. (2019), with  
25 minor modifications. 0.2 g of frozen fresh weight (FW) plant material homogenised in liquid  
26 N<sub>2</sub> was used for extraction. Samples were spiked with 20 ng of labelled [<sup>2</sup>H<sub>6</sub>](+)-*cis,trans-*  
27 *abscisic acid* (OlChemIm s.r.o. Olomouc, Czech Republic) as an internal standard. The  
28 extraction was performed with 1 ml of methanol:water (2:1), followed by 5 sec of vigorous  
29 vortexing; then, samples were shaken with a Spex Mini G 1600 in a cryo-cooled rack at 1.500  
30 rpm for 3 min. After centrifugation, the supernatants were collected, and the remaining pellets  
31 were re-extracted by repeating the extraction procedure once more. The respective supernatants  
32 were pooled to a final sample ratio of 0.1 g FW ml<sup>-1</sup>, and the methanol-water sample solution  
33 was liquid-liquid partitioned by adding 1 ml of n-hexane to remove apolar matrix components  
34 such as lipids. Afterwards, centrifugation at 10.000 g (at 4 °C for 10 min) was addressed to  
35 collect the lower methanol-water phase that was finally filtered through a 0.22 μm PTFE  
36 syringe filter, then submitted directly for UPLC-US-MS/MS analysis according to Cseh et al.  
37 (2024). Separation was achieved on a Waters HSS T3 column (1.8 μm, 100 mm × 2.1 mm)  
38 using an Acquity I class UPLC system (Waters Corp., Milford, MA, USA). Gradient elution  
39 was used with 0.1% (v/v) formic acid, both in water (A) and acetonitrile (B). Tandem mass  
40 spectrometry detection was performed in multiple reaction monitoring (MRM) mode on a Xevo  
41 TQ-XS (Waters) equipped with a UniSpray™ source, with the following settings: impactor  
42 voltage was 2 kV in both positive and negative modes; nebuliser gas, 6 bar; desolvation  
43 temperature, 550°C; cone gas flow, 450 l h<sup>-1</sup>; desolvation gas flow, 1000 l h<sup>-1</sup>. For collision gas,  
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1 argon (5.0 purity) was used with a gas flow of 0.15 ml min<sup>-1</sup>. A unit resolution was applied to  
2 each quadrupole. Dwell time set to be automatically calculated to take at least 20 points across  
3 each peak for quantitation. Where possible, at least three MRM transitions were used for data  
4 acquisition and the transition having the highest S : N ratio was used for quantitation.  
5 Quantitative traces and retention times were listed in Suppl. Table 1.  
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## 8 9 10 **2.5. Gene expression analysis**

11 To conduct gene expression studies, the third, fully developed leaves and roots of plants  
12 were collected. The procedures for total RNA extraction and cDNA synthesis were conducted  
13 following the methodology described by Tajti et al. (2021). The qRT-PCR measurements were  
14 conducted using a BioRad CFX96 Touch Real-Time Detection System (Bio-Rad Laboratories  
15 Inc., Hercules, CA, USA). The experimental setup included the use of 1.0 µl of four-fold diluted  
16 cDNA, 200 nM forward and reverse primers (the primer sequences can be found in Suppl. Table  
17 2), 2.5 µl of PCR BIO Mastermix (PCR Biosystem Ltd., London, United Kingdom), and 2.5 µl  
18 of molecular grade water. The 2<sup>-ΔΔCt</sup> method, as described by Livak and Schmittgen (2001),  
19 was employed to ascertain the relative transcript levels.  
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## 30 **2.6. Statistical analysis**

31 Three independent experiments were performed, and the most representative data are  
32 presented here. The results represented at least three biological replicates. The data were  
33 visualised using the Microsoft Windows® Excel program. Duncan's post-hoc test was  
34 performed using SPSS 16.0.  
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## 40 **3. Results**

### 41 **3.1. Genotype-dependent differences in PA metabolism in the presence of PUT excess**

#### 42 **3.1.1. Changes in polyamine contents in the roots and leaves of three wheat** 43 **genotypes**

44 In the roots of the investigated wheat genotypes, PUT was the most abundant polyamine,  
45 followed by SPD and SPM (Fig. 1. and Suppl. Figs. 2-3.). The basal levels of these polyamines  
46 were similar in the three genotypes. The PUT content did not alter substantially due to  
47 conditions changing from dark to light, or during the day, even senescence did not influence it  
48 remarkably according to the 73h control (Figs. 1A, C, E). Exogenous PUT induced a substantial  
49 accumulation of PUT at comparable levels in the three genotypes. However, the pattern of  
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changes was partly distinct. In Mv Béres roots, a continuous increase was observed with a peak at 13h (Fig. 1A), while in Mv Emese roots, a peak was observed at 5h, followed by a slow, tendentious decrease (Fig. 1C). In TC33, the accumulation reached its maximum immediately after 1h of PUT treatment and remained nearly unchanged until 13h, after 25h it decreased (Fig. 1E). The PUT levels in roots of all genotypes nearly returned to the control levels after 25, 49, and 73 hours of PUT treatment.

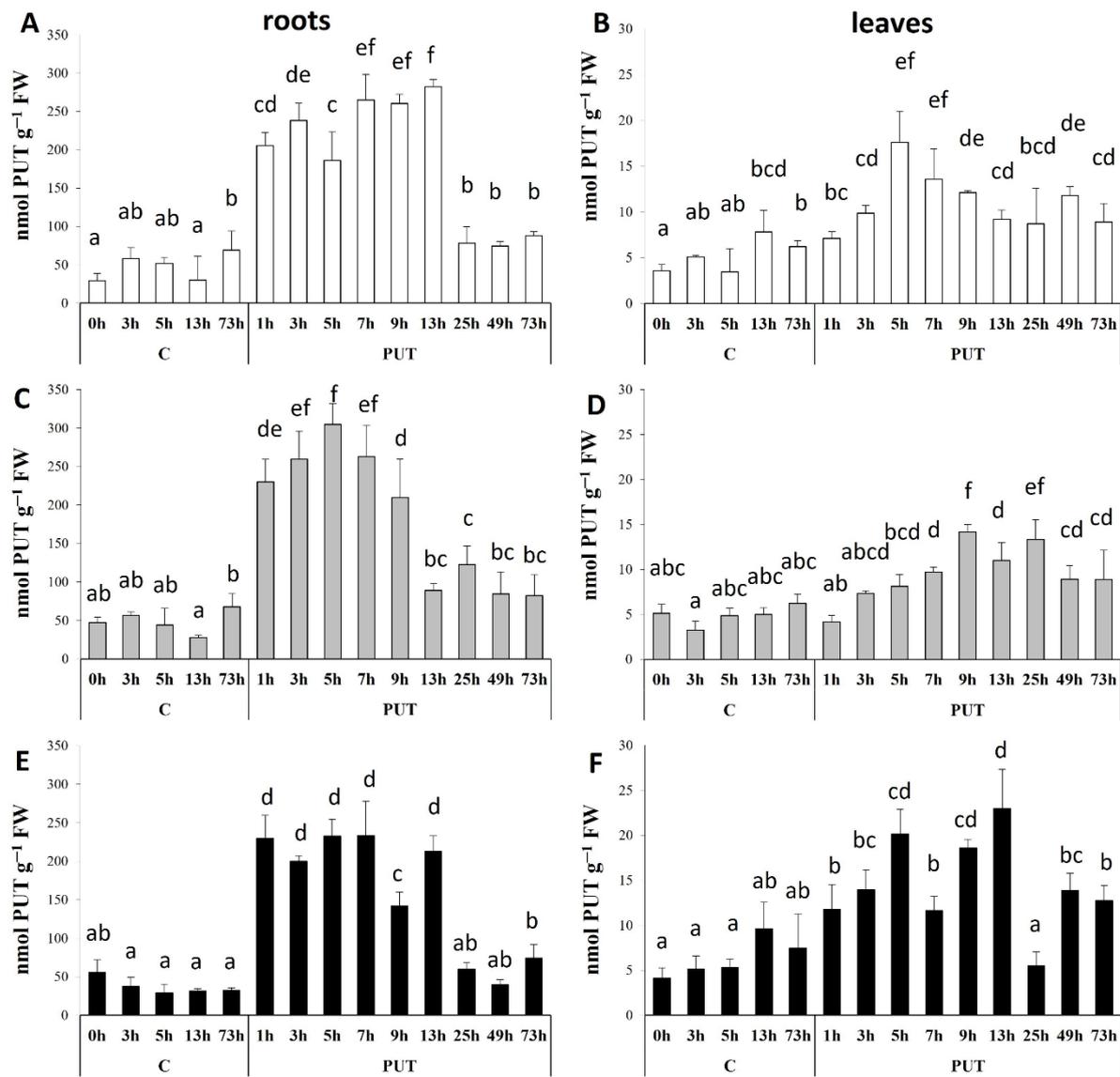


Fig. 1. The effect of 0.5 mM putrescine (PUT) treatment compared to the control conditions (C) on endogenous putrescine (PUT) contents in the roots (A, C, E) and leaves (B, D, F) of Mv Béres (A, B), Mv Emese (C, D) and TC33 (E, F) wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

1 After dark conditions (0h) without PUT treatment, a tendentious slight increase was  
2 observed in PUT content in the leaves of the Mv Béres genotype. In contrast, in the other  
3 genotypes no significant changes were detected during the day (Fig. 1B, D, F). Based on the  
4 data of control samples after 73 hours, senescence alone did not induce pronounced alterations  
5 in the PUT levels. In Mv Béres and TC33 genotypes, statistically significant PUT accumulation  
6 was induced by PUT treatment after 1 hour, whereas in Mv Emese, it was observed after 7  
7 hours. The highest PUT accumulation was observed in TC33, followed by Mv Béres, and the  
8 lowest in Mv Emese, despite the initial similarity of the PUT contents in the leaves of the three  
9 genotypes. In conjunction with this, the investigated wheat genotypes exhibited diverse  
10 dynamics regarding the accumulation of PUT and its subsequent decrease. In Mv Béres, after  
11 a PUT accumulation peak at 5h, the PUT level decreased back to the level that was detected at  
12 1h (Fig. 1 B). The highest PUT accumulation was observed in Mv Emese at 9 hours, and the  
13 level of PUT remained higher than it was at 1 hour of PUT treatment even after 1, 2, and 3 days  
14 (Fig. 1D). While in TC33, the PUT content increased steadily from 1h onward, and a late PUT  
15 accumulation peak was observed at 13h (Fig. 1F). The quantity of PUT returned to the level  
16 observed at 1 hour after 1 day; however, a new increase was observed after 2 and 3 days (Fig.  
17 1F)

18 In the roots, 3 hours later after changing from dark to light conditions, the SPD (Suppl.  
19 Fig. 2A, C, E) and SPM (Suppl. Fig. 3A, C, E) levels increased under control conditions in Mv  
20 Béres and Mv Emese. Interestingly, these peaks were also detected in PUT-treated Mv Béres  
21 plants (Suppl. Fig. 2A), while in Mv Emese PUT application shifted this accumulation peak  
22 from 3h to 5h (Suppl. Fig. 2C). However, PUT treatment alone did not further influence the  
23 SPD and SPM contents in these genotypes. In TC33 exogenous PUT induced a slight, early  
24 increase in SPD level, but it decreased back to the control level after 5 hours (Suppl. Fig. 2E).  
25 Similar tendency was observed in the case of the root SPM content in the TC33 genotype  
26 (Suppl. Fig. 3E).

27 The most abundant PA in the leaves was SPD, and its initial level was similar in the  
28 three investigated genotypes (Suppl. Fig. 2B, D, F). In contrast, the basal level of SPM was  
29 similar in Mv Béres and TC33, but compared to these it was more than double in Mv Emese  
30 (Suppl. Fig. 3B, D, E). The levels of SPD and SPM in the leaves did not change remarkably  
31 during the experiment, as neither the light nor the PUT treatment could substantially influence  
32 them in the leaves of the three genotypes (Suppl. Figs. 2-3). The only noticeable exception was  
33 a peak in Mv Béres and Mv Emese after 3h exposure to light, and it was not only observed in  
34 the leaves but also in the roots.

1 The level of root DAP did not show pronounced changes in the investigated genotypes  
2 (Suppl. Fig. 4A, C, E). In the case of the leaf DAP content, only in Mv Emese could be detected  
3 changes after PUT treatment, namely after 5h the DAP level increased, at 9h reached a peak,  
4 and after then it decreased back to the control level (Suppl. Fig. 4D).  
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### 8 **3.1.2. Changes at gene expression level in the roots and leaves of Mv Béres and Mv** 9 **Emese varieties**

10 The expression levels of certain PA metabolism-related genes were measured only in Mv Béres  
11 and Mv Emese, at dedicated sampling hours. After PUT treatment, the expression level of the  
12 arginine decarboxylase 2 gene (*TaADC2*) in Mv Béres roots decreased, with the lowest  
13 transcript level detected at 5 hours. Subsequently, the transcript level returned to approximately  
14 the control value (Fig. 2A). Similarly, the transcript levels of spermidine synthase (*TaSPDS*)  
15 and peroxisomal polyamine oxidase (*TapxPAO*) decreased in roots at the early hours of PUT  
16 application. However, these levels subsequently returned to the control level (Fig. 2C-E). The  
17 expression level of *TaPUT1* increased in roots from the 5<sup>th</sup> hour of PUT treatment (Fig. 2G).  
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27 The patterns of changes in gene expression levels in the leaves were partly similar to  
28 those described in the roots of Mv Béres. The *TaADC2* expression decreased after the PUT  
29 treatment, and about after 7h it increased back to the control level (Fig. 2B). No pronounced  
30 changes were observed in the case of *TaSPDS* (Fig. 2D). The expression level of *TapxPAO*  
31 decreased under light condition during the day, but the PUT treatment did not influence it (Fig.  
32 2F). The most remarkable changes were detected in case of *TaPUT1*, as its expression increased  
33 continuously and dramatically after PUT treatment from 7h (Fig. 2H).  
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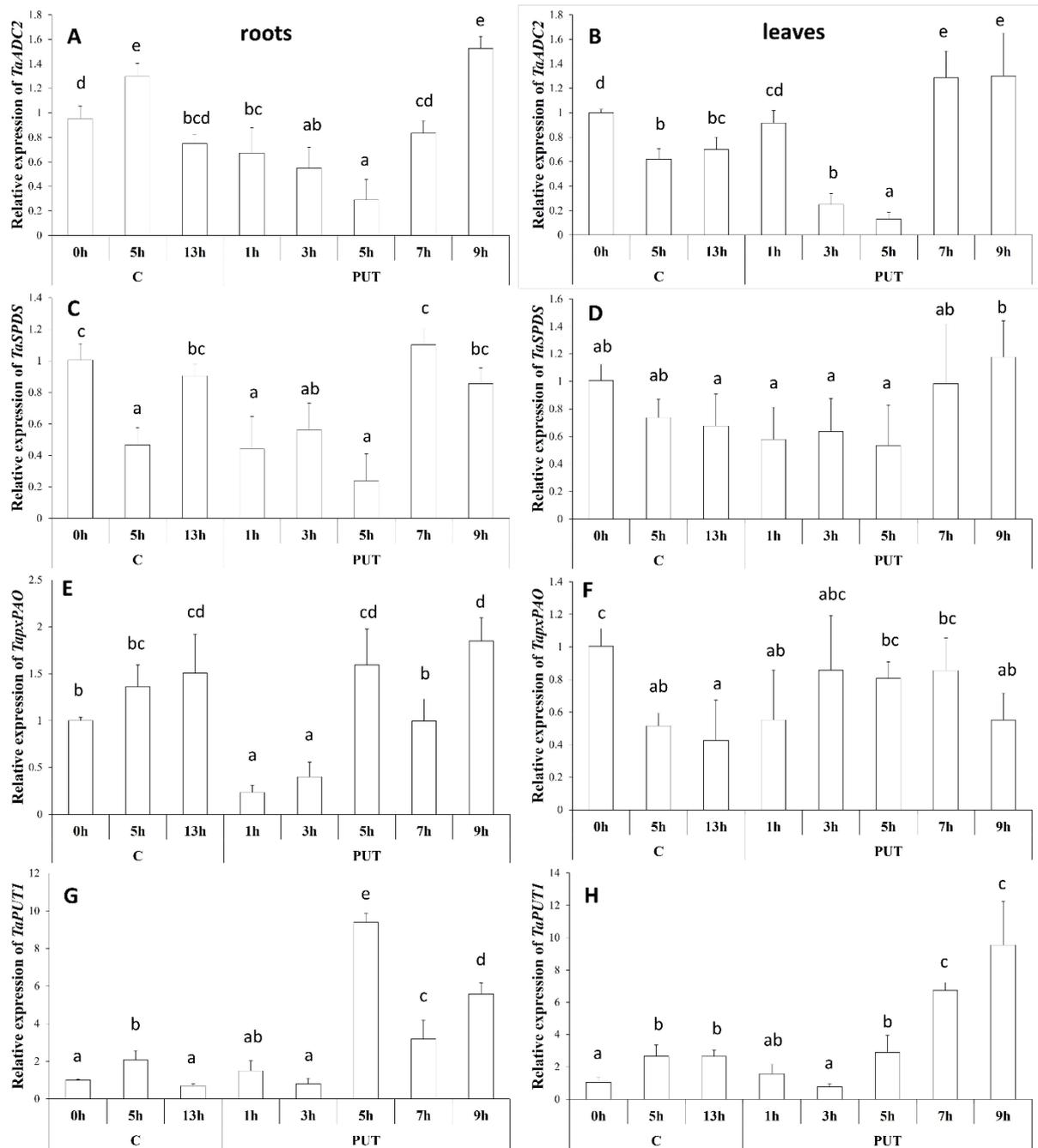
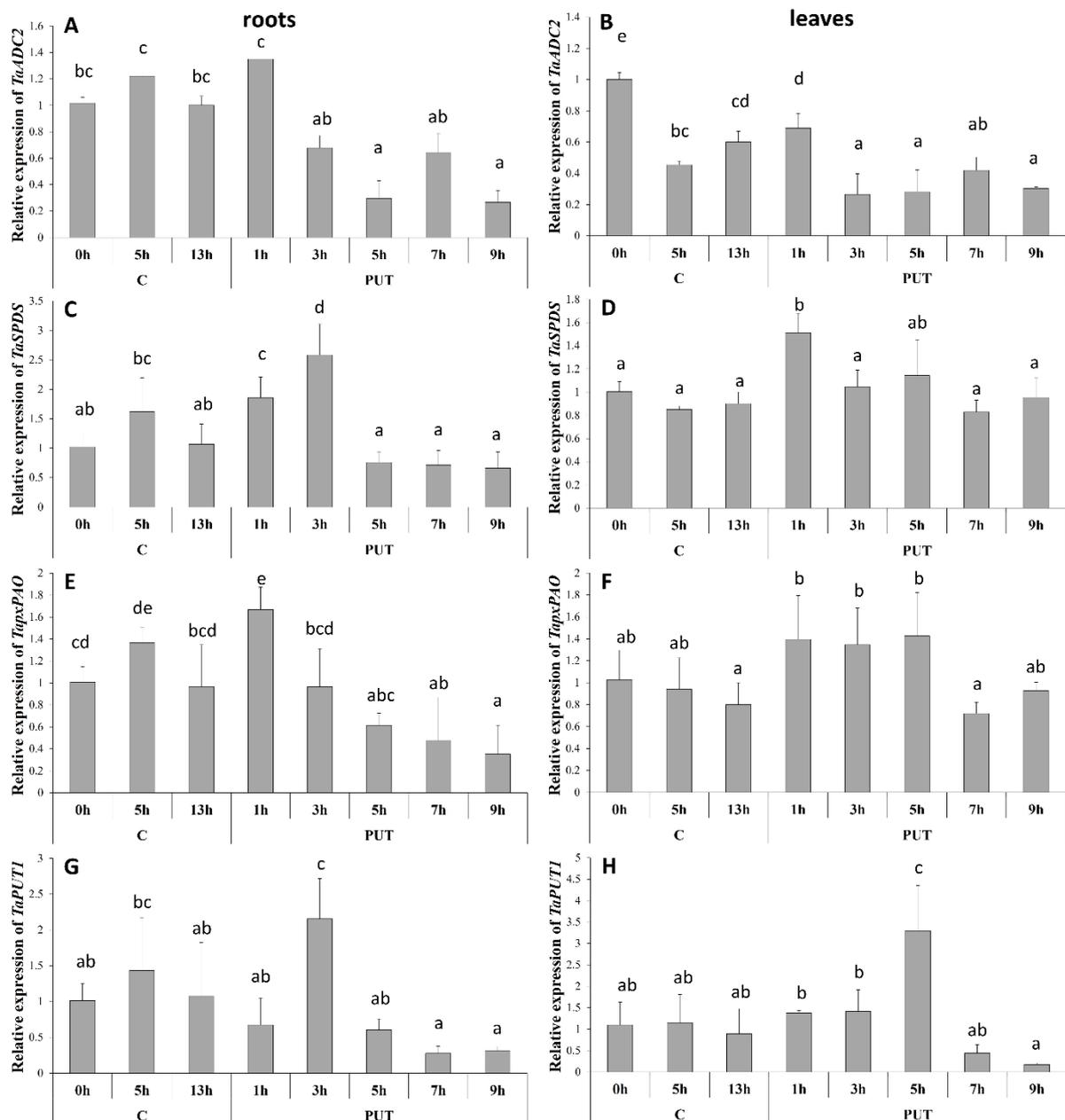


Fig. 2. The effect of 0.5 mM putrescine (PUT) compared to the control (C) on the gene expression level of arginine decarboxylase 2 (*TaADC2*) (A-B), spermidine synthase (*TaSPDS*) (C-D) peroxisomal polyamine oxidase (*TapxPAO*) (E-F) and polyamine uptake transporter (*TaPUT*) (G-H) genes in the roots (A, C, E, G) and leaves (B, D, F, H) of Mv Béres wheat plants. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

The PUT treatment also inhibited the expression of *TaADC2* in the roots of Mv Emese; however, the decrease was still discernible after 9 hours (Fig. 3A). The transcript levels of *TaSPDS* exhibited a modest, early increase during the day, but it is unlikely that this increase was influenced by the PUT treatment (Fig. 3C). The PUT application resulted in a consistent

1 decrease in the transcript level of *TapxPAO* (Fig. 3E). The expression of *TaPUT1* did not  
 2 exhibit any remarkable changes in the control plants; however, it showed a significant,  
 3 temporary increase after 3 h in the PUT-treated plants (Fig. 3G).  
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5 Light conditions decreased the transcript level of *TaADC2* in the leaves of Mv Emese  
 6 in comparison to the dark (0h) conditions, even in the absence of PUT treatment (Fig. 3B).  
 7 However, exogenous PUT also decreased its expression level even after 9h. No significant  
 8 alterations in the expression pattern of *TaSPDS* or *TapxPAO* were observed as a result of the  
 9 light or PUT treatments (Fig. 3D-F). *TaPUT1* expression levels increased at 5 hours following  
 10 PUT treatments, however, in contrast to Mv Béres, they were reduced to a lower level at 7 and  
 11 9 hours (Fig. 3H).  
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1 Fig. 3. The effect of 0.5 mM putrescine (PUT) compared to the control (C) on the gene  
2 expression level of arginine decarboxylase 2 (*TaADC2*) (A-B), spermidine synthase (*TaSPDS*)  
3 (C-D) peroxisomal polyamine oxidase (*TapxPAO*) (E-F) and polyamine uptake transporter  
4 (*TaPUT*) (G-H) genes in the roots (A, C, E, G) and leaves (B, D, F, H) of Mv Emese wheat  
5 plants. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with  
6 different letters at  $p < 0.05$  level.  
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### 9 **3.2. Light-dependent differences in PA metabolism in the presence of PUT or SPD** 10 **excess**

#### 11 **3.2.1. Changes in polyamine contents in Mv Emese after polyamine treatments** 12 **under continuous dark or light**

13 In the second experiment, a more detailed investigation was carried out focusing on the  
14 light-dependent changes in the processes regulating the PA levels in a selected variety, Mv  
15 Emese. Daily changes in the PA pool were monitored under continuous dark or light conditions  
16 with or without PUT or SPD treatments. In the leaves, PUT content showed statistically no  
17 significant fluctuation during the day under dark or light conditions even without any PA  
18 treatment (Suppl. Fig. 5A). The highest PUT levels were detected in the leaves of PUT-treated  
19 plants both under dark and light conditions after 7h and 9 hours. In the roots, the PUT  
20 application induced the accumulation of the endogenous PUT already after 1 hour, but with a  
21 different pattern under dark or light conditions (Suppl. Fig. 5B). Under continuous illumination,  
22 the increase in PUT level was also continuous, while under dark the highest increment was  
23 observed at 5<sup>th</sup> hour, after which a decrease was found. The exogenous SPD did not induce  
24 pronounced changes in PUT content.  
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39 The SPD content did not change during the day either with or without PA treatments in the  
40 leaves, however, it was higher under light conditions than under continuous dark (Fig. 4A). In  
41 the roots, exogenous SPD increased the SPD content both under light and dark conditions  
42 already after 1 hour, after which, especially in illuminated plants, the SPD level decreased (Fig.  
43 4B).  
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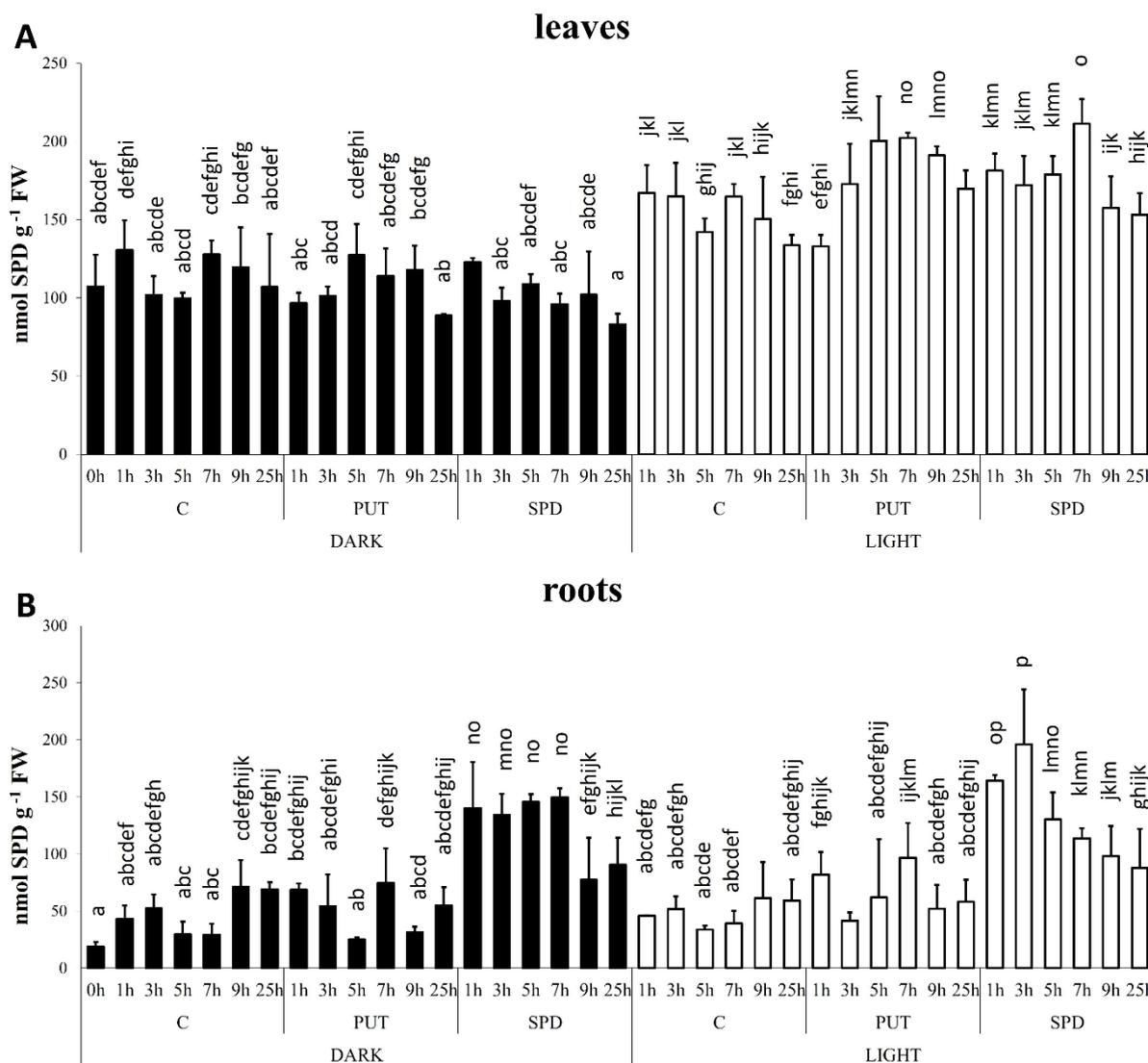


Fig. 4. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on spermidine (SPD) contents in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

In the case of SPM, although its level was lower in the dark than in the light (Suppl. Fig. 6A), a permanent increase was detected until the last sampling time (25h), regardless of PA application, while only a slightly increasing tendency was observed during the day in illuminated plants. In the roots, the SPM levels fluctuated during the day, and neither the PA treatments nor the light regime influenced it in an explainable way (Suppl. Fig. 6B).

Compared to the tendency described for leaf SPM content, an opposite pattern was found for leaf DAP content (Suppl Fig. 7A), namely during the day it was decreased under both dark and light conditions, regardless of PA application. Changes in the root DAP content were rather

similar to those found for root SPD content, as SPD treatment increased its levels under both light regimes (Suppl Fig. 7B).

### 3.2.2. Changes at gene expression level in *Mv Emese* after PA treatments under continuous dark or light

Even though the SPD content in the leaves was not affected by the daily rhythm or PA application under either dark or light conditions, the transcript level of *TaSPDS* exhibited an early increasing and then decreasing tendency during the day at dark, regardless of PA treatments. However, it did not change significantly at light, regardless of PA excess (Fig. 5A).

In the roots, the expression level of *TaSPDS* fluctuated during the day in the dark, but a pronounced decrease of it was observed during the day in the illuminated plants both in control and PA-treated ones (Fig. 5B).

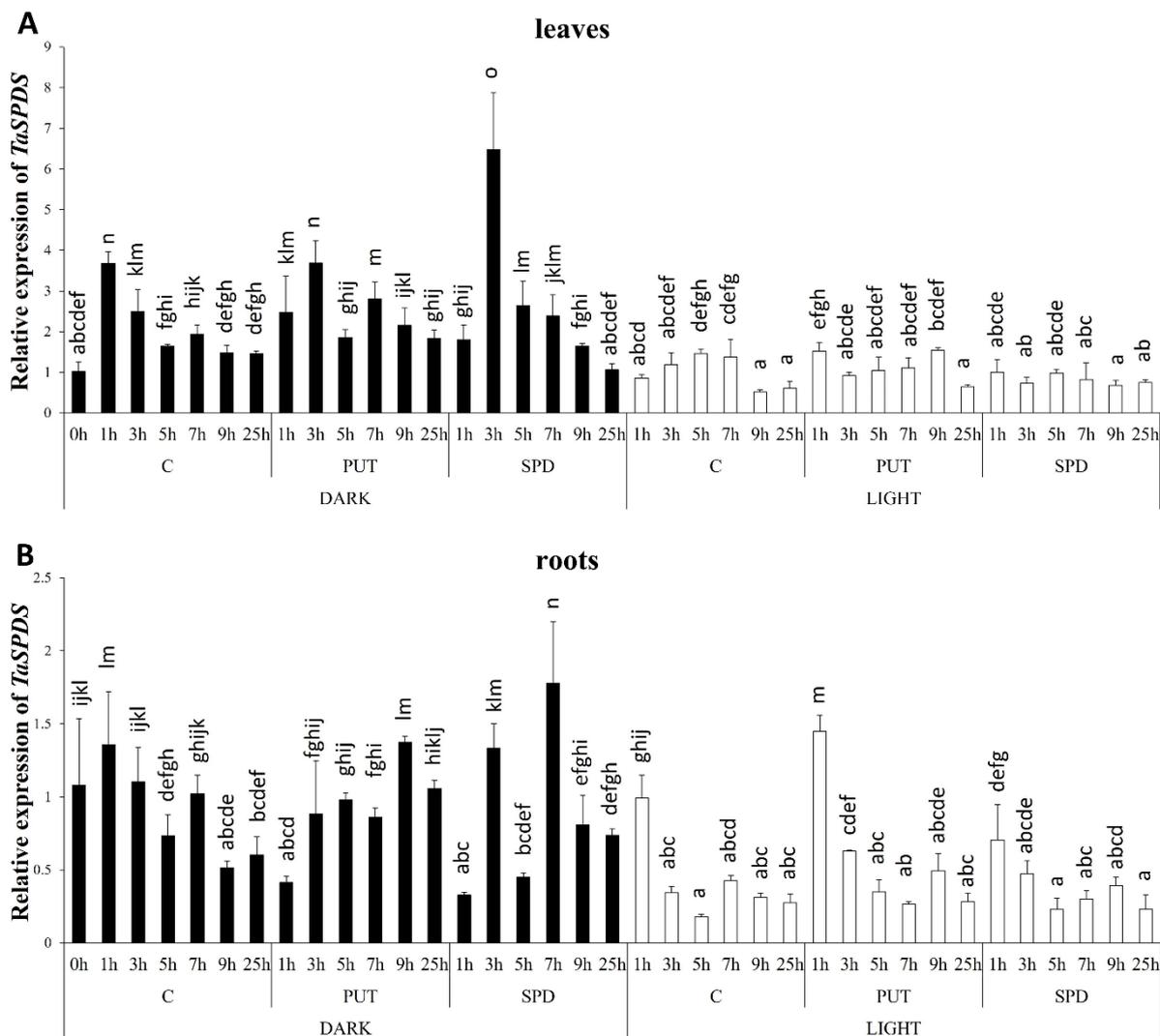


Fig. 5. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on gene expression level spermidine synthase (*TaSPDS*) genes in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

The transcript level of *TapxPAO*, which is responsible for the back-conversion of SPD or SPM, was initially lower in the leaves and roots under dark conditions (Suppl. Fig. 8A-B). The *TapxPAO* expression level in the leaves was nearly constant in the absence of PA application at dark. However, it was induced in the early hours following PUT or SPD treatments and subsequently declined. A daily increase in *TapxPAO* expression was observed in the leaves of control or PA-treated plants under light conditions, particularly after SPD treatment. The daily rhythm of the expression profile of *TapxPAO* was observed in the roots under both dark and light conditions. The pattern of expression increased in the early hours, but it subsequently decreased (Suppl. Fig. 8B).

As excess of PA may influence the PA conjugation processes, certain conjugated PAs were also analysed in dedicated sampling times. Changes in N-*p*-coumaroyl-*agmatine* and N-*p*-coumaroyl putrescine levels were monitored. The level of N-*p*-coumaroyl-*agmatine* increased in continuous dark in the leaves, and this increase was the same for all the treatments (Table 1).

Table 1. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on certain conjugated polyamine content (N-*p*-coumaroyl-*agmatine* and N-*p*-coumaroyl putrescine) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences indicated are with different letters at  $p < 0.05$  level.

	nmol g <sup>-1</sup> FW	leaves		roots	
		N- <i>p</i> -coumaroyl agmatine	N- <i>p</i> -coumaroyl putrescine	N- <i>p</i> -coumaroyl agmatine	N- <i>p</i> -coumaroyl putrescine
<b>DARK</b>	0h C	1.05 $\pm$ 0.9 a	0.3 $\pm$ 0.01 a	4.33 $\pm$ 0.55 c	43.39 $\pm$ 6.17 e
	25h C	2.27 $\pm$ 0.2 cd	n.d.	6.15 $\pm$ 0.68 e	34.03 $\pm$ 2.49 cd
	25h PUT	2.18 $\pm$ 0.3 c	0.66 $\pm$ 0.15 b	6.16 $\pm$ 0.33 e	44.36 $\pm$ 6.86 e
	25h SPD	2.2 $\pm$ 0.15 c	0.36 $\pm$ 0.01 a	5.55 $\pm$ 0.81 de	35.2 $\pm$ 3.63 d
<b>LIGHT</b>	25h C	1.07 $\pm$ 0.07 a	1.54 $\pm$ 0.2 c	0.12 $\pm$ 0.01 a	5.9 $\pm$ 1.41 a
	25h PUT	1.44 $\pm$ 0.14 b	1.49 $\pm$ 0.22 c	2.21 $\pm$ 0.09 b	27.19 $\pm$ 1.76 bc
	25h SPD	2.55 $\pm$ 0.1 d	1.39 $\pm$ 0.16 c	4.46 $\pm$ 1.22 cd	24.9 $\pm$ 2.09 b

1 Under continuous light conditions PA treatments, especially SPD, increased its level to  
2 a greater extent. Similarly, its level in the roots was higher under continuous dark conditions  
3 compared to continuous light, and PA treatments could not influence it. While under permanent  
4 illumination, PUT and SPD application highly increased its level (Table 1). In the case of N-*p*-  
5 coumaroyl putrescine, the most characteristic differences were also observed in the roots and  
6 were similar to those that were described for N-*p*-coumaroyl-*agmatine*. Briefly, continuous  
7 light alone decreased the accumulation of N-*p*-coumaroyl putrescine in the roots, compared to  
8 the dark regime, but under illumination, the PA treatments could increase its level.

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10 In order to reveal how PA conjugation depends on the availability of phenolic  
11 compounds, the levels of certain phenolic compounds, namely cinnamic acid, *p*-coumaric acid,  
12 caffeic acid and ferulic acid were also determined. The list of these phenolic compounds here  
13 also shows the order of their synthesis from each other. Under dark conditions, the cinnamic  
14 acid could not be detected either in the leaves or roots, in addition in the roots only *p*-coumaric  
15 acid could be detected (Table 2). The levels of other phenolic compounds in the leaves were  
16 also lower under dark conditions compared to the light, and no pronounced daily changes or  
17 PA-induced changes were found, rather fluctuation was typical. In the leaves, under the light  
18 regime, the levels of cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid increased  
19 during the day, with the highest accumulation after 25 hours, and PA treatments could hardly  
20 influence them. In the roots, more fluctuation was observed, however, under illumination, the  
21 level of cinnamic acid was also above the detection limit indicating that light signal also induced  
22 its accumulation not only in the leaves but also in the roots (Table 2.).

Table 2. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on the levels of phenolic compounds (cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

ng g <sup>-1</sup> FW	leaves				roots			
	cinnamic acid	<i>p</i> -coumaric acid	caffeic acid	ferulic acid	cinnamic acid	<i>p</i> -coumaric acid		
<b>DARK</b>	<b>C</b>	0h	nd	15.7 $\pm$ 1.65 def	12.87 $\pm$ 0.67 i	813.67 $\pm$ 45.54 f	nd	105.83 $\pm$ 9.7 ijklm
		1h	nd	15.27 $\pm$ 1.75 cdef	9.73 $\pm$ 0.32 efghi	691.33 $\pm$ 36.2 e	nd	112.3 $\pm$ 29.41 jklm
		3h	nd	9.46 $\pm$ 1.18 ab	7.91 $\pm$ 0.31 defg	297.67 $\pm$ 14.22 c	nd	140.33 $\pm$ 23.86 nop
		5h	nd	11.27 $\pm$ 0.91 abcd	6.75 $\pm$ 1.65 bcde	457 $\pm$ 38.04 d	nd	78.4 $\pm$ 2.4 cdefgh
		7h	nd	8.73 $\pm$ 0.65 ab	5.76 $\pm$ 0.32 bcd	300 $\pm$ 13 c	nd	86.15 $\pm$ 3.89 efghij
		9h	nd	7.09 $\pm$ 0.47 a	6.28 $\pm$ 2.22 bcd	316 $\pm$ 43 c	nd	65.53 $\pm$ 3.81 bcdef
		25h	nd	11.8 $\pm$ 0.08 abcd	6.84 $\pm$ 0.26 bcdef	173.67 $\pm$ 6.42 ab	nd	22.07 $\pm$ 1.75 a
		<b>PUT</b>	1h	nd	13.5 $\pm$ 2.35 bcde	12.63 $\pm$ 0.35 hi	484 $\pm$ 17.77 d	nd
	3h		nd	7.44 $\pm$ 2.02 a	4.58 $\pm$ 1.31 abc	238.33 $\pm$ 3.79 abc	nd	65.13 $\pm$ 5.31 bcde
	5h		nd	13.07 $\pm$ 1.22 bcd	12.27 $\pm$ 1.45 hi	335.67 $\pm$ 16.2 c	nd	81.2 $\pm$ 10.8 defghi
	7h		nd	10.61 $\pm$ 0.84 abc	7.72 $\pm$ 0.85 cdef	304 $\pm$ 19.67 c	nd	164 $\pm$ 40.04 p
	9h		nd	8.82 $\pm$ 0.43 ab	6.08 $\pm$ 0.75 bcd	211 $\pm$ 24.25 abc	nd	111.67 $\pm$ 8.5 jklm
	25h		nd	6.92 $\pm$ 0.75 a	2.48 $\pm$ 0.85 a	143 $\pm$ 17.69 a	nd	99.23 $\pm$ 1.53 hijkl
	<b>SPD</b>	1h	nd	10.79 $\pm$ 0.96 abcd	12 $\pm$ 0.5 hi	447.67 $\pm$ 7.37 d	nd	68.4 $\pm$ 4.98 bcdefg
		3h	nd	13.4 $\pm$ 3.25 bcde	6.19 $\pm$ 0.85 bcd	538 $\pm$ 57.98 d	nd	61.35 $\pm$ 3.04 bcde
		5h	nd	7.21 $\pm$ 0.23 a	9.65 $\pm$ 0.93 efgh	292 $\pm$ 21.62 c	nd	60.77 $\pm$ 14.49 bcde
		7h	nd	9.29 $\pm$ 0.6 ab	9.97 $\pm$ 1.2 fghi	264.67 $\pm$ 10.69 bc	nd	54.7 $\pm$ 5.22 bcd
		9h	nd	9.9 $\pm$ 0.26 ab	10.97 $\pm$ 0.66 ghi	302 $\pm$ 21.28 c	nd	53.07 $\pm$ 8.2 bc
		25h	nd	9.37 $\pm$ 1.39 ab	4.23 $\pm$ 0.23 ab	153.33 $\pm$ 1.52 ab	nd	117.17 $\pm$ 22.58 klmn
<b>LIGHT</b>	<b>C</b>	1h	5.81 $\pm$ 0.69 ef	31.47 $\pm$ 0.96 jk	31.13 $\pm$ 4.51 o	1843.33 $\pm$ 47.26 n	2.53 $\pm$ 0.63 a	60.77 $\pm$ 6.48 bcde
		3h	6.94 $\pm$ 0.81 fg	39.23 $\pm$ 4.6 l	29.6 $\pm$ 0.65 o	1530 $\pm$ 60.83 kl	5.47 $\pm$ 0.17 b	93.23 $\pm$ 7.51 ghijk
		5h	4.37 $\pm$ 0.29 cd	48.73 $\pm$ 6.5 m	30.8 $\pm$ 2.55 o	1500 $\pm$ 88.88 kl	9.5 $\pm$ 0.56 d	99.1 $\pm$ 3 hijkl
		7h	2.58 $\pm$ 0.23 a	24.03 $\pm$ 2.42 hi	23.63 $\pm$ 1.53 klm	995 $\pm$ 59.25 g	13.47 $\pm$ 1.16 f	153 $\pm$ 11.79 op
		9h	2.03 $\pm$ 0.21 a	28.2 $\pm$ 1.27 ijk	25.95 $\pm$ 1.48 mn	1380 $\pm$ 14.14 ij	6.05 $\pm$ 0.37 b	158 $\pm$ 3.46 p
		25h	18.23 $\pm$ 0.85 j	59.83 $\pm$ 4.3 n	62.23 $\pm$ 4.03 t	1853.33 $\pm$ 196.04 n	11.8 $\pm$ 0.26 ef	91.7 $\pm$ 10.48 fghijk
	<b>PUT</b>	1h	3.21 $\pm$ 0.31 abc	18.63 $\pm$ 0.21 fg	24.57 $\pm$ 2.06 lm	1460 $\pm$ 26.45 jkl	2.4 $\pm$ 0.55 a	122.33 $\pm$ 16.2 lmn
		3h	7.28 $\pm$ 0.32 g	32.83 $\pm$ 1.5 k	24.27 $\pm$ 0.87 lm	1446.67 $\pm$ 11.55 jk	5.1 $\pm$ 0.52 b	78.77 $\pm$ 5.05 cdefgh
		5h	5.9 $\pm$ 0.53 ef	27.4 $\pm$ 1.4 ij	30.4 $\pm$ 1.28 o	1373.33 $\pm$ 61.1 ij	8.55 $\pm$ 1.95 cd	115.67 $\pm$ 8.08 klmn
		7h	5.16 $\pm$ 0.82 de	29.2 $\pm$ 2.86 jk	34.53 $\pm$ 1.6 p	1683.33 $\pm$ 75.05 m	7.15 $\pm$ 1.2 bc	131 $\pm$ 23.26 mno
		9h	2.85 $\pm$ 0.18 ab	24.33 $\pm$ 4.41 hi	20.97 $\pm$ 0.42 k	1243.33 $\pm$ 77.67 h	2.86 $\pm$ 1.02 a	46.8 $\pm$ 11.6 b
		25h	12.93 $\pm$ 0.25 h	41.2 $\pm$ 1.67 l	45.43 $\pm$ 1.64 r	1976.67 $\pm$ 104.08 o	13.3 $\pm$ 2.8 f	124 $\pm$ 6.24 lmn
	<b>SPD</b>	1h	3.01 $\pm$ 0.27 abc	22.5 $\pm$ 3.6 gh	16.87 $\pm$ 1.51 j	1573.33 $\pm$ 68.07 l	2.54 $\pm$ 0.36 a	61.97 $\pm$ 6.16 bcde
		3h	4.13 $\pm$ 2.3 bcd	29.13 $\pm$ 5.59 jk	21.83 $\pm$ 2.14 kl	1706.67 $\pm$ 153.08 m	5.98 $\pm$ 0.67 b	102.03 $\pm$ 23.95
		5h	2.84 $\pm$ 0.37 ab	27.67 $\pm$ 3.45 ij	28.5 $\pm$ 1.31 no	1516.67 $\pm$ 11.55 kl	5.87 $\pm$ 1.4 b	94.37 $\pm$ 4.3 ghijk
		7h	2.27 $\pm$ 0.5 a	18.03 $\pm$ 0.83 efg	17.43 $\pm$ 0.92 j	927.33 $\pm$ 34.31 g	5.65 $\pm$ 1.36 b	69.8 $\pm$ 17.32 bcdefg
		9h	4.32 $\pm$ 0.45 cd	32.8 $\pm$ 5.12 k	24.53 $\pm$ 2.19	1270 $\pm$ 87.18 hi	2.27 $\pm$ 0.05 a	59.53 $\pm$ 4.65 bcde
		25h	16.2 $\pm$ 0.98 i	49.43 $\pm$ 2.62 m	55.07 $\pm$ 3.23 s	2613.33 $\pm$ 130.51 p	10.36 $\pm$ 1.54 de	123 $\pm$ 11.13 lmn

## 4. Discussion

The levels of individual PA compounds may follow a circadian rhythm and they can fluctuate depending on the plant developmental stage and environmental conditions, including light intensities (Kolesnikov et al., 2024; Pál et al., 2021). Maintaining of PA homeostasis involves the complex regulation of synthesis and back-conversion, according to the PA-cycle, and it is completed with terminal catabolism, conjugation, root-to-shoot transport, uptake and efflux mechanisms. The success of a PA treatment thus strongly depends on the fine-tuning of PA metabolism. In the present study, investigations were partly focused on the early effects of PUT treatment on PA pool and metabolism in the leaves and roots of three wheat genotypes, to reveal whether there is a genotype-dependent difference in coping with PA excess. These genotypes were chosen based on previous experiments, where 0.5 mM PUT treatment had positive effects under certain stress conditions (Szalai et al., 2017; Tajti et al., 2019; Rahman et al., 2024b). The other main question of our research was, how the lack of light affects the uptake and metabolism of PUT or SPD excess in a single one-day interval experimental setup. A similar, detailed investigation has not yet been carried out.

### 4.1. Genotype-dependent differences in PA metabolism in the presence of PUT excess

PA treatments may influence PA metabolism at gene expression level. For example, in rice SPD treatment increased the expression level of ornithine decarboxylase 1 and *OsSPDS3*, but decreased the transcript level of *OsADC1* and S-adenosylmethionine decarboxylase 2 (Zhou et al., 2020). PUT, SPD and SPM treatments in wheat inhibited the expression of *TaSPDS*, but induced that of *TapxPAO* (Gondor et al., 2021). In Arabidopsis, only PUT treatment could increase the *AtADC2* expression, while SPD inhibited it (Yariuchi et al., 2021). A comparison study on wheat, maize and rice plants, revealed that 7 days of 0.3 mM PUT treatment induced different expression changes of genes involved in PA metabolism (Altafur et al., 2024).

In the present study, the most characteristic changes after PUT treatments were observed in the roots. Monitoring the changes in PA homeostasis of the three wheat genotypes revealed that in the roots, parallel with similarly high accumulation of PUT, the expression levels of the PA metabolism-related genes altered differently. In Mv Béres, the genes responsible for the PUT and SPD synthesis (*TaADC2* and *TaSPDS*, respectively), and for the back-conversion (*TapxPAO*) were down-regulated in the early hours of PUT treatment, after which returned to the control level. In contrast, in Mv Emese the expression levels of these genes did not change or even increased in the early period, but in long-term, they also pronouncedly decreased. This opposite pattern was also true for *TaPUT1*.

1 Genotype-dependent differences observed in the present study at gene expression level may  
2 be responsible for the earlier decreasing accumulation of PUT in the roots of Mv Emese  
3 compared to the Mv Béres. Interestingly, in TC33 the root PUT level decreased back to the  
4 control level already after 25 hours, while in the leaves, the highest PUT accumulation was  
5 found in TC33, compared to the other two genotypes. The increasing PUT accumulation was  
6 not associated with such remarkable changes at gene expression levels in the leaves in Mv Béres  
7 and Mv Emese. In Mv Béres, a temporarily lower level of the expression of *TaADC2* was  
8 detected at 3 and 5 hours of PUT treatment, while in Mv Emese the decrease was sustained.  
9 The pattern of the *TaPUT1* gene expression also showed genotype dependence as was described  
10 in the roots.  
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12 Although the transcript level of *TaPUT1* increased in Mv Béres, it was not followed by  
13 further PUT accumulation. But it cannot be ruled out that the uptaken PUT was restored as  
14 conjugated form. Changes in DAP content were only detectable in the leaves of Mv Emese  
15 following PUT treatment. Specifically, the DAP level increased after 5 hours, peaked at 9 hours,  
16 and subsequently diminished to the control level. This tendency was similar to that was  
17 observed in the case of PUT level in the leaves of this genotype. These changes indicated that  
18 the PUT to SPD/SPM synthesis pathway was intensive without the accumulation of SPD and  
19 SPM but with the activation of the terminal catabolism in the leaves of Mv Emese. More  
20 intensive PUT to SPM canalisation and subsequent apoplastic oxidation has been demonstrated  
21 to have an important role in the elimination of excess PUT (Szalai et al., 2017).  
22

23 In summary, daily changes in PA levels of different genotypes can be partly generalised.  
24 PUT treatment induced the accumulation of PAs, but after a maximum at different times for the  
25 three genotypes, plants could metabolise the excess of PUT. However, different PA metabolism  
26 strategies were observed in the three genotypes. In Mv Béres, the early inhibition of expression  
27 of PA-metabolism-related genes was observed, which was only partly efficient against the  
28 extreme PUT accumulation in both the roots and leaves. In Mv Emese, the PUT synthesis and  
29 absorption decreased, while the terminal catabolism was initiated, leading to the successful  
30 early metabolism of excess PUT.  
31

#### 32 **4.2. Light-dependent changes in the PA metabolism in the presence of PUT excess**

33 The lack of light did not inhibit the PUT accumulation in the root of Mv Emese. Indeed,  
34 the PUT content was higher in the early period of PUT treatment, reached a maximum earlier,  
35 and even began to decrease earlier under dark conditions than under illumination. The amounts  
36 of the investigated conjugated PAs (*N-p-coumaroyl-agmatine* and *N-p-coumaroyl-putrescine*)  
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in the roots were initially higher in the dark than at light and PA treatments did not influence it, while under light conditions its lower basic level increased by PUT or SPD treatments. Simultaneously, the degree of conjugation increased not only after PUT treatment but also after SPD application, indicating that PUT accumulation is not a direct cause of PA conjugation. This is because endogenous PUT content increased exclusively in the roots of PUT-treated plants. It is also substantiated by the fact that agmatine conjugation occurred in addition to PUT. The conjugation was inhibited by light in the roots, and it was only activated in the presence of an excess of PA.

As the levels of all the cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid showed similar changes in the leaves, it suggests that these changes were rather related to the light conditions and daily changes than to PA excess, and *p*-coumaric acid were not used up for PA conjugation, thus did not directly influence the level of *N-p*-coumaroyl-agmatine and *N-p*-coumaroyl-putrescine. The light could hardly influence the conjugation process in the leaves, in addition, PA treatments had fewer effects than in the roots. In the roots, the daily fluctuation in the amounts of phenolic compounds indicated that neither daily rhythm nor PA excess had a dominant effect on them under dark conditions. Under light conditions, the slightly increasing tendency during the day after PA treatments also could not be responsible for the observed higher accumulation of the investigated conjugated PAs in PA-treated plants.

SPD treatment resulted in SPD accumulation in the roots under both dark and light conditions, which may induce the PA metabolism, and the level of SPD started to decrease back after 5-7 hours. As the expression level of *TaSPDS* in the roots showed a similar pattern in control or PA-treated plants, under dark or light conditions, respectively, it is unlikely that the SPD accumulation in SPD-treated plants had an inhibitory feed-back effect on its expression level. This is also true for the expression level of *TapxPAO*, as it exhibited a consistent pattern in the roots during the day, irrespective of the light regime or PA application level. The query that arises is how the SPD was metabolised so rapidly, without the synthesis being inhibited and the back-conversion being activated. It is evident, that terminal catabolism is responsible for the metabolism of SPD excess when the variations in root DAP content are examined. The accumulation of SPD in the roots did not increase the leaf SPD content or SPD synthesis (*TaSPDS*). These results supported the fact that the level of SPD, the most stable, central intermediate in the PA cycle, is controlled at the synthesis side and at the catabolism/back-conversion side, too. In addition, the SPD pool could also be regulated at the post-transcriptional level (Martin-Tanguy, 2001).

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There were no variations in the SPM content of the leaves or roots of the plants as a result of PA treatments or light conditions. In the leaves a characteristic increasing tendency was observed both in dark and light conditions, irrespective of the presence of excessive PA, which showed a close, negative correlation with the daily changes in leaf DAP content.

## 5. Conclusion

Based on the results, the three investigated wheat genotypes responded partly differently to the PUT treatment. Although PUT excess induced changes in PA metabolism in all the genotypes, these changes were different to a certain extent. The distinctly induced elements of the PA metabolism were responsible for the observed differences in the patterns of the changes in the PA pool, especially in the roots. It was also demonstrated that the leaf PA pool was more significantly influenced by the extant daily fluctuation or rhythm than by the PA treatments, regardless of whether the conditions were dark or light. It supports the view that the PA pool is strictly regulated, and it follows the changes in the environmental signals. In the roots, the PA treatments induced the accumulation of themselves, but the process was characterised by varying tendencies under dark and light conditions. Additionally, the conjugation process was activated following PA treatments under light conditions. At the gene expression level (*TapxPAO* and *TaSPDS*) more differences were observed under the two light regimes, but these are unlikely to be responsible for the changes at the metabolite level. In the case of SPD treatment, the induced terminal catabolism helped cope with SPD excess in the roots regardless of light conditions. Based on these results further investigations can be conducted to find a treatment method and concentration that can be used for several genotypes in general.

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## Author Contributions

Conception and design: M.P.; Analysis and interpretation of the data: M.P.; A.R, K.Á.H.; K.N.; M.D.; G.Sz.; Statistical analyses: M.P.; Drafting of the article: M.P., A.R.; Visualization: M.P.; Critical revision of the article: T.J.; G.Sz; Final approval of the article: K.Á.H.; K.N.; M.D.; Obtaining of funding: M.P. All authors have read and agreed to the published version of the manuscript.

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## Legends:

**Fig. 1.** The effect of 0.5 mM putrescine (PUT) treatment compared to the control conditions (C) on endogenous putrescine (PUT) contents in the roots (A, C, E) and leaves (B, D, F) of Mv Béres (A, B), Mv Emese (C, D) and TC33 (E, F) wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Fig. 2.** The effect of 0.5 mM putrescine (PUT) compared to the control (C) on the gene expression level of arginine decarboxylase 2 (*TaADC2*) (A-B), spermidine synthase (*TaSPDS*) (C-D) peroxisomal polyamine oxidase (*TapxPAO*) (E-F) and polyamine uptake transporter (*TaPUT*) (G-H) genes in the roots (A, C, E, G) and leaves (B, D, F, H) of Mv Béres wheat plants. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Fig. 3.** The effect of 0.5 mM putrescine (PUT) compared to the control (C) on the gene expression level of arginine decarboxylase 2 (*TaADC2*) (A-B), spermidine synthase (*TaSPDS*) (C-D) peroxisomal polyamine oxidase (*TapxPAO*) (E-F) and polyamine uptake transporter (*TaPUT*) (G-H) genes in the roots (A, C, E, G) and leaves (B, D, F, H) of Mv Emese wheat plants. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Fig. 4.** The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on spermidine (SPD) contents in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Fig. 5.** The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on gene expression level spermidine synthase (*TaSPDS*) genes in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Table 1.** The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on certain conjugated polyamine content (N-*p*-coumaroyl-*agmatine* and N-*p*-coumaroyl putrescine) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences indicated are with different letters at  $p < 0.05$  level.

**Table 2.** The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on the levels of phenolic compounds (cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

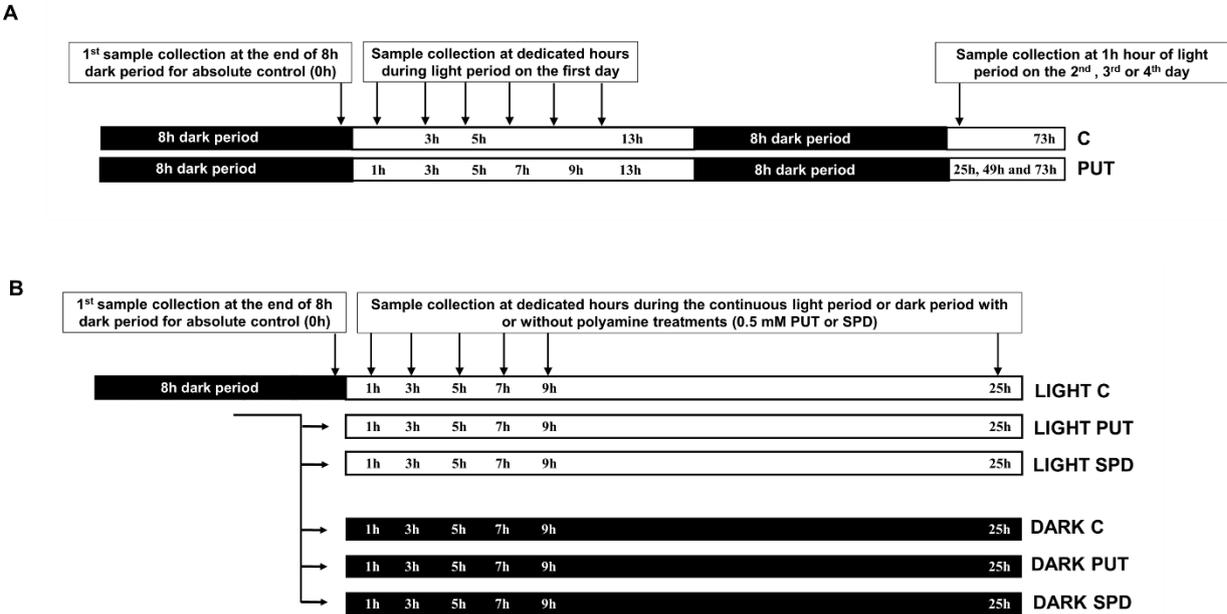
Table 1. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on certain conjugated polyamine content (N-*p*-coumaroyl-*agmatine* and N-*p*-coumaroyl *putrescine*) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences indicated are with different letters at  $p < 0.05$  level.

	nmol g <sup>-1</sup> FW	leaves		roots	
		N- <i>p</i> -coumaroyl <i>agmatine</i>	N- <i>p</i> -coumaroyl <i>putrescine</i>	N- <i>p</i> -coumaroyl <i>agmatine</i>	N- <i>p</i> -coumaroyl <i>putrescine</i>
<b>DARK</b>	0h C	1.05 $\pm$ 0.9 a	0.3 $\pm$ 0.01 a	4.33 $\pm$ 0.55 c	43.39 $\pm$ 6.17 e
	25h C	2.27 $\pm$ 0.2 cd	n.d.	6.15 $\pm$ 0.68 e	34.03 $\pm$ 2.49 cd
	25h PUT	2.18 $\pm$ 0.3 c	0.66 $\pm$ 0.15 b	6.16 $\pm$ 0.33 e	44.36 $\pm$ 6.86 e
	25h SPD	2.2 $\pm$ 0.15 c	0.36 $\pm$ 0.01 a	5.55 $\pm$ 0.81 de	35.2 $\pm$ 3.63 d
<b>LIGHT</b>	25h C	1.07 $\pm$ 0.07 a	1.54 $\pm$ 0.2 c	0.12 $\pm$ 0.01 a	5.9 $\pm$ 1.41 a
	25h PUT	1.44 $\pm$ 0.14 b	1.49 $\pm$ 0.22 c	2.21 $\pm$ 0.09 b	27.19 $\pm$ 1.76 bc
	25h SPD	2.55 $\pm$ 0.1 d	1.39 $\pm$ 0.16 c	4.46 $\pm$ 1.22 cd	24.9 $\pm$ 2.09 b

Table 2. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on the levels of phenolic compounds (cinnamic acid, *p*-coumaric acid, caffeic acid and ferulic acid) in the leaves and roots of wheat plants at given sampling times. Data are mean values  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

ng g <sup>-1</sup> FW	leaves				roots				
	cinnamic acid	<i>p</i> -coumaric acid	caffeic acid	ferulic acid	cinnamic acid	<i>p</i> -coumaric acid			
<b>C</b>	0h	nd	15.7 $\pm$ 1.65 def	12.87 $\pm$ 0.67 i	813.67 $\pm$ 45.54 f	nd	105.83 $\pm$ 9.7 ijklm		
	1h	nd	15.27 $\pm$ 1.75 cdef	9.73 $\pm$ 0.32 efghi	691.33 $\pm$ 36.2 e	nd	112.3 $\pm$ 29.41 jklm		
	3h	nd	9.46 $\pm$ 1.18 ab	7.91 $\pm$ 0.31 defg	297.67 $\pm$ 14.22 c	nd	140.33 $\pm$ 23.86 nop		
	5h	nd	11.27 $\pm$ 0.91 abcd	6.75 $\pm$ 1.65 bcde	457 $\pm$ 38.04 d	nd	78.4 $\pm$ 2.4 cdefgh		
	7h	nd	8.73 $\pm$ 0.65 ab	5.76 $\pm$ 0.32 bcd	300 $\pm$ 13 c	nd	86.15 $\pm$ 3.89 efghij		
	9h	nd	7.09 $\pm$ 0.47 a	6.28 $\pm$ 2.22 bcd	316 $\pm$ 43 c	nd	65.53 $\pm$ 3.81 bcdef		
	25h	nd	11.8 $\pm$ 0.08 abcd	6.84 $\pm$ 0.26 bcdef	173.67 $\pm$ 6.42 ab	nd	22.07 $\pm$ 1.75 a		
	<b>DARK</b>	<b>PUT</b>	1h	nd	13.5 $\pm$ 2.35 bcde	12.63 $\pm$ 0.35 hi	484 $\pm$ 17.77 d	nd	64.03 $\pm$ 5.86 bcde
3h			nd	7.44 $\pm$ 2.02 a	4.58 $\pm$ 1.31 abc	238.33 $\pm$ 3.79 abc	nd	65.13 $\pm$ 5.31 bcde	
5h			nd	13.07 $\pm$ 1.22 bcd	12.27 $\pm$ 1.45 hi	335.67 $\pm$ 16.2 c	nd	81.2 $\pm$ 10.8 defghi	
7h			nd	10.61 $\pm$ 0.84 abc	7.72 $\pm$ 0.85 cdef	304 $\pm$ 19.67 c	nd	164 $\pm$ 40.04 p	
9h			nd	8.82 $\pm$ 0.43 ab	6.08 $\pm$ 0.75 bcd	211 $\pm$ 24.25 abc	nd	111.67 $\pm$ 8.5 jklm	
25h			nd	6.92 $\pm$ 0.75 a	2.48 $\pm$ 0.85 a	143 $\pm$ 17.69 a	nd	99.23 $\pm$ 1.53 hijkl	
<b>SPD</b>			1h	nd	10.79 $\pm$ 0.96 abcd	12 $\pm$ 0.5 hi	447.67 $\pm$ 7.37 d	nd	68.4 $\pm$ 4.98 bcdefg
			3h	nd	13.4 $\pm$ 3.25 bcde	6.19 $\pm$ 0.85 bcd	538 $\pm$ 57.98 d	nd	61.35 $\pm$ 3.04 bcde
	5h	nd	7.21 $\pm$ 0.23 a	9.65 $\pm$ 0.93 efgh	292 $\pm$ 21.62 c	nd	60.77 $\pm$ 14.49 bcde		
	7h	nd	9.29 $\pm$ 0.6 ab	9.97 $\pm$ 1.2 fghi	264.67 $\pm$ 10.69 bc	nd	54.7 $\pm$ 5.22 bcd		
	9h	nd	9.9 $\pm$ 0.26 ab	10.97 $\pm$ 0.66 ghi	302 $\pm$ 21.28 c	nd	53.07 $\pm$ 8.2 bc		
	25h	nd	9.37 $\pm$ 1.39 ab	4.23 $\pm$ 0.23 ab	153.33 $\pm$ 1.52 ab	nd	117.17 $\pm$ 22.58 klmn		
<b>LIGHT</b>	<b>C</b>	1h	5.81 $\pm$ 0.69 ef	31.47 $\pm$ 0.96 jk	31.13 $\pm$ 4.51 o	1843.33 $\pm$ 47.26 n	2.53 $\pm$ 0.63 a	60.77 $\pm$ 6.48 bcde	
		3h	6.94 $\pm$ 0.81 fg	39.23 $\pm$ 4.6 l	29.6 $\pm$ 0.65 o	1530 $\pm$ 60.83 kl	5.47 $\pm$ 0.17 b	93.23 $\pm$ 7.51 ghijk	
		5h	4.37 $\pm$ 0.29 cd	48.73 $\pm$ 6.5 m	30.8 $\pm$ 2.55 o	1500 $\pm$ 88.88 kl	9.5 $\pm$ 0.56 d	99.1 $\pm$ 3 hijkl	
		7h	2.58 $\pm$ 0.23 a	24.03 $\pm$ 2.42 hi	23.63 $\pm$ 1.53 klm	995 $\pm$ 59.25 g	13.47 $\pm$ 1.16 f	153 $\pm$ 11.79 op	
		9h	2.03 $\pm$ 0.21 a	28.2 $\pm$ 1.27 ijk	25.95 $\pm$ 1.48 mn	1380 $\pm$ 14.14 ij	6.05 $\pm$ 0.37 b	158 $\pm$ 3.46 p	
		25h	18.23 $\pm$ 0.85 j	59.83 $\pm$ 4.3 n	62.23 $\pm$ 4.03 t	1853.33 $\pm$ 196.04 n	11.8 $\pm$ 0.26 ef	91.7 $\pm$ 10.48 fghijk	
	<b>PUT</b>	1h	3.21 $\pm$ 0.31 abc	18.63 $\pm$ 0.21 fg	24.57 $\pm$ 2.06 lm	1460 $\pm$ 26.45 jkl	2.4 $\pm$ 0.55 a	122.33 $\pm$ 16.2 lmn	
		3h	7.28 $\pm$ 0.32 g	32.83 $\pm$ 1.5 k	24.27 $\pm$ 0.87 lm	1446.67 $\pm$ 11.55 jk	5.1 $\pm$ 0.52 b	78.77 $\pm$ 5.05 cdefgh	
		5h	5.9 $\pm$ 0.53 ef	27.4 $\pm$ 1.4 ij	30.4 $\pm$ 1.28 o	1373.33 $\pm$ 61.1 ij	8.55 $\pm$ 1.95 cd	115.67 $\pm$ 8.08 klmn	
		7h	5.16 $\pm$ 0.82 de	29.2 $\pm$ 2.86 jk	34.53 $\pm$ 1.6 p	1683.33 $\pm$ 75.05 m	7.15 $\pm$ 1.2 bc	131 $\pm$ 23.26 mno	
		9h	2.85 $\pm$ 0.18 ab	24.33 $\pm$ 4.41 hi	20.97 $\pm$ 0.42 k	1243.33 $\pm$ 77.67 h	2.86 $\pm$ 1.02 a	46.8 $\pm$ 11.6 b	
		25h	12.93 $\pm$ 0.25 h	41.2 $\pm$ 1.67 l	45.43 $\pm$ 1.64 r	1976.67 $\pm$ 104.08 o	13.3 $\pm$ 2.8 f	124 $\pm$ 6.24 lmn	
<b>SPD</b>	1h	3.01 $\pm$ 0.27 abc	22.5 $\pm$ 3.6 gh	16.87 $\pm$ 1.51 j	1573.33 $\pm$ 68.07 l	2.54 $\pm$ 0.36 a	61.97 $\pm$ 6.16 bcde		
	3h	4.13 $\pm$ 2.3 bcd	29.13 $\pm$ 5.59 jk	21.83 $\pm$ 2.14 kl	1706.67 $\pm$ 153.08 m	5.98 $\pm$ 0.67 b	102.03 $\pm$ 23.95		
	5h	2.84 $\pm$ 0.37 ab	27.67 $\pm$ 3.45 ij	28.5 $\pm$ 1.31 no	1516.67 $\pm$ 11.55 kl	5.87 $\pm$ 1.4 b	94.37 $\pm$ 4.3 ghijk		
	7h	2.27 $\pm$ 0.5 a	18.03 $\pm$ 0.83 efg	17.43 $\pm$ 0.92 j	927.33 $\pm$ 34.31 g	5.65 $\pm$ 1.36 b	69.8 $\pm$ 17.32 bcdefg		
	9h	4.32 $\pm$ 0.45 cd	32.8 $\pm$ 5.12 k	24.53 $\pm$ 2.19	1270 $\pm$ 87.18 hi	2.27 $\pm$ 0.05 a	59.53 $\pm$ 4.65 bcde		
	25h	16.2 $\pm$ 0.98 i	49.43 $\pm$ 2.62 m	55.07 $\pm$ 3.23 s	2613.33 $\pm$ 130.51 p	10.36 $\pm$ 1.54 de	123 $\pm$ 11.13 lmn		

Supplementary information:



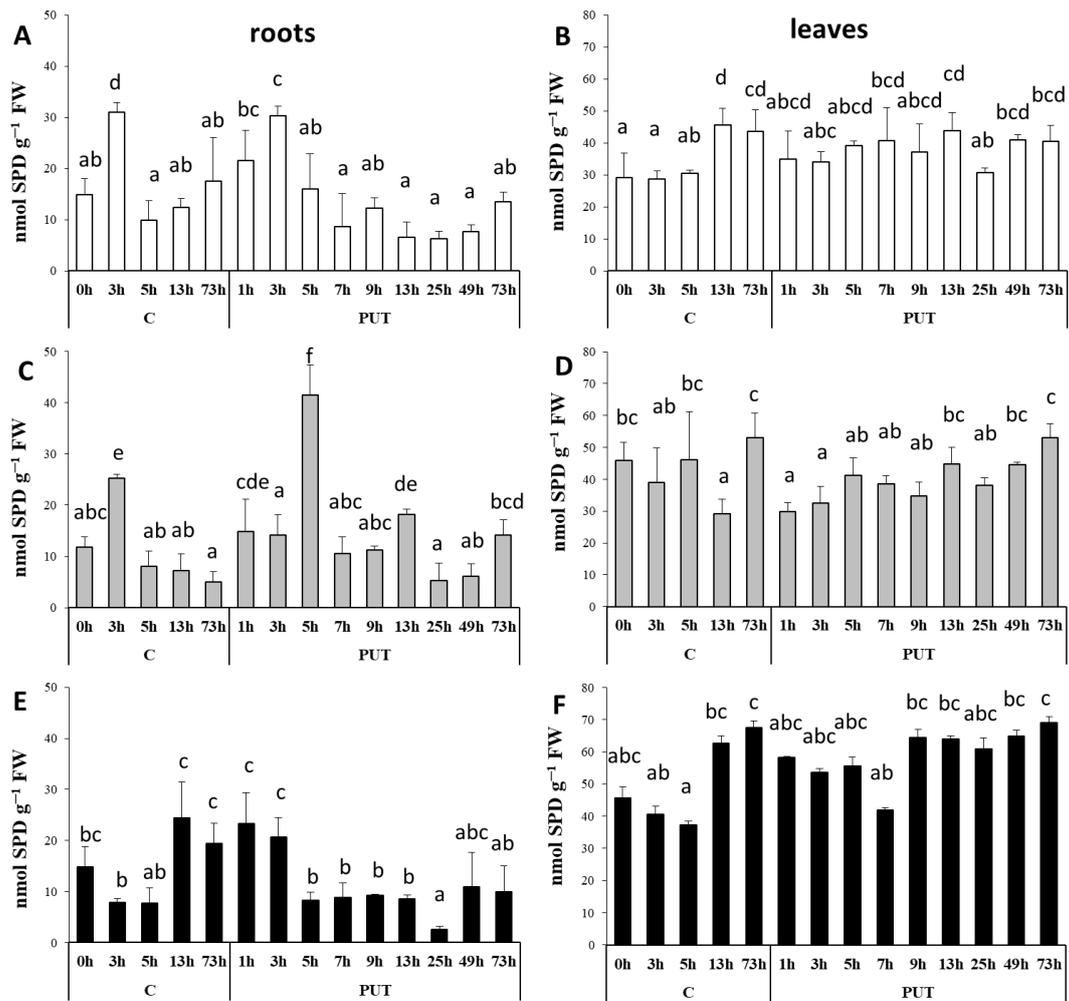
**Suppl. Fig. 1.** Experimental setup with the description of treatments and sample collections in the first experiment (A) and the second experiment (B).

**Suppl. Table 1.** MRM transitions used for LC-MS/MS analysis.

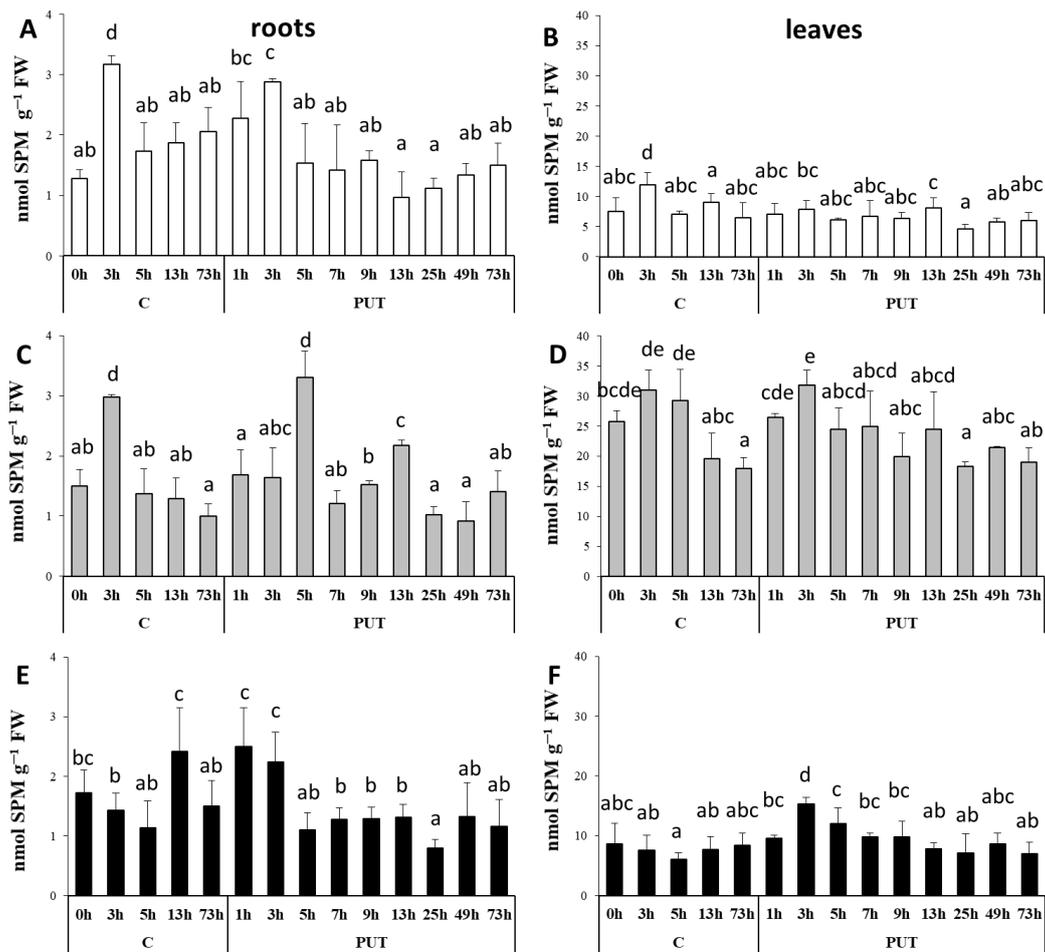
<b>Name</b>	<b>Trace (<i>m/z</i>) Q1 &gt; Q3</b>	<b>RT (min)</b>	<b>US polarity</b>
<b>caffeic acid</b>	179 > 134	3.23	negative
<b><i>p</i>-coumaric acid</b>	163 > 93	4.07	negative
<b>ferulic acid</b>	193 > 134	4.56	negative
<b>cinnamic acid</b>	131 > 103	7.54	positive

**Suppl. Table 2.** Primer sequences for qRT-PCR analysis of reference and gene of interest genes in wheat plants.

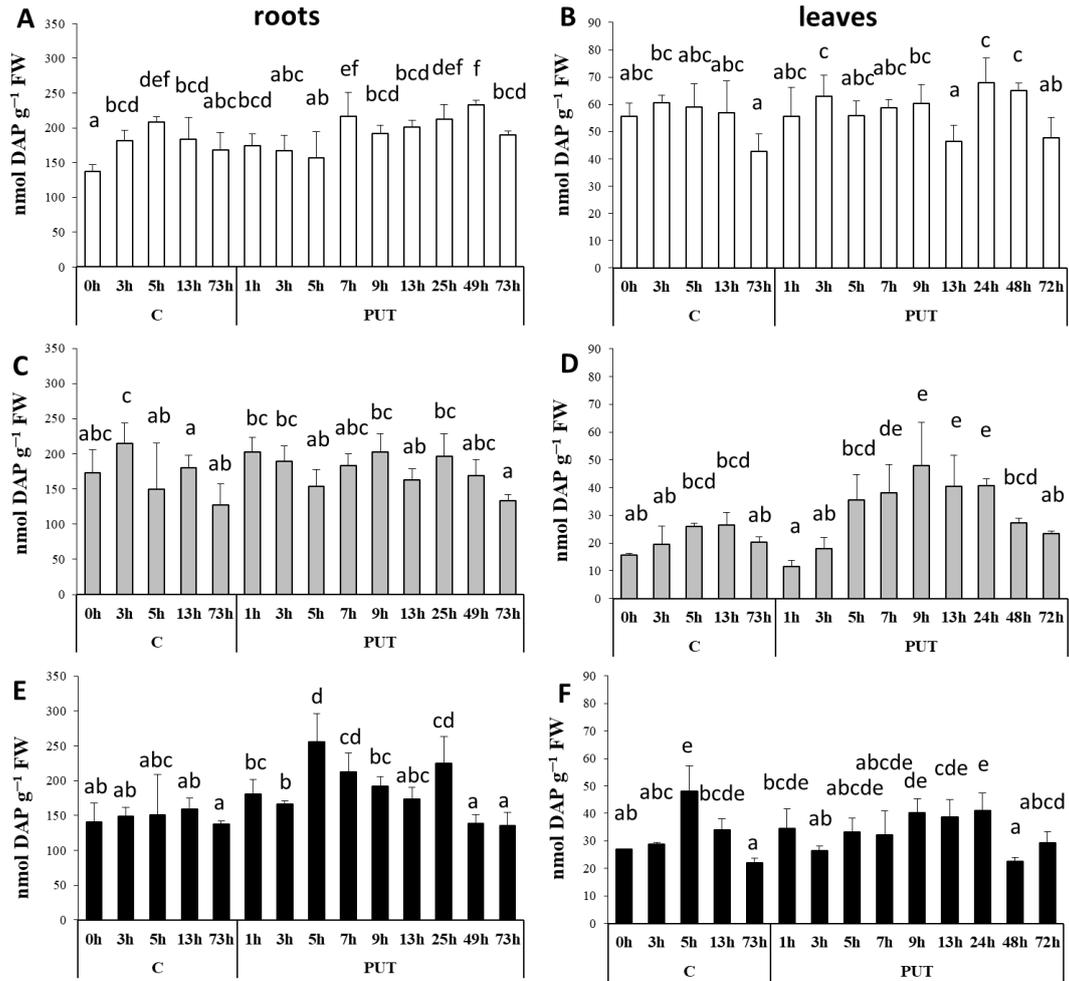
<i>Ta35497</i>	Forward	GTGTGTCCCGTGTCGTGTC	131 bp	Paolacci et al. 2009
	Reverse	TCCAGCAGCCCAAAGAGTCC		
<i>TaADC2</i>	Forward	AGGAGGAGGAGCTCGACATT	137 bp	Gardiner et al. 2010
	Reverse	GCCGAACTTGCCCTTCTC		
<i>TaSPDS</i>	Forward	AGGTATTCAAGGGTGGCGTG	137 bp	Pál et al. 2018
	Reverse	TGGGTTCACAGGAGTCAGGA		
<i>TapxPAO</i>	Forward	GTCATAAATCAGCCCAATTCCA	125bp	Xiong et al. 2017
	Reverse	TTCGCCATTTGTTGAGCTCT		
<i>TaPUT1</i>	Forward	GGTCTTCTCCCTCTTGCCTT	156 bp	XM_044548016.1
	Reverse	GTGCTGATCGAGTCCCAGTA		



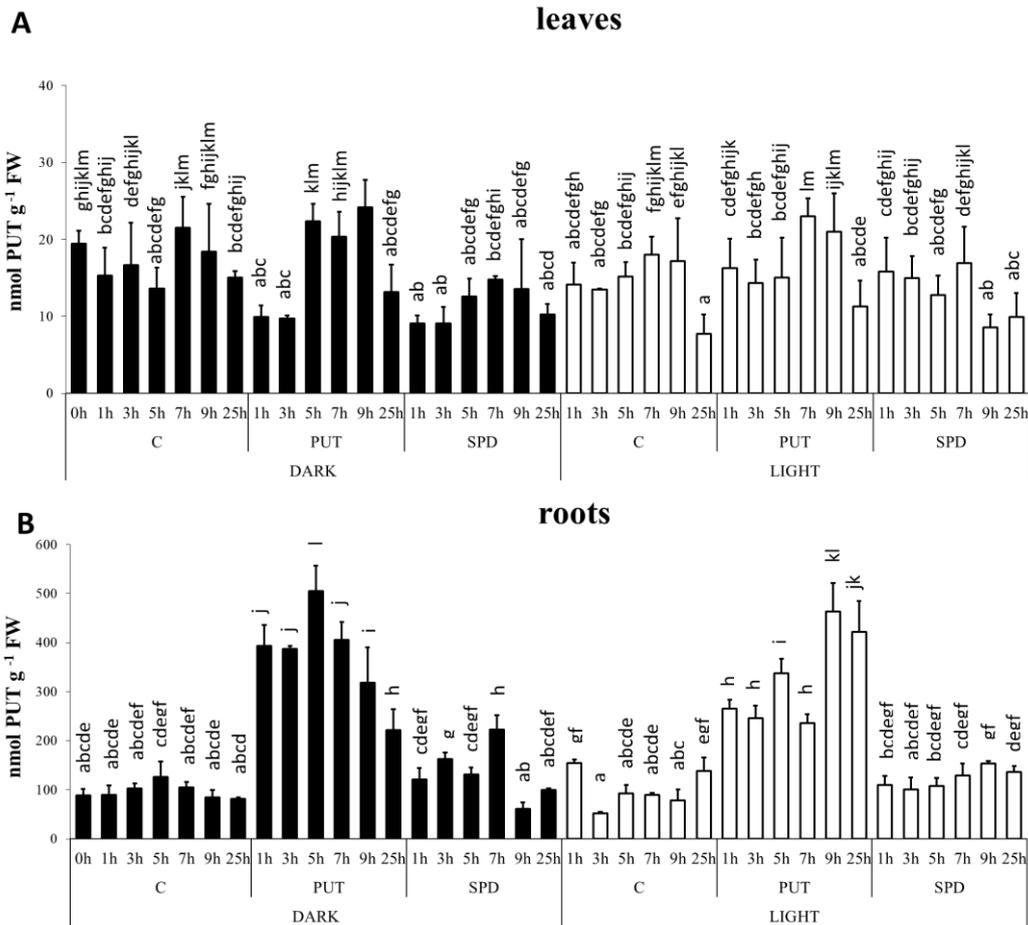
Suppl. Fig. 2. The effect of 0.5 mM putrescine (PUT) treatment compared to the control conditions (C) on endogenous spermidine (SPD) contents in the roots (A, C, E) and leaves (B, D, F) of Mv Bérés (A, B), Mv Emese (C, D) and TC33 (E, F) wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



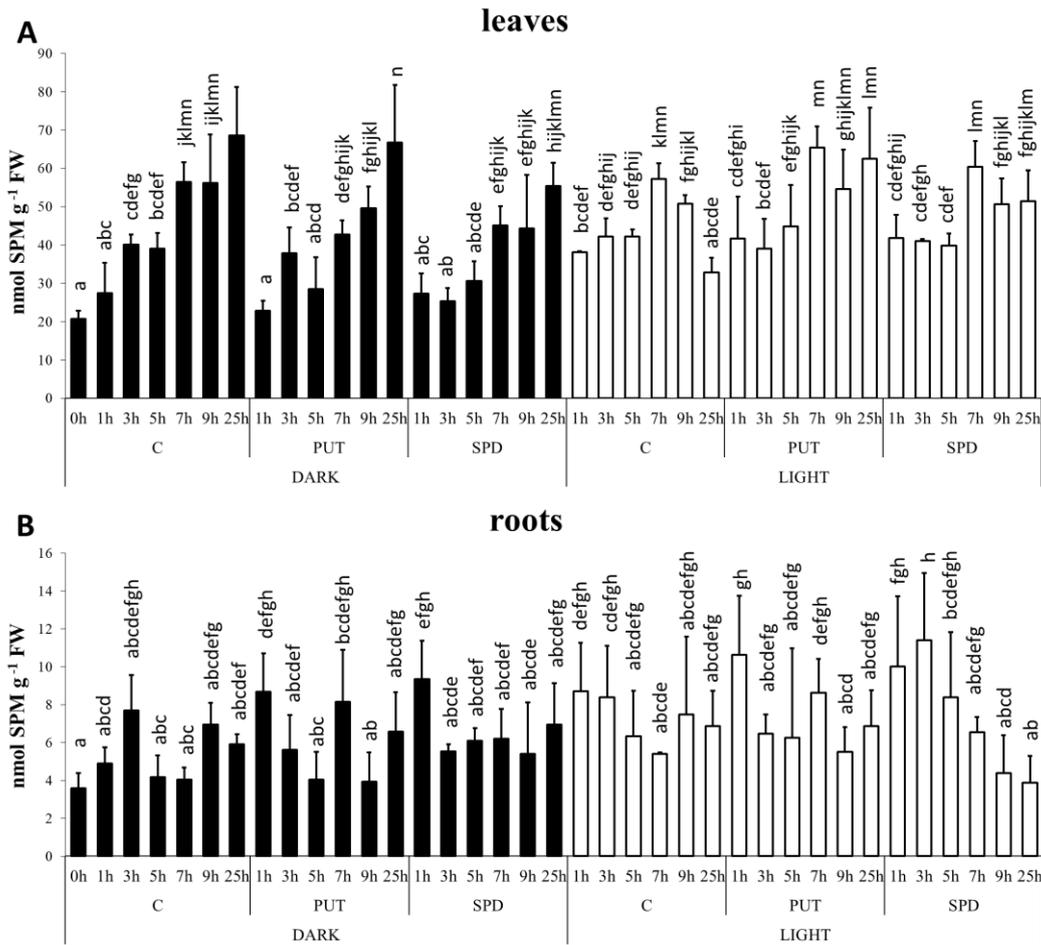
Suppl. Fig. 3. The effect of 0.5 mM putrescine (PUT) treatment compared to the control conditions (C) on endogenous spermine (SPM) contents in the roots (A, C, E) and leaves (B, D, F) of Mv Bérés (A, B), Mv Emese (C, D) and TC33 (E, F) wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



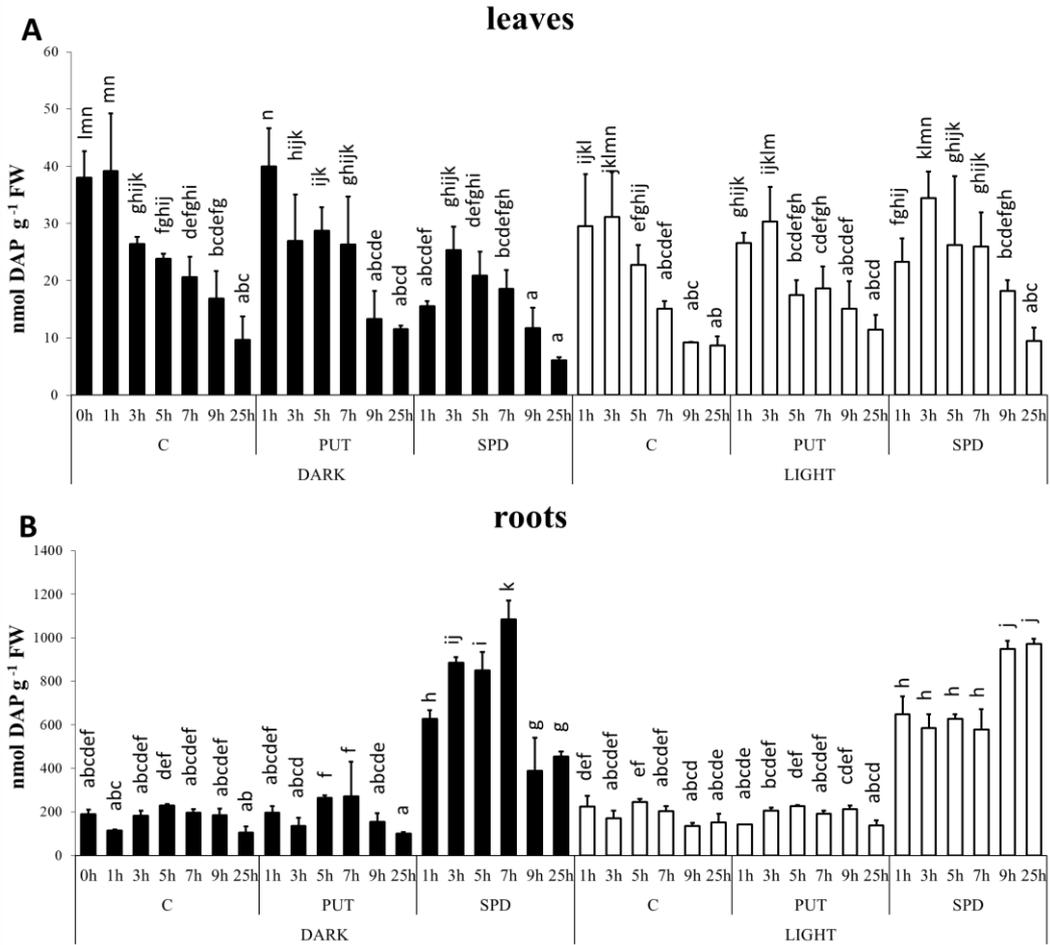
Suppl. Fig. 4. The effect of 0.5 mM putrescine (PUT) treatment compared to the control conditions (C) on endogenous 1,3-diaminopropane (DAP) contents in the roots (A, C, E) and leaves (B, D, F) of Mv Bérés (A, B), Mv Emese (C, D) and TC33 (E, F) wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



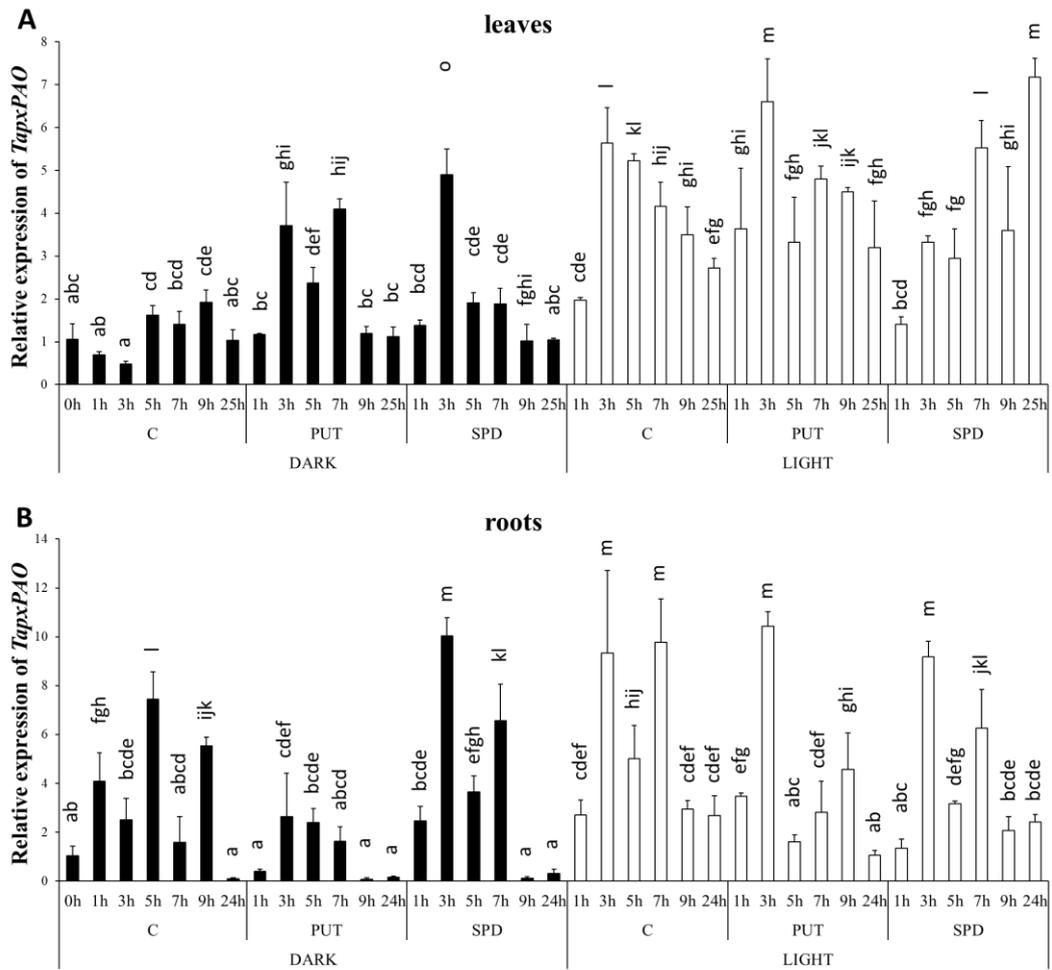
Suppl. Fig. 5. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on putrescine (PUT) contents in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



Suppl. Fig. 6. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on spermine (SPM) contents in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



Suppl. Fig. 7. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on 1,3-diaminopropane (DAP) contents in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD,  $n=3$ . Statistically significant differences are indicated with different letters at  $p < 0.05$  level.



Suppl. Fig. 8. The effect of 0.5 mM putrescine (PUT) or spermidine (SPD) treatments compared to the control (C) under continuous dark or light conditions on the expression level of peroxisomal polyamine oxidase (*TapxPAO*) genes in the leaves (A) and roots (B) of Mv Emese wheat plants at given sampling times. Values are means  $\pm$  SD, n=3. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Magda Pal reports financial support was provided by National Research Development and Innovation Office. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

# **Appendix C**



Article

# Light Spectral Composition Modifies Polyamine Metabolism in Young Wheat Plants

Magda Pál <sup>1,\*</sup> , Kamirán Áron Hamow <sup>1</sup> , Altafur Rahman <sup>1,2</sup> , Imre Majláth <sup>1</sup>, Judit Tajti <sup>1</sup> , Orsolya Kinga Gondor <sup>1</sup> , Mohamed Ahres <sup>1</sup>, Fatemeh Gholizadeh <sup>1,3</sup> , Gabriella Szalai <sup>1</sup> and Tibor Janda <sup>1</sup>

<sup>1</sup> Eötvös Loránd Research Network, Centre for Agricultural Research, 2462 Martonvásár, Hungary; hamow.kamiran@atk.hu (K.Á.H.); altafk013@gmail.com (A.R.); majlath.imre@atk.hu (I.M.); tajtijuci@gmail.com (J.T.); gondor.kinga@atk.hu (O.K.G.); mohamed.ahres@atk.hu (M.A.); fatima.gholizadeh64@gmail.com (F.G.); szalai.gabriella@atk.hu (G.S.); janda.tibor@atk.hu (T.J.)

<sup>2</sup> Doctoral School of Horticultural Sciences, Hungarian University of Agriculture and Life Sciences, 2100 Gödöllő, Hungary

<sup>3</sup> Department of Plant Production and Genetics, Faculty of Agriculture, University of Kurdistan, Sanandaj 66177-15175, Iran

\* Correspondence: pal.magda@atk.hu; Tel.: +36-22-569-502

**Abstract:** Although light-emitting diode (LED) technology has extended the research on targeted photomorphogenic, physiological, and biochemical responses in plants, there is not enough direct information about how light affects polyamine metabolism. In this study, the effect of three spectral compositions (referred to by their most typical characteristic: blue, red, and the combination of blue and red [pink] lights) on polyamine metabolism was compared to those obtained under white light conditions at the same light intensity. Although light quality induced pronounced differences in plant morphology, pigment contents, and the expression of polyamine metabolism-related genes, endogenous polyamine levels did not differ substantially. When exogenous polyamines were applied, their roborative effect were detected under all light conditions, but these beneficial changes were correlated with an increase in polyamine content and polyamine metabolism-related gene expression only under blue light. The effect of the polyamines on leaf gene expression under red light was the opposite, with a decreasing tendency. Results suggest that light quality may optimize plant growth through the adjustment of polyamine metabolism at the gene expression level. Polyamine treatments induced different strategies in fine-tuning of polyamine metabolism, which were induced for optimal plant growth and development under different spectral compositions.

**Keywords:** blue light; light quality; putrescine; red light; spermidine; spermine; wheat



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## 1. Introduction

Light is the major energy source for plants and one of the most important environmental factors influencing plant morphology, physiology, and development [1]. Several studies have already been published on the effects of light intensity not only on plant growth and development but also on metabolite accumulation [2–6]. However, besides light intensity, the spectral composition also influences plant growth, development, and stress tolerance. In addition, the specific wavelengths of light have different effects on plants [7–19]. The synergistic effects of an optimum ratio of red and blue light on the control of plant growth and development have also been demonstrated [20–24]. Besides primary metabolism, light spectra also influence secondary metabolism, including the accumulation of anthocyanins, carotenoids, and flavonols [25–28]. However, other groups of protective compounds are also influenced, as has been reported in the case of ascorbic acid or proline [29–31]. Changes in the spectral composition also modified both the amount and the ratio of free amino acids and the total glutathione pool in wheat plants [32–34]. Most of the results are available on

the effect of changes in the red/far-red light ratio, but blue light-induced responses are less well known.

Polyamines (PAs) are generally considered plant growth regulators, and they are involved in a range of growth and developmental processes from embryogenic development, stem elongation, root growth, and flower induction to fruit ripening, in addition to having a role in several cell processes, such as gene expression, translation, cell proliferation, membrane stabilization, and even programmed cell death [35,36]. There is also several evidence on the close relationship between PA metabolism and photosynthesis. High level of PAs in chloroplasts, especially in light-harvesting and PS II complexes and the high activity of the transglutaminase enzyme, which is responsible for the covalent binding of PAs to pigment proteins, suggests the involvement of PAs in plant growth via photosynthesis [37–40]. It has been also proven that light itself controls PA metabolism, both on the synthesis side [33,41–43] and on the catabolism side [43–45]. Nevertheless, PAs are able to support photosynthesis at several levels: modulating chlorophyll destruction and/or biosynthesis, stabilizing the conformation of photosynthetic complexes, and increasing chemiosmotic ATP synthesis [46]. Earlier, it was accepted that interactions of PAs with macromolecules constituted the main mechanism by which these compounds play their role. Now, the picture is becoming clearer, and it is evident that PAs also interact with phytohormones and other small protective compounds, and thus they have a distinctive role in a complex metabolite and signaling pathway.

Increased nitrate metabolism under blue light conditions has been found to be parallel with higher chlorophyll content and net photosynthetic rate compared to white light conditions [47]. The effects of different light spectra on protein profile and PA content have been found in *Cedrela fissilis*, which in turn affects in vitro shoot development [48]. Similarly, LED lamp treatment with white plus low blue and deep red resulted in greater elongation and higher free PA contents than a fluorescent lamp in *Cariniana legalis* [49]. In addition, when endogenous PA contents were compared between lettuce cotyledon explants cultured under different light qualities, it was found that during shoot primordium production, white or red light conditions resulted in a higher accumulation of free or perchloric acid (PCA)-soluble conjugated PAs and a lower proportion of PCA-insoluble conjugated PAs than under blue light. The ratio of putrescine (PUT) to spermidine (SPD) is lower under white or red light and higher under blue light [50]. Red light also promoted the formation of embryogenic calli in cotton at the induction phase, where the highest total PA contents were detected, while blue light inhibited embryogenic callus formation and resulted in lower PA contents than red light [51].

The relationship between PAs and plant growth and development is evident [52]. In addition, light-related regulation of the PA metabolism in plants was partly reviewed recently [53]. However, there is still a lack of detailed information on how light quality influences PA metabolism. Few studies are available on the comparison of changes in dedicated components of PA metabolism under different spectral conditions. Nevertheless, it is difficult to compare these studies, as different plant species were studied under different light intensities and spectral compositions. The effect of light sources high in blue, blue and red, or far-red irradiance on PA content can be dependent on the light intensity [54]. In addition, in most of the studies only a short period or a pulse of different light spectral conditions were applied on etiolated plants or after dark adaptation [44,55,56], and the results were obtained mainly from *Arabidopsis* plants (as reviewed in [53]).

We have demonstrated that, different light quantities (either the differences in the hours of daily illumination or the light intensities) induced different changes in polyamine metabolism. Briefly, light distinctly induced the PUT level and reduced the 1,3-diamino propane (DAP) content in the leaves, the latter result suggesting the inhibition of the terminal catabolism of higher PAs under higher light conditions. In addition, exogenous PA treatments influenced the PA metabolism differently under altered light intensities [57]. However, despite the above-described information about the relationship between PA metabolism and light, there is still little direct information available about light-influenced

PA metabolism. Until now, there has been no literature about the relationship between light and PA metabolism under not only different spectral conditions but together with the application of exogenous PAs. In the present study, the effects of three spectra were studied using wheat plants and compared to the control—white light at the same light intensity—to answer the following questions: (1) How do the light conditions, referred to by their typical characteristics (blue, red + far-red, and the combination of blue and red + far-red, called pink lights) influence PA metabolism?; (2) How do these light conditions influence the effects of exogenous PAs compared to each other?; and (3) How do PA treatments modify the effect of different spectral conditions at the metabolite and gene expression levels?

## 2. Results

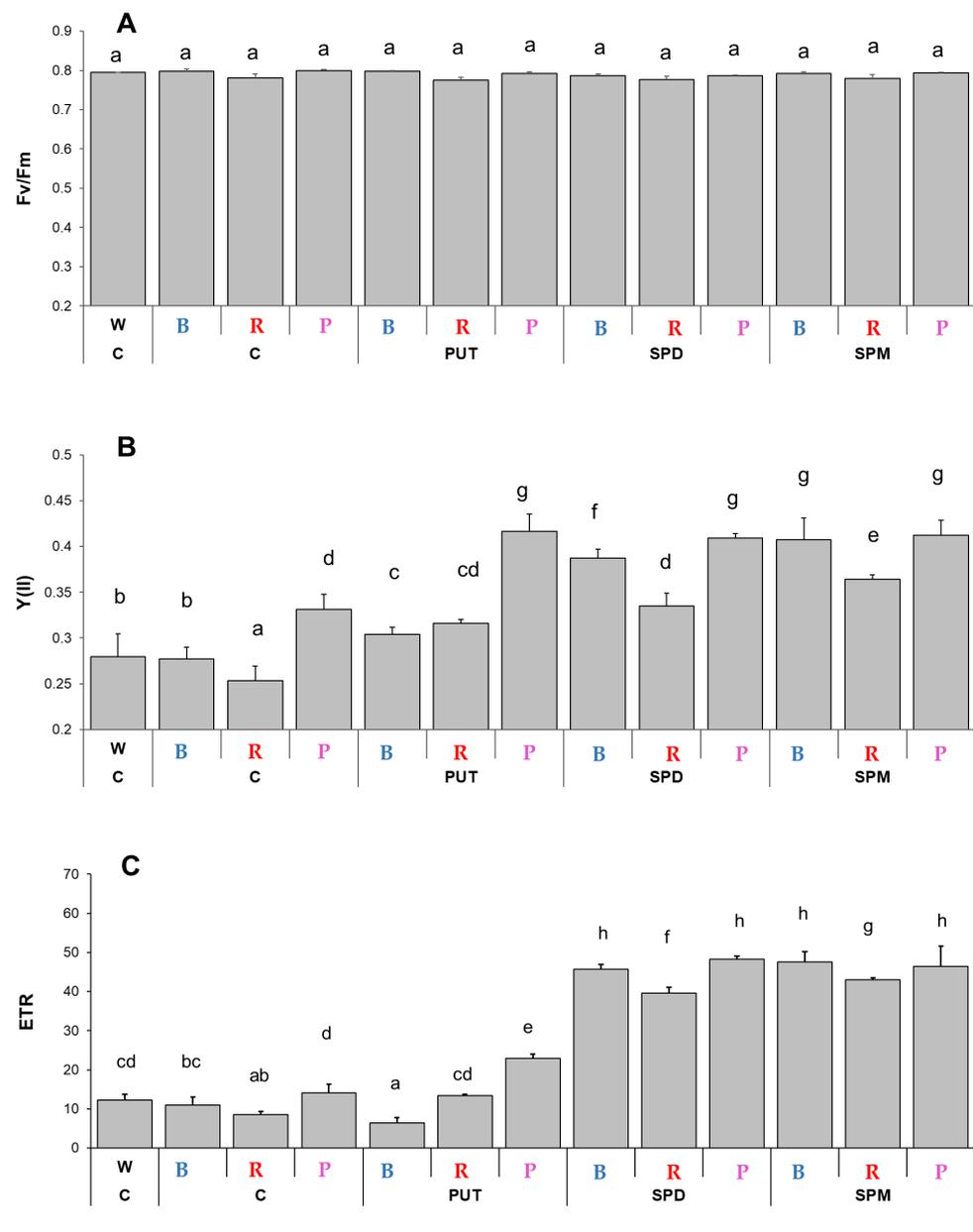
Four light conditions were used in the present experiment. The light source for white light (W) was a continuous wide-spectrum LED. In the case of the blue regime (B), blue light was dominant (82.55%) and its ratio was approximately 5 times greater than the red light. In the case of the red regime (R), the red light was dominant (66.28% red and 6.59% far-red), as its ratio was approximately 5 times greater than that of the blue light, while in the pink (P) regimen, the ratio of blue and red was approximately 1 (see in the Materials and Methods section for the main characteristics of light regimes). Results were compared, with special regard to highlighting the differences between the B-, R-, and P light-induced changes in comparison to the white light-related ones. PA treatments, namely PUT, SPD, and spermine (SPM) were applied at 0.3 mM concentration only under B, R, and P light regimes. In a previous study, the effects of these PA compounds were investigated under white light conditions at different light intensities [57]. In the present study PUT, SPD, and SPM treatments were applied only under B, R, and P regimes.

### 2.1. The Effect of Different Spectral Compositions in Combination with Polyamine Treatments on Physiological Parameters, Proline Content, and Plant Hormone Composition

#### 2.1.1. Changes in Chlorophyll-*a* Fluorescence Induction Parameters and Pigment Contents

Chlorophyll-*a* fluorescence quenching analyses revealed that although the light quality did not influence the maximum quantum yield of PS II (Fv/Fm parameter, Figure 1A), it affected the photosynthetic activity of PS II, as significant differences were detected in the actual quantum yield [Y(II)] and the electron transport rate (ETR) (Figure 1B,C). Blue light illumination resulted in similar Y(II) value, as it was detected under W light, while the lowest value of Y(II) was found under R light and the highest under P light (Figure 1B). The positive effect of 0.3 mM PA treatments was detected under all of the B, R, and P light conditions, and it was the most pronounced in the case of SPM under B and R lights, with 147% and 143% increments, respectively, compared to the controls. Similar changes and differences were found in the case of the ETR parameter (Figure 1C). The lowest ETR value was detected under R conditions. SPM treatment induced the highest increase in the ETR parameter under both B and R light conditions compared to the control ones, as the ETR value reached the highest level at P illumination during control conditions (Figure 1C).

The results on fluorescence induction parameters showed similar changes with the plant pigment contents (Table 1). Light quality differently affected all the presented pigment levels. B light increased all of them except for *trans*-neoxanthin, while R light caused similar or lower concentrations as W itself. In almost all cases, under the P regime, the diminishing effect of red light was partly compensated by the presence of blue light. The roborative effect of exogenous PAs has been also recognized here in the results of plant pigment content, especially in the cases of *trans*-lutein, chlorophyll *a*, and chlorophyll *b* after SPM treatment.



**Figure 1.** Chlorophyll-*a* fluorescence induction parameters. (A): Maximum quantum yield of PSII ( $F_v/F_m$ ), (B): actual quantum yield of PS II [ $Y(II)$ ], and (C): the electron transport rate (ETR) determined at the steady state level of photosynthesis on the third fully expanded leaves of plants grown under different light regimes [(white light (W), blue light (B), red + far-red: R and the combination of blue and red + far-red, called pink (P)] treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD ( $n = 6$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

**Table 1.** Color-map comparison of the pigment contents (*trans*-violaxanthin, *trans*-neoxanthin, *trans*-lutein, chlorophyll a, chlorophyll b, *trans*- $\beta$ -carotene, and 9-*cis*- $\beta$ -carotene) of leaves of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD of 3 measurements per treatment. Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

$\mu\text{g g}^{-1}$ FW		<i>Trans</i> -Violaxanthin	<i>Trans</i> -Neoxanthin	<i>Trans</i> -Lutein	Chlorophyll a	Chlorophyll b	<i>Trans</i> - $\beta$ -Carotene	9- <i>Cis</i> - $\beta$ -Carotene
C	W	57.7 $\pm$ 0.4 a	52.4 $\pm$ 0.5 g	189 $\pm$ 1 d	639.7 $\pm$ 3 bc	280.7 $\pm$ 1.2 a	59.3 $\pm$ 0.7 c	6.4 $\pm$ 0.1 ab
	B	77.8 $\pm$ 1.8	48.5 $\pm$ 1.7 ef	193 $\pm$ 3.6 d	714.3 $\pm$ 7.5 e	309.7 $\pm$ 2.5 fg	72.2 $\pm$ 1.6 e	8.8 $\pm$ 0.1 e
	R	54.9 $\pm$ 0.8 a	33 $\pm$ 0.8 a	157.7 $\pm$ 1.5 a	622 $\pm$ 11.1 ab	288.3 $\pm$ 5.5 ab	51.8 $\pm$ 0.6 a	6.7 $\pm$ 0.1 bc
	P	67.7 $\pm$ 1.4 d	39.3 $\pm$ 0.5 b	178.7 $\pm$ 2.1 c	691 $\pm$ 8.7 d	304 $\pm$ 2.6 ef	64.2 $\pm$ 1.2 d	8.3 $\pm$ 0.1 d
PUT	B	74.5 $\pm$ 1.8 e	43.8 $\pm$ 1.7 cd	190 $\pm$ 4.6 d	746.3 $\pm$ 4 fg	323 $\pm$ 1 h	69.4 $\pm$ 2.8 de	8.8 $\pm$ 0.2 e
	R	57.5 $\pm$ 1.9 a	38.8 $\pm$ 1.3 b	165.7 $\pm$ 5 ab	605 $\pm$ 12.5 a	282 $\pm$ 5.6 a	51.4 $\pm$ 1 a	6.2 $\pm$ 0.1 a
	P	75.3 $\pm$ 0.6 e	45.9 $\pm$ 0.8 de	192 $\pm$ 1.7 d	725 $\pm$ 7 e	316.3 $\pm$ 3 gh	70.4 $\pm$ 1.7 de	8.4 $\pm$ 0.2 d
SPD	B	86.8 $\pm$ 2 f	52.5 $\pm$ 1.4 g	204.7 $\pm$ 4.6 ef	772 $\pm$ 15.9 h	331.7 $\pm$ 6.7 i	76.4 $\pm$ 0.4 f	9.4 $\pm$ 0.1 f
	R	62.7 $\pm$ 3.3 b	41.5 $\pm$ 2.5 bc	176.3 $\pm$ 7 c	651.3 $\pm$ 11 c	294.7 $\pm$ 4.5 bc	56.9 $\pm$ 2.7 bc	6.8 $\pm$ 0.7 c
	P	74.3 $\pm$ 0.6 e	44.9 $\pm$ 1 cd	190.7 $\pm$ 1.5 d	731.3 $\pm$ 4 ef	318 $\pm$ 2 h	70.6 $\pm$ 2.4 de	8.3 $\pm$ 0.1 d
SPM	B	84.2 $\pm$ 2.5 f	51.5 $\pm$ 1.7 fg	209.3 $\pm$ 4.9 f	790.3 $\pm$ 12.9 i	346.7 $\pm$ 4.7 j	75.8 $\pm$ 1.1 f	9.3 $\pm$ 0.2 f
	R	62 $\pm$ 0.9 b	39.9 $\pm$ 0.8 b	171.3 $\pm$ 2.5 bc	656 $\pm$ 11.1 c	298.3 $\pm$ 4.5 cd	54.4 $\pm$ 0.9 ab	6.6 $\pm$ 0.04 bc
	P	74 $\pm$ 6.5 e	44.1 $\pm$ 5.5 cd	204 $\pm$ 12 de	755.7 $\pm$ 20.5 gh	330.7 $\pm$ 7.5 i	68.1 $\pm$ 4 d	8.6 $\pm$ 0.4 de

lowest value  highest value

## 2.1.2. Differences in Biomass Parameters

Wheat plants grown under W light conditions exhibited the highest elongation in both the shoots and roots (Table 2). Blue light treatment inhibited elongation, but due to the resulting compact morphology, the shoot and root weights did not differ pronouncedly compared to the control plants grown under W light. Compared to the W regime, the R light without PA treatment had minor effects on elongation parameters, whereas under P light, the effect of the blue component in the spectral composition was still dominant in the reduction of elongation, which could not be mitigated by increase of the red light ratio. The effect of blue light on shoot and root weights was also dominant over the effect of red under the P regime, as similar biomass values were measured in the case of B light alone. Interestingly, PA treatments in several cases induced significant changes in the biomass parameters, but these differences were slight only. In addition, shoots and roots responded partly differently to the various treatments. In general, biomass values increased in the shoots, especially after SPM treatment, while in the roots higher PAs (SPD and SPM) induced no change or even decrease in these parameters.

**Table 2.** Color-map comparison of biomass parameters of plants treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM) under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P). Values are means  $\pm$  SD ( $n = 30$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

		Shoot Length (cm)	Root Length (cm)	Shoot Weight ( $\text{g}^{-1}$ plant)	Root Weight ( $\text{g}^{-1}$ plant)
C	W	27.04 $\pm$ 1.45 h	18.84 $\pm$ 1.93 e	0.31 $\pm$ 0.02 a	0.15 $\pm$ 0.02 a
	B	19.4 $\pm$ 0.96 a	16.56 $\pm$ 1.44 bc	0.34 $\pm$ 0.02 a	0.23 $\pm$ 0.02 bc
	R	25.8 $\pm$ 2.18 g	17.88 $\pm$ 4 de	0.38 $\pm$ 0.06 abc	0.22 $\pm$ 0.04 abc
	P	21.56 $\pm$ 1.33 c	15.88 $\pm$ 2.11 ab	0.32 $\pm$ 0.02 a	0.21 $\pm$ 0.03 abc
PUT	B	20.56 $\pm$ 1.36 b	17.84 $\pm$ 1.62 dc	0.35 $\pm$ 0.08 ab	0.22 $\pm$ 0.02 abc
	R	25.84 $\pm$ 1.37 g	18.24 $\pm$ 2.22 de	0.45 $\pm$ 0.04 d	0.31 $\pm$ 0.03 c
	P	22.04 $\pm$ 1 cd	16.16 $\pm$ 2.37 ab	0.35 $\pm$ 0.04 a	0.28 $\pm$ 0.04 cd

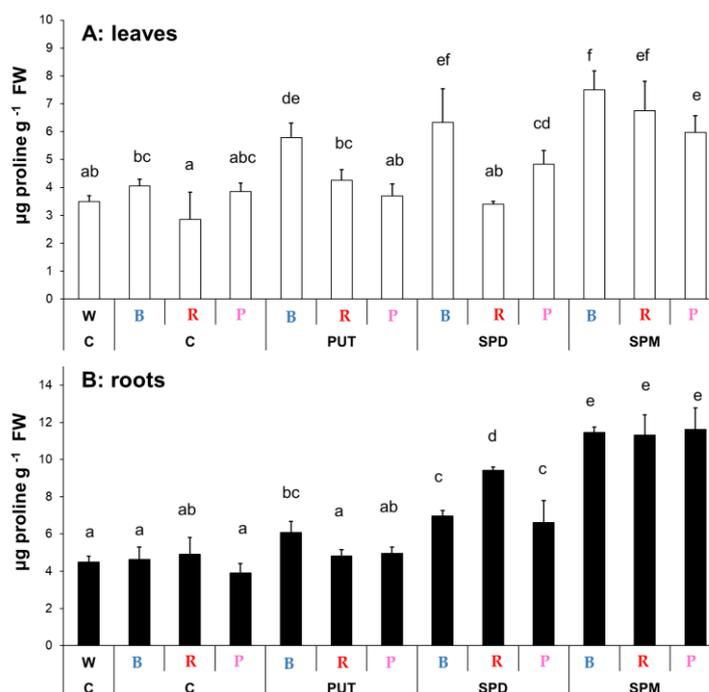
Table 2. Cont.

		Shoot Length (cm)	Root Length (cm)	Shoot Weight ( $\text{g}^{-1}$ plant)	Root Weight ( $\text{g}^{-1}$ plant)
SPD	B	20.52 $\pm$ 1.85 b	14.96 $\pm$ 3.12 a	0.33 $\pm$ 0.06 a	0.19 $\pm$ 0.04 ab
	R	24.52 $\pm$ 1.69 f	15.76 $\pm$ 1.83 ab	0.44 $\pm$ 0.03 cd	0.28 $\pm$ 0.03 cd
	P	22.32 $\pm$ 1.63 d	17.6 $\pm$ 2.5 cd	0.37 $\pm$ 0.0 abc	0.23 $\pm$ 0.04 abc
SPM	B	21.43 $\pm$ 1.14 c	15.53 $\pm$ 2.26 ab	0.38 $\pm$ 0.03 abc	0.2 $\pm$ 0.02 ab
	R	25.27 $\pm$ 1.68 g	14.93 $\pm$ 2.36 a	0.47 $\pm$ 0.04 d	0.22 $\pm$ 0.01 abc
	P	23.13 $\pm$ 1 e	15.47 $\pm$ 1.5 ab	0.379 $\pm$ 0.02 abc	0.16 $\pm$ 0.01 ab

lowest value  highest value

### 2.1.3. Changes in Proline Content

Light quality (B, R, or P) had no significant effect on the proline content in the leaves or roots compared to the W light condition (Figure 2). However, the proline content of the leaves was lower in plants grown under R light condition, in contrast to those, which were treated with blue light. Under blue light, all the PA treatments increased the proline level in the leaves, but under R or P regimens, significant increases were only detected after SPM application. In the roots, under B light, PUT, SPD and SPM increased the proline level, while under R and P lights, only SPD and SPM treatments could increase it (Figure 2A,B).



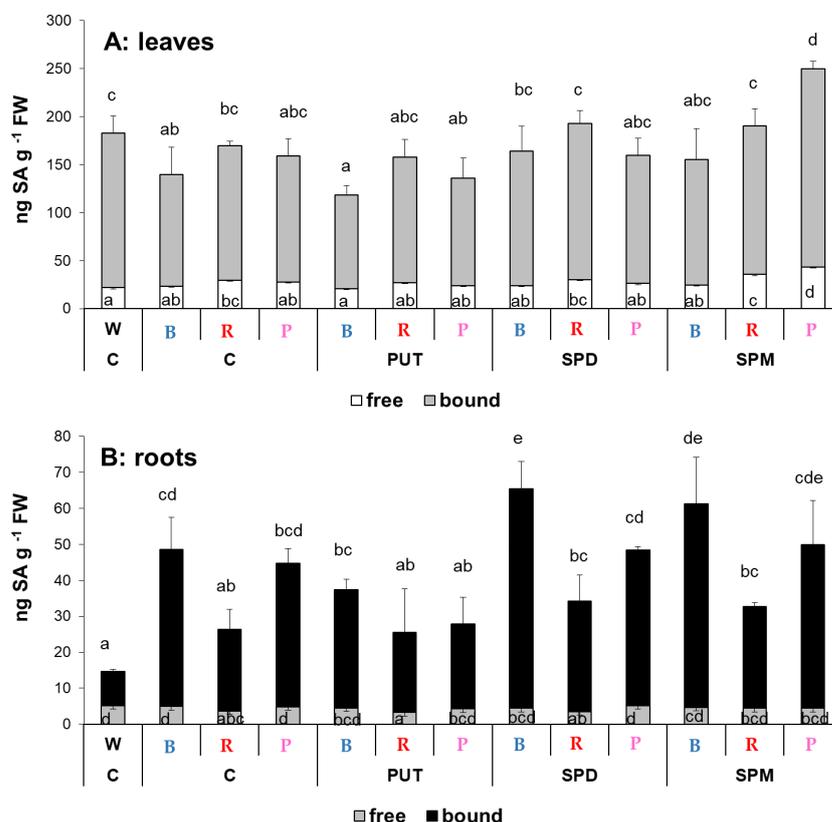
**Figure 2.** Changes in proline contents in the leaves (A) and roots (B) of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

### 2.1.4. Changes in Salicylic Acid and Abscisic Acid Contents

Previous studies demonstrated, that PA treatments influence the synthesis of certain plant hormones. In addition, light may have also influence on their levels. In order to further characterize the physiological status of wheat plants, the levels of two plant hormones, salicylic acid (SA) and abscisic acid (ABA), were determined.

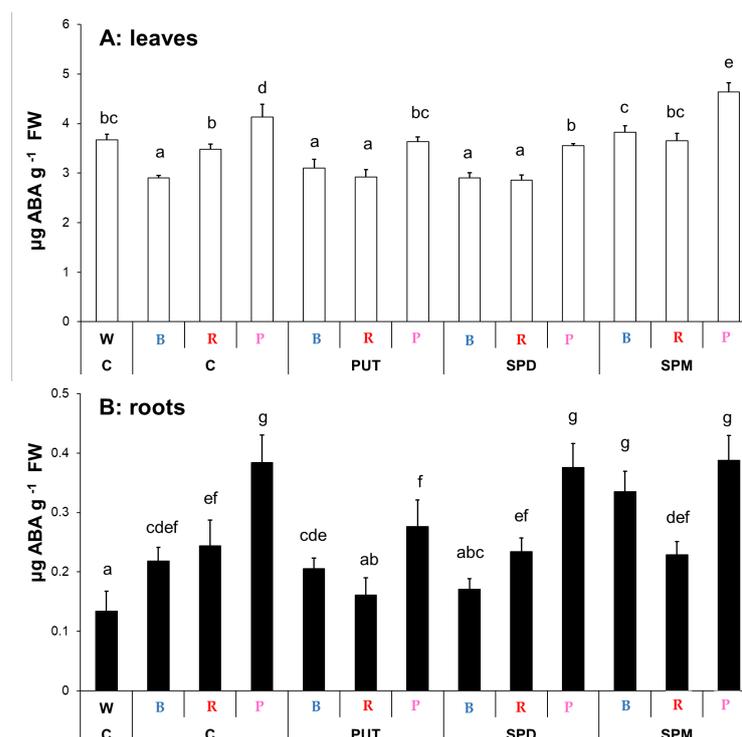
Although the light composition has no pronounced effect on the free or bound SA content in the leaves, interestingly in the roots significant differences were detected in

the bound fraction, resulting in increased total SA content either under B, R, or P light compared to the W light condition (Figure 3A). Statistically significant effects of exogenous PAs on total leaf SA level could not be recognized, except for when the SPM-treated plants were compared to the control under the P regime. The root SA amount increased after SPD and SPM treatments under the B regime, and no significant changes were detected under R or P light conditions (Figure 3B).



**Figure 3.** Salicylic acid (SA) contents in the leaves (A) and roots (B) of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

The effect of light composition affected the leaf ABA content. The lowest level was found with the B light treatment, and the highest level was detected under the P regime. The W and R regimes had similar ABA levels (Figure 4A). PA treatments also modified the leaf ABA content depending on the light conditions, indicating an interaction between light and PA signaling. Under B light, only SPM could increase its level, under R light PUT and SPD treatments decreased it, while under the P regime PUT and SPD also decreased, but SPM increased it (Figure 4A). In the roots, ABA content was also influenced by the light conditions compared to the W light. All of the special light regimes increased it, with the highest accumulation under P light (Figure 4B). Interestingly, only SPM treatment increased its level under B light, while under R and P conditions a decrease in it was detected after PUT treatment. Changes in the levels of ABA showed similar patterns under R and P regimes after PA treatments in both the leaves and roots, respectively (Figure 4).



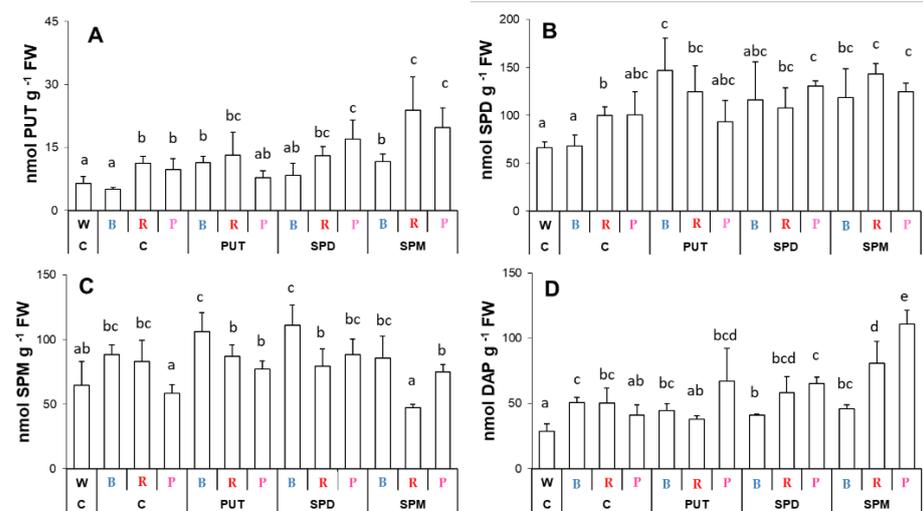
**Figure 4.** Abscisic acid (ABA) contents in the leaves (A) and roots (B) of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

## 2.2. Effects of Different Spectral Conditions in Combination with Polyamine Treatments on Polyamine Metabolism

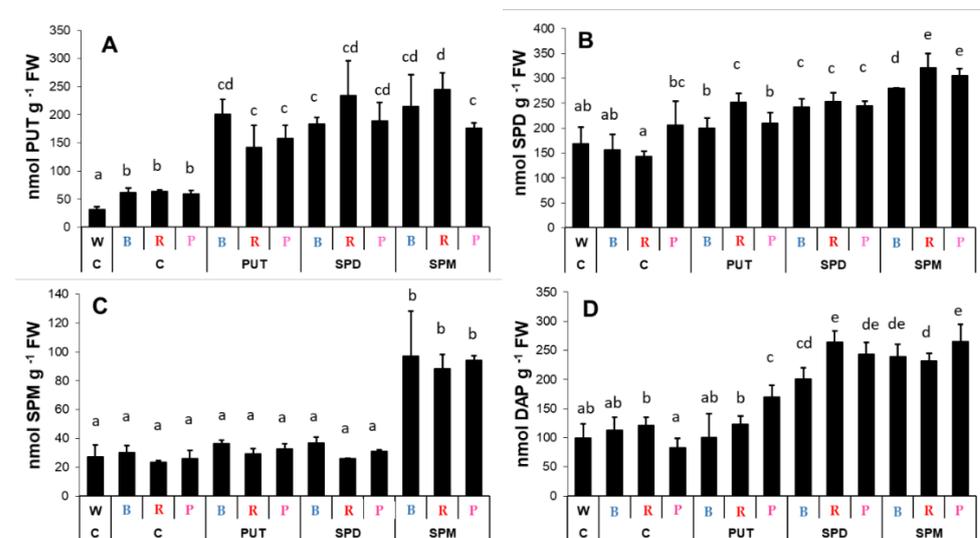
### 2.2.1. Changes in Polyamine Contents

Compared to the white light conditions, B light treatment did not influence the PUT, SPD, or SPM contents, but increased DAP level in the leaves (Figure 5A–D). Though besides DAP content, R light also significantly increased the amount of PUT and SPD, the P light condition did not modify significantly the PA levels. The exogenous PAs induced changes in the endogenous PA levels with a different efficiency under B, R, or P light treatment. The PUT level slightly increased after all of the applied PA treatments under B light, but only after SPM treatment under R light condition compared to their controls. P light condition combined with SPD or SPM treatment also increased PUT level (Figure 5A). Pronounced increment of SPD amount was also found after PA treatments under B condition, while only slight increases were observed under R or P conditions (Figure 5B). Leaf SPM content did not change significantly after PA treatments under B light, but increased after PA treatment under P and decreased by SPM treatment under R conditions (Figure 5C). Interestingly, the amount of DAP showed an increasing tendency after SPM treatment only under R and P conditions (Figure 5D), suggesting that the catabolism induced by the excess of higher PAs in the leaves is a result of the presence of a higher proportion of red light.

Light treatment itself did not influence the endogenous PA levels in the roots, while exogenous PAs had pronounced effects on them (Figure 6). Characteristic changes were induced in the PUT, SPD, SPM, and DAP contents after PA applications (Figure 6A–D), especially after SPM treatment. However, these tendencies were very similar to each other under all light conditions.



**Figure 5.** Polyamine contents ((A): putrescine: PUT; (B): spermidine: SPD; (C): spermine: SPM; (D): 1,3-diaminopropane: DAP) in the leaves of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means ± SD (*n* = 3). Different letters indicate statistically significant differences at *p* < 0.05 level, using Duncan’s post hoc test.

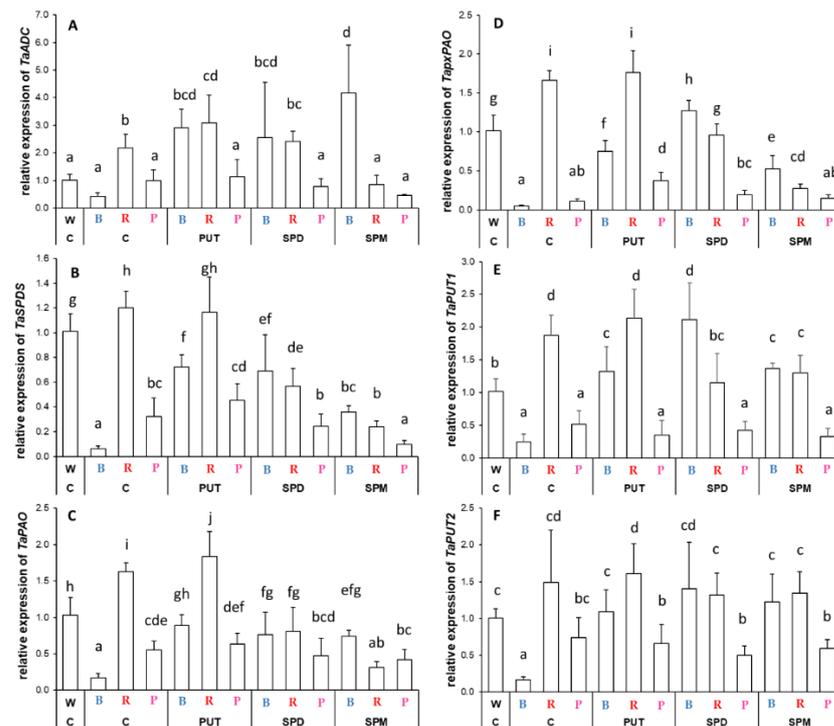


**Figure 6.** Polyamine contents ((A): putrescine: PUT; (B): spermidine: SPD; (C): spermine: SPM; (D): 1,3-diaminopropane: DAP) in the roots of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means ± SD (*n* = 3). Different letters indicate statistically significant differences at *p* < 0.05 level, using Duncan’s post hoc test.

### 2.2.2. Differences in the Expression Levels of Genes Involved

Different light compositions caused statistically significant and characteristic patterns in the expression pattern of PA metabolism-related genes in the leaves (Figure 7). Without PA treatments, only R light could influence arginine decarboxylase (*ADC*) expression, increasing it (Figure 7A). Under R condition, the PUT content slightly increased *ADC* expression, too. Under B light, all the PA treatments induced its transcription to a similar level as was found in the control plants under R conditions, whereas under R conditions, the SPM treatment decreased its transcription. Interestingly, the PA treatments had no

effect under P light (Figure 7A). B light or P light decreased spermidine synthase (*SPDS*) expression—with higher decrease in the case of the B regime—compared to that observed in plants grown under W light conditions, while R slightly increased it (Figure 7B). According to these, the influence of PA treatments on *SPDS* expression was also different under the three light regimes. Under B light, all the PAs increased *SPDS* expression, but this activation was not enough to reach the level of expression found under W light conditions. Under R light, SPD and SPM treatments decreased it, while under P light, SPM application also decreased its expression with a similar pattern (Figure 7B).



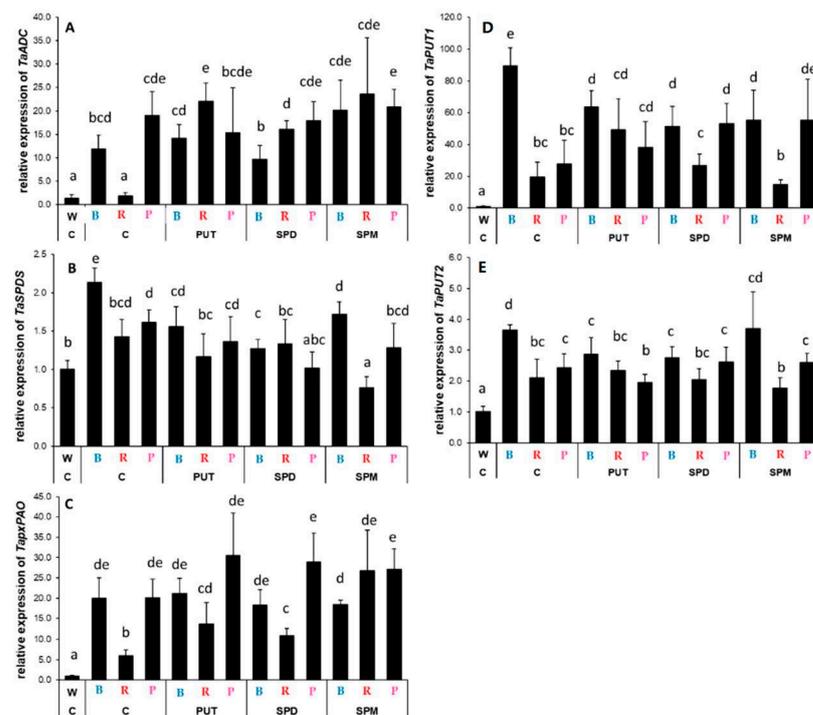
**Figure 7.** Gene expression patterns of arginine decarboxylase ((A): *ADC*), spermidine synthase ((B): *SPDS*), peroxisomal polyamine oxidase ((C): *pxPAO*), polyamine oxidase ((D): *PAO*) and polyamine uptake transporter genes ((E): *PUT1* and (F): *PUT2*) in the leaves of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD. All reactions for gene expression analyses were performed in triplicate using 3 biological and 3 technical repetitions. Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

The transcript level of *PAO*, responsible for the terminal catabolism of SPD and SPM, also showed basal light dependence in the leaves (Figure 7C). As B light decreased, R light increased it, while P light also decreased it compared to those determined in the plants grown under W light. Its expression level increased by all PA treatments under the B regime, resulting in similar values as in plants grown under W light, but it was decreased by SPD and SPM treatments under R regime. Neither of the PA treatments could change the expression level significantly under P light conditions.

A partly similar expression pattern was observed for peroxisomal *PAO* (*pxPAO*) as was described for *ADC*, *SPDS*, and *PAO* (Figure 7D). B light alone decreased the transcription of *pxPAO*, but after PA treatments it was activated, and almost resulted in similar expression to that found in plants under W light. Under the R regime, the initially higher expression was inhibited by SPD and SPM application, while P light alone decreased it, and only PUT treatment could increase its expression (Figure 7D).

The effect of B light was also characteristic on the polyamine uptake transporter genes (*PUT1* and *PUT2*) (Figure 7E,F). Under B and P light conditions, the transcript level of *PUT1* and *PUT2* was lower than under W or R conditions, but PA treatments could only induce their expression under B light treatment.

Interestingly, a completely opposite effect of blue light was observed on PA metabolism-related gene expression in the roots compared to that found in the leaves. Without any PA treatment among light treatments, the B light induced the expression of *ADC*, *SPDS*, *pxPAO*, *PUT1*, and *PUT2* genes compared to that detected under W or R conditions, while the effects of the P light treatment were rather similar in cases of *ADC* and *pxPAO* to that of B light treatment, but in cases of *SPDS*, *PUT1*, and *PUT2* the transcript levels were similar to those measured under R light conditions (Figure 8A–E). Even though the effect of blue light on gene expression levels could be detected in the leaves under P light treatment, it was only true for *ADC* and *pxPAO* in the roots. Nevertheless, the PA treatments had different effects depending on the investigated gene and the applied spectral composition. Exogenous PA application could induce the *ADC* gene expression under R conditions (Figure 5A) and the transcription of *SPDS* slightly, but it was tendentially decreased by PAs under all the light treatments (Figure 8B). The level of *pxPAO* gene expression increased with the PA treatments under R and P light conditions, but it was more pronounced with R treatment (Figure 8B). The expression of *PAO* was not detectable in the roots.



**Figure 8.** Gene expression patterns of arginine decarboxylase ((A): *ADC*), spermidine synthase ((B): *SPDS*), peroxisomal polyamine oxidase ((C): *pxPAO*) and polyamine uptake transporter genes ((D): *PUT1* and (E): *PUT2*) in the leaves of plants grown under different light regimes (white light: W, blue light: B, red + far-red: R and the combination of blue and red + far-red, pink: P) treated with or without polyamines (control: C, putrescine: PUT, spermidine: SPD and spermine: SPM). Values are means  $\pm$  SD. All reactions for gene expression analyses were performed in triplicate using 3 biological and 3 technical repetitions. Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

The transcript levels of *PUT1* were greatly increased by blue light, but it was decreased after PA treatments (Figure 8D). PUT application under R treatment, while all the PA treatments under P condition increased it. Similarly, *PUT2* expression was induced especially

by blue light compared to the W light conditions, while PA treatments had no pronounced influence on it under either light condition (Figure 8E).

### 3. Discussion

Besides the light intensity and duration of illumination, i.e., light quantity, specific light qualities also have deep effects on plants. Light spectral composition can influence plant morphology, physiology, development, and stress responses by impacting on processes ranging from photosynthesis to secondary metabolism [58]. As blue and red light are the most effectively utilized wavelengths during photosynthesis, the understanding of their regulatory role in other plant processes is an important aspect.

Generally, it can be said that red light plays an important role in controlling chloroplast functions, stem and petiole growth, and reproductive development, while blue light influences plant growth, leaf expansion, photomorphogenesis, stomatal opening, photosynthesis, and pigment accumulation [22]. Far-red light accelerating plant flowering can also modulate plant height and leaf size, thus regulating plant morphology and photosynthetic capacity, enabling plants to capture more light and in turn indirectly increase growth [59]. Although several studies have focused on evaluating the effects of light composition, some specific processes affected by spectral changes remain largely unknown.

Despite the numerous studies on the role of PAs in plant growth and development and on the protective and beneficial effects of various PA treatments (seed soaking, hydroponically or spraying) in stress responses and tolerance [60–65], there is still little direct and detailed information available about how light influences PA metabolism and consequently its effects. Most of the studies on the investigation of the effect of light quantity were performed on *Arabidopsis* mutants, under different light intensities or with only one spectral composition compared to white light conditions for a short period or after dark adaptation. In addition, even fewer studies are available focusing directly on PA metabolism under different spectral conditions, as above [reviewed in 53]. In the present study, the effect of three different spectral compositions were studied in comparison with white light conditions. Besides some photosynthesis related and biomass parameters, the contents of proline and plant hormones (SA and ABA)—which usually show a close relationship with PA levels—were also determined, but the present study focused mainly on PA metabolism at metabolite and gene expression levels. In our previous study, the effects of PA treatment under white light conditions was investigated under different light intensities, where it was demonstrated that in the leaves, light distinctly induced the PUT level and reduced the DAP content, which suggests that higher light conditions inhibit the terminal catabolism of higher PAs. In addition, exogenous PA treatments influenced the PA metabolism differently under different light intensities [57]. In the present study the effects of PUT, SPD, and SPM treatments were only investigated under B, R, and P regimes.

#### 3.1. Light Quality Modifies Certain Photosynthesis- and Biomass-Related Parameters and Plant Hormone Levels

Under the present conditions, B light had no significant effect on the investigated fluorescence induction parameters, Y(II) and ETR, indicating the presence of a control mechanism that regulates the electron transport process. However, shorter and more compact plant morphology was observed (as indicated by the biomass parameters), as well as higher pigment concentrations, particularly chlorophyll a and b, *trans*-violaxanthin, and  $\beta$ -carotene contents. In contrast, the dominant ratio of red decreased the PSII actual quantum yield and the electron transport rate, although the pigment content pattern was similar, and the biomass parameters were also almost the same as found in the plants grown under white light conditions. At the same time, P light treatment increased the photosynthesis-related Y(II) and ETR parameters, the chlorophyll a and b contents, and the  $\beta$ -carotene levels, although decreased shoot and root length values were detected compared to plants grown under white light conditions. These findings are in accordance with the results of other studies on barley [66], sweet pepper [22], and wheat [32]. However,

responses of different plant species can be partly different. Various combinations of red and blue light have been investigated [24].

The spectral composition did not influence pronouncedly the proline content in the leaves and roots or the SA levels in the leaves compared to the W light conditions. However, the root SA content was increased under B and P light treatments compared to the W or R conditions. In addition, although the ABA concentration slightly decreased in the leaves under B light, it increased remarkably under P light treatment in both the leaves and roots. In *Drosera peltata* and *Dionaea muscipula* in vitro cultures, blue–red LED light (spectral composition ratio 6:1) treatment resulted in high accumulation of SA in both plant species, but did not influence the proline contents compared to the fluorescent light conditions [67]. However, in wheat flag leaves blue light induced and far-red light decreased the proline content [32], but an opposite tendency was observed in the leaves of 10-day-old wheat plants [33]. In watermelon, comparison of LEDs emitting narrow-band red, narrow-band blue, or combination of red and blue light (88:12) revealed that the combined treatment induced the highest ABA content, but the red light condition resulted in the highest SA accumulation in both the scion and the rootstock [68]. Different combinations of blue and red light induced the growth of *Salvia miltiorrhiza*, which was related to enhanced accumulation of phenolic acids and activation of *PAL1* gene expression, the gene encoding phenylalanine ammonia-lyase enzyme, which also has a key role in SA synthesis [69]. Similarly to our results, the increasing ratio of blue light also decreased the ABA content in rose leaves under light conditions [70], while far-red supplementation increased ABA levels in barley [71]. However, due to the widespread use of LED light sources, knowledge of the effects of spectral composition on phytohormone metabolism is an emerging field.

### 3.2. Light Quality May Influence the Expression of Genes Related to Polyamine Metabolism without Affecting the Polyamine Levels

Despite the pronounced differences observed visually and detected in the biomass parameters, plant pigment contents, and fluorescence induction parameters, B, R, or P light treatments caused only slight changes in the endogenous PA contents in the leaves and did not influence them in the roots. The lowest leaf PUT level was detected under B light treatment and was higher under R or P treatments than under W light. The content of SPD was similar under B and W treatments, while it was higher under R and P conditions. The SPM levels were higher under B and R conditions than under W or P light conditions, while the DAP amount increased especially during B and P treatments. These results suggest that due to dynamic PA metabolism, the balance of the synthesis, catabolism, or back-conversion resulted in such similar PA levels under light conditions that are optimal for the adequate plant growth conditions.

Investigation on the effect of different light sources on in vitro shoot development in *Cariniana legalis* revealed that the combination of white, low-blue, and deep-red without far-red spectra resulted in higher levels of PUT, SPD, and total free PAs compared to the fluorescent light conditions [50]. In *Cedrela fissilis* Vell., LED light with white plus medium blue and deep red spectral composition induced only a slight increase in PUT content, but did not influence the SPD or SPM content compared to the fluorescent light treatment [49]. Also in wheat plants, the level of ornithine, one of the precursor compounds of PUT synthesis, was hardly influenced by the spectral composition, while the amount of arginine, another precursor of PUT, was highest under far-red light conditions [33]. However, the level of arginine was not in accordance with the gene expression level of *ADC*, as it was decreased by both blue and far-red light treatment compared to that measured under white light conditions [33]. In *Arabidopsis*, the comparative gene expression analysis of RNA isolated from the plants grown under LED light supplemented with blue, red, or the combination of blue and red revealed that PA synthesis was not affected [72]. In contrast, under the present conditions, the pattern of the expression level of the genes involved in the PA metabolism showed pronounced differences depending on the spectral composition. In addition, the leaves and roots responded differently. In the leaves, the

most characteristic effect was observed for the B treatment, as the transcript level of all the investigated genes was the lowest there compared to the other light conditions. Growth under R light resulted in similar gene expression levels as found under white light. Only slight increases were detected, especially in the cases of *ADC*, *PAO*, and *PUT1*. Under P light conditions, the additive effect of blue and red light treatments could be observed, as similar or a slightly lower levels of gene expression were detected compared to those found under W light treatment, so the decreasing effect of blue light on gene expression was partly alleviated by the higher ratio of red in the composition. Interestingly, in the roots B, R, or P light treatments induced the expression of almost all the investigated genes compared to the white light condition. However, here the effect of B light was positive, and the R light induced only similar or slightly higher expression levels as white light. Under the combined P light conditions, the dominant, gene expression-inducing effect of the blue light component was observed for *ADC* and *pxPAO*.

According to these results, it seems that plants try to maintain the same optimal concentration and ratio of PA under different spectral compositions. In addition, the effects of light quantity or time of illumination or the lack of light may be more dominant on the actual PA pool than the light quality [32,33,43,54,57]. However, it should also be taken into consideration that red and far-red light supplementation can result in similar changes as growth under white light conditions, especially under low light intensity [33,50].

### 3.3. PA Treatments Have Roborative Effects under Different Spectral Compositions and Their Effects on PA Metabolism Depend on the Light Quality

According to the present results, the effects of different combination of light spectra on endogenous PA metabolism are still not clear. In addition, the level of PA can alter during development or due to environmental factors leading to different morphological responses [73]. Thus, a well-maintained dynamic balance of PAs is necessary for plant development and stress tolerance. The positive effect of exogenous PAs has been reported in different plant species under control or various stress conditions, and the roborative or protective effect was always related to fine-tuning of PA metabolism [60,61,63,65]. Under the present conditions, the PA treatments increased the electron transport rate around PSII, the photosynthesis-related pigment contents, and in some cases the biomass parameters compared to the control under the same light conditions. Increased chlorophyll-a fluorescence induction parameters and chlorophyll contents showed similar patterns as PUT content in the leaves. Several studies with different plant species provided evidence of the positive effect of PUT on photosynthetic pigments, photosynthetic CO<sub>2</sub> assimilation, or ATP production [35]. Similarly, the positive effect of PAs has also been demonstrated under different light quantity conditions, where also in wheat, PUT, SPD, or SPM treatment under low or medium light resulted in similar actual quantum yield as was detected under the elevated light condition, where the leaf PUT content also correlated with the Y(II) parameter [57]. The exogenous PA treatments, especially higher PAs (SPD and SPM) also increased the proline content, and especially in the roots, the changes were in relation to the increased total PA level. As biosynthesis of PAs and proline use glutamate as a common precursor, considerable changes in the pool of PAs could cause a shift between the synthesis pathways from PA to proline. Besides acting as an ROS scavenger or osmolite under stress conditions, proline is also a source of nitrogen and carbon, and thus may increase plant growth and development. Positive effects of exogenous PA treatments on biomass parameters have been also reported to be parallel with changes in proline content in the dwarf wheat genotype [63]. Close correlations have been found between SA and PA contents [63], and PA treatments, especially higher PAs, induced the accumulation of SA in wheat and maize leaves and roots [61]. Under the present conditions, a significant increase in leaf SA content was observed under P light in SPM-treated plants, while the root SA level showed an increasing tendency under B and P light after SPD and SPM application. Similarly, a greater effect on roots SA production has been described after PA application in the same wheat genotypes under white light conditions [57]. A positive feedback loop between ABA

and PAs has been suggested by several findings [57,64]. Under the present conditions, the ABA level increased under B or P light conditions after SPM treatment in the leaves, and in the roots under B light also after SPM treatment. Under white light, all the PA treatments induced ABA accumulation in the leaves and roots of wheat plants [58]. As ABA and SA synthesis is also regulated by light conditions [74,75], the different spectral compositions may influence the modulating effect of PA treatments on plant hormones differently.

Although the initial leaf PA levels were only slightly different under W, B, R, and P light conditions, the excess of PAs had pronouncedly different effects on PA metabolism under the three spectral compositions. In the leaves, the SPM treatment increased the PUT level under R and P light treatments. However in these treatments, *ADC* expression showed a decreasing tendency as a feedback mechanism. Interestingly, the transcript level of *ADC* under B light increased after all the PA treatments, which was in accordance with the slight increase in PUT levels. Similarly, under B light, excess PA increased leaf SPD levels, and parallel with this, *SPDS* expression also increased. Under R and P light treatment, SPM application increased SPD content, but decreased the *SPDS* transcript level. The SPM level could hardly be influenced, except for SPM-treated plants growth under R conditions, where it decreased. The DAP content also increased after SPM treatment under R and P light conditions. The *PAO* gene, which encodes the apoplasmic PAO enzyme, responsible for the terminal catabolism of SPD and SPM, was induced after PA treatments under B light, resulting in similar DAP level as in the control plants, while it was inhibited after SPM treatment under R light where high DAP concentration was found, though no changes in *PAO* expression were observed under P light, maybe as an additive effect of the combination of blue and red light. A similar expression pattern was found for the gene encoding the peroxisomal PAO, responsible for the back-conversion of higher PAs to PUT or SPD. These results suggest that the applied PAs are taken up by the plants, metabolized by terminal catabolism, and back-converted, in addition to being translocated into the leaves. However, as under B light the basal gene expression levels were very low, PA treatments could cause drastic changes in them, both on the synthesis and the catabolism sides. In addition, the same was true for the PA uptake transporter genes (*PUT1* and *PUT2*).

In the roots, due to the hydroponic application, more pronounced changes were detected in the endogenous PA content, especially after SPM treatment. The exogenous SPD and SPM not only elevated their own levels, but due to the back-conversion, the levels of PUT and SPD also increased, leading to terminal catabolism and increased DAP content. However, in contrast to the leaves, the lowest expression levels for most of the investigated genes were found under R treatment. PA treatments induced high expression of *ADC* and *pxPAO* genes only under R light. The transcript level of *SPDS* showed only a slight decreasing tendency after PA treatments under any spectral condition. Expression of *PUT1* decreased under B light after all the PA treatments, increased in PUT-treated and decreased in SPD or SPM treated ones under R light, and increased by the applied PAs under P light conditions.

## 4. Materials and Methods

### 4.1. Plant Materials, Growth Conditions, and Treatments

The wheat seeds (winter wheat (*Triticum aestivum* L.) variety “Mv Béres”) were germinated for 3 days at 26 °C, and thereafter were grown in modified Hoagland solution [76], 15 seedlings per plastic container, and changed every 2 days. Containers were placed in a Conviron PGR-15 plant-growth chamber (Controlled Environments Ltd., Winnipeg, Canada) in a randomized manner, using the following settings: 22/20 °C day/night temperature with 16/8-h light/dark periodicity, with relative humidity of 75%, as described in [76]. Plants from six containers were used for the detailed analyses for every treatment.

Plants were grown under different spectral conditions at the same light intensity ( $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Four light regimes were established using modules equipped with a continuous wide-spectrum LED (Philips Lumileds, LXZ2-5790-y) and three narrow bands of LEDs with dominant wavelengths of 448 nm (Philips Lumileds, LXZ1-PR01), 655 nm

(Philips Lumileds, LXZ1-PA01), and 750 nm (Edison Edixeon, 2ER101FX00000001). All LED modules were equipped with these LEDs, and each type of LED was independently controlled within the module. The spectral composition used in the experiments—composed of different combinations of LEDs—is described in [32]. The four light conditions are referred to according to their typical characteristic. The light source for white light (W) was a continuous wide-spectrum LED (Philips Lumileds, LXZ25790-y). In the blue regimen (B), the blue light intensity was 5 times that of the red light. In the red + far-red regimen (R), the red light was dominant with elevated far-red component compared with the other light conditions (with 66.28% red and 6.59% far-red light components), and its intensity is approximately 5 times that of the blue one. In the pink (P) regimen, the ratio of blue and red was approximately 1 [32]. The most important characteristics of the light conditions are summarized in Table 3.

**Table 3.** Characteristics of light regimens.

Treatments	Intensity PAR ( $\mu\text{mol}$ )	Blue $\mu\text{W}/\text{cm}^2$ (400–500 nm)	Green $\mu\text{W}/\text{cm}^2$ (500–600 nm)	Red $\mu\text{W}/\text{cm}^2$ (600–700 nm)	Far-red $\mu\text{W}/\text{cm}^2$ (700–800 nm)	Blue%	Green%	Red%	Far-red%	Blue/Red	Red/Far-red
White (W)	250	1120	1840	2240	130	21	34.52	42.03	2.44	0.5	17.23
Blue (B)	250	5060	50	1010	10	82.55	0.82	16.48	0.16	5	101
Red + Far-red (R)	250	720	680	3420	340	13.95	13.18	66.28	6.59	0.21	10.06
Pink (P)	250	2720	20	2690	20	49.91	0.37	49.36	0.37	1.01	134.5

The 7-day-old plants (4 leaf development stages) were treated with nutrition solution containing 0.3 mM PUT, SPD, or SPM (based on [50]). After 1-week exposure to different PA treatments, the fully developed leaves and roots were sampled.

#### 4.2. Chlorophyll a Fluorescence Induction (FI) Analysis

The FI analysis was carried out using a pulse amplitude modulated fluorometer (PAM) with a blue LED-array illumination unit IMAG-MAX/L ( $\lambda = 450$  nm) (Imaging-PAM MSeries, Walz, Effeltrich, Germany) on the fully expanded leaves of wheat, which were exposed to dark for 15 min in order to reach the open state of the acceptor side of electron transport chain. Application of a short (800 ms) saturation light (PPFD:  $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) led to the measurement of  $F_0$  and  $F_m$  fluorescence and the maximum quantum yield ( $F_v/F_m$ ) of photosystem II (PSII) was calculated. The kinetic (quenching) analysis was carried out to determine actual quantum yield  $[Y(\text{II})]$  and electron transport rate (ETR), as described in [77]. The blue actinic light intensity was PPFD:  $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ . The saturation pulses were set with 30 s frequency in order to record the  $Y(\text{II})$  at light-adapted state. The IMAG-MAX/L illumination unit was used throughout the entire quenching analysis (675 s).

#### 4.3. Polyamine Analysis

Leaf and root samples were homogenized in 2 mL 0.2 N  $\text{HClO}_4$  and left on ice for half an hour. The homogenates were centrifuged at  $4^\circ\text{C}$  in a centrifuge for 10 min at  $10,000\times g$ . The supernatant was used for the precolumn derivatization with dansyl chloride, according to [78]. DAP, PUT, SPD, and SPM were analyzed on a reverse phase Kinetex column (C18,  $100 \times 2.1$  mm,  $5 \mu\text{m}$ , Phenomenex, Torrance, CA, USA) by HPLC consisting of a W2690 separation module and a W474 scanning fluorescence detector with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA).

#### 4.4. Extraction of Plant Hormones and Analytical Procedure

The extraction procedure and both the separation and detection for ABA with tandem mass spectrometry (UPLC-MS/MS) were carried out according to [57]. Briefly, leaf or root samples were homogenized in liquid  $\text{N}_2$  and extracted with methanol:water (2:1) to a final sample ratio of  $100 \text{ mg FW mL}^{-1}$ . UPLC-MS/MS analysis was performed on

a Waters Acquity I class UPLC system coupled to a Waters Xevo TQ-XS (Milford, MA, USA), equipped with a UniSpray ion source (US) operated in timed MRM mode, with argon (Gruppo SIAD, Bergamo, Italy) as a collision gas. Separation was performed on a Waters Acquity HSS T3 column (1.8  $\mu\text{m}$ , 100 mm  $\times$  2.1 mm) at 40 °C. For gradient elution, water and acetonitrile containing 0.1  $v/v\%$  formic acid were used. Data processing was performed using Waters MassLynx 4.2 and Target-Lynx software (Milford, MA, USA).

SA extraction was performed according to Pál et al. [76]. After separation on a reverse-phase column (Synergi Fusion-RP, 80A, 150  $\times$  4.6 mm, 4 $\mu\text{m}$ , Phenomenex, Torrance, CA, USA) SA was quantified fluorometrically (W474 scanning fluorescence detector, Waters, Milford, CT, USA), with excitation at 305 nm and emission at 407 nm for SA.

#### 4.5. Pigment Extraction and Analyses

Pigment extraction and chromatographic analyses was performed as described in [57]. Briefly, liquid N<sub>2</sub>-homogenized 200 mg fresh weight leaf tissue was spiked with beta-apo-8'-carotenal (Merck-Sigma, Darmstadt, Germany) as an internal standard at 2.5 or 5  $\mu\text{g}$  100  $\text{mg}^{-1}$ . Samples were extracted twice with 1 mL of acetone:methanol 80:20  $v/v\%$  by vortexing for 10 s, followed by shaking in a MiniG 1600 instrument (SPEX SamplePrep.; Metuchen, NJ, USA). After centrifugation at 14,000  $\times g$  (at 4 °C for 10 min), supernatants were collected, pooled, and filtered through 0.22  $\mu\text{m}$  PTFE syringe filters and analyzed immediately. For LC-PDA-MS analysis, a Waters Acquity I-class UPLC coupled to a Xevo TQ-XS mass-spectrometry system and a Thermo Accucore C30 2.6  $\mu\text{m}$ , 4.6  $\times$  150 mm column was used. Eluent system A was methanol:water:tert-butyl methyl ether (TBME) 70:30:30  $v/v\%$ , while eluent B was methanol:TBME 50:50  $v/v\%$ . Solvents used were all at least HPLC grade and were purchased from VWR International (Radnor, Pennsylvania, United States). Absorbance was recorded at 250–700 nm with 1.2 nm resolution and 20 Hz with a PDA detector.

#### 4.6. Proline Measurement

The proline content was measured according to the method of Bates et al. [79]. Briefly, the samples were homogenized with distilled water. The extract was centrifuged at 10,000  $\times g$  for 10 min at 4 °C. The supernatant was mixed in a 1:1:1 ratio with ninhydrin acid and glacial acetic acid. The mixture was incubated at 100 °C for 1 h. The reaction was arrested in an ice bath, the chromophore was extracted with toluene, and its absorbance was determined at 520 nm using a UV-visible spectrophotometer (160A, Shimadzu, Kyoto, Japan).

#### 4.7. Gene Expression Analysis

For gene expression studies, the second, fully developed leaves and roots of 14-day-old wheat plants were taken and immediately stored in liquid nitrogen. Total RNA extraction and cDNA synthesis were performed as described in Tajti et al. [80]. Briefly, total RNA was extracted from samples using TRI reagent. The samples were treated with DNase I and cleaned with a Direct-Zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. For RT-qPCR measurements, a BioRad CFX96 Touch Real-Time Detection System was used with 1  $\mu\text{L}$  4-fold diluted cDNA, 200 nM forward and reverse primers, 2.5  $\mu\text{L}$  PCR BIO Mastermix (PCR Biosystem, London, UK) and 2.5  $\mu\text{L}$  molecular grade water. Relative transcript levels were determined with the  $2^{-\Delta\Delta\text{Ct}}$  method [81], with *Ta2291* as internal control gene [82]. Primer sequences are available in Table S1 [64,82–84].

#### 4.8. Statistical Analysis

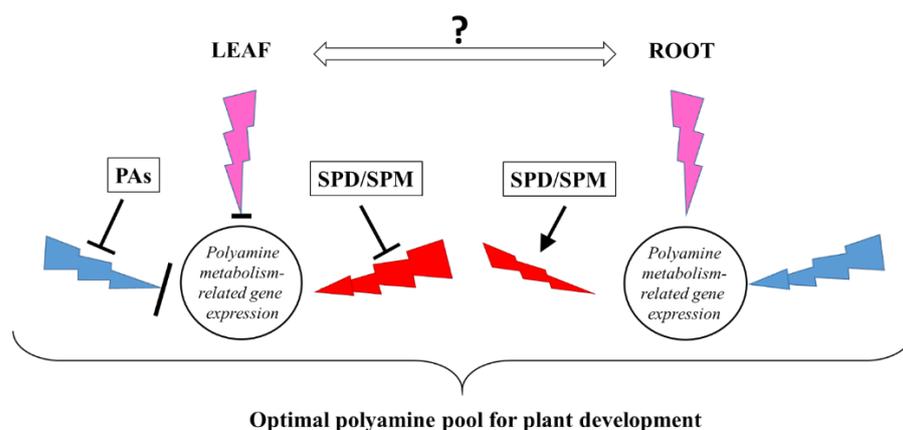
Data are presented for the most representative repetition of the four independent biological experiments. The results are the means of 30 replicates for biomass parameters, six replicates for chlorophyll *a* fluorescence induction measurement, and at least three replicates for chromatographic and spectrophotometric determinations. All reactions for

gene expression analyses were performed in triplicate using 3 biological and 3 technical repetitions. The data were statistically evaluated using the standard deviation in Microsoft Excel (STDEV.S function) with  $n \geq 3$ . Different letters indicate statistically significant differences ( $p < 0.05$ ) between multiple groups (one-way ANOVA with Duncan post hoc test was performed using SPSS 16.0).

## 5. Conclusions

Based on the present findings, light composition under the same light intensity has no pronounced effect on PA content. However, the nearly identical PA levels were accompanied by completely opposite effects of B and R lights on the expression levels of PA metabolism-related genes, with a significant inhibiting effect under the B light, which was only partially compensated under P light conditions due to the presence of a higher proportion of the red component. Although a positive effect of PA treatment was observed under B, R, and P light conditions, as evidenced by leaf biomass parameters and photosynthetic performance, these beneficial changes were correlated only with the increase in endogenous PUT content, especially under B light. Here, PA treatments increased the leaf PUT and SPD contents, as well as the expression levels of genes involved in PA metabolism, confirming that the intensive uptake and metabolism of PAs were responsible for the roborative effects. Despite the fact that initial differences in the expression pattern of PA metabolism-related genes were observed between the three spectral regimes, the effect of PA treatments was opposite under B and R conditions, with a partially intermediate influence under the P light condition. Our results also demonstrated that the effects of exogenous PAs on PA levels were nearly identical under R and P treatments in the leaves. In addition, especially in SPM-treated plants, the leaf transcript levels of *ADC*, *SPDS*, *PAO*, and *pxPAO* genes showed similar changes under R and P light conditions. In the roots, no effect of light spectral composition was observed on the PA treatments regarding PA content, and no similar tendencies in gene expression levels were observed after PA treatments between the three spectral compositions.

Taken together, light quality optimizes plant growth under given conditions and may influence plant growth via the adaptive adjustment of PA metabolism at the gene expression level, which results in a similar spectrum-independent PA pattern (Figure 9). Nevertheless, after PA treatments, the excess of PAs that disturbs the fine tuning of PA metabolism induces different strategies under B, R, or P light conditions for maintaining the optimal PA pool for plant growth and development.



**Figure 9.** Schematic figure on the effect of polyamines (PAs) (SPD: spermidine; SPM: spermine) treatments in the leaves and roots of plants grown under three light regimes (blue lightning arrows indicate blue light, red lightning arrows indicate red + far-red light, and the pink ones the combination of blue and red + far-red, called pink light) compared to white light conditions without any polyamine treatment.

**Supplementary Materials:** The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23158394/s1>.

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# **Appendix D**

## Article

# Influence of a *phyA* Mutation on Polyamine Metabolism in *Arabidopsis* Depends on Light Spectral Conditions

Altafur Rahman <sup>1</sup>, Judit Tajti <sup>1</sup>, Imre Majláth <sup>1</sup>, Tibor Janda <sup>1</sup>, Sylva Prerostova <sup>2</sup>, Mohamed Ahres <sup>1</sup> and Magda Pál <sup>1,\*</sup>

<sup>1</sup> Agricultural Institute, Centre for Agricultural Research, Eötvös Loránd Research Network, 2462 Martonvásár, Hungary

<sup>2</sup> Laboratory of Hormonal Regulations in Plants, Institute of Experimental Botany, Czech Academy of Sciences, 11720 Prague, Czech Republic

\* Correspondence: pal.magda@atk.hu

**Abstract:** The aim of the study was to reveal the influence of *phyA* mutations on polyamine metabolism in *Arabidopsis* under different spectral compositions. Polyamine metabolism was also provoked with exogenous spermine. The polyamine metabolism-related gene expression of the wild type and *phyA* plants responded similarly under white and far-red light conditions but not at blue light. Blue light influences rather the synthesis side, while far red had more pronounced effects on the catabolism and back-conversion of the polyamines. The observed changes under elevated far-red light were less dependent on PhyA than the blue light responses. The polyamine contents were similar under all light conditions in the two genotypes without spermine application, suggesting that a stable polyamine pool is important for normal plant growth conditions even under different spectral conditions. However, after spermine treatment, the blue regime had more similar effects on synthesis/catabolism and back-conversion to the white light than the far-red light conditions. The additive effects of differences observed on the synthesis, back-conversion and catabolism side of metabolism may be responsible for the similar putrescine content pattern under all light conditions, even in the presence of an excess of spermine. Our results demonstrated that both light spectrum and *phyA* mutation influence polyamine metabolism.

**Keywords:** blue light; phytochrome; far-red light; spermine



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## 1. Introduction

Light, as the major energy source for plants, is one of the most important environmental factors, and its characteristics, namely the intensity, the spectral composition, and even the direction affect plant growth and development, including net photosynthesis and chemical composition. These changes, in turn, modify plant morphology and tissue anatomy and, in the long term, influence plant biomass parameters and reproduction-related rates [1]. Plants sense different wavelengths using distinct photoreceptors; among them, one superfamily is phytochromes (Phys), the receptors of red/far-red light [2]. Angiosperm Phys are divided into two groups; type I is characterized as light labile, while the members of type II are light stable [3]. In *Arabidopsis*, there are five Phy genes encoding the apoproteins of Phy A-E [4]. The PhyA protein is considered primarily as a far-red sensor, while the PhyB is rather a red sensor [5]. The effects of *phyA* mutation have been studied with the usage of, for example, T-DNA insertion *Arabidopsis* mutants. Besides the effect of far-red light and darkness in mutant plants on dormancy and germination or hypocotyl elongation [6–8], the role of *phyA* mutation has also been investigated in jasmonic acid-mediated defense response against *Botrytis cinerea* [9], in peroxisome proliferation during photomorphogenesis [10], and in cold acclimation under different light quality and intensity conditions in *Arabidopsis* [11].

PhyA-regulated responses involve metabolite and gene expression fine-tuning mechanisms [12–15]. Investigation on *phyA* and *phyB* mutant *Arabidopsis* plants also revealed that PhyA and PhyB signaling has essential roles in the control of primary metabolism, including starch and sugar content, and other metabolites involved amino acids, polyamines (PAs) and the members of the tricarboxylic cycle in response to light [14], and phytohormone levels [11]. The most detailed study on the relationship between *phyA* mutation and PA metabolism performed under white and far-red light conditions revealed the involvement of PhyA in putrescine (PUT) biosynthesis and its role in the regulation of S-adenosylmethionine decarboxylase 2 and 4 in response to far-red light [12]. Some interesting results also revealed the role of PhyA in mediating the blue light/UV-A photoresponses during chloroplast biogenesis [16] and phototropism [17]. Mesophyll-specific PhyA is involved in the blue-light-dependent regulation of anthocyanin levels [18]. Investigation on quintuple *phy* mutant (*phyA phyB phyC phyD phyE*) *Arabidopsis* plants also revealed that Phys play a role in blue-light-mediated stem elongation and the associated shade-avoidance responses [19] or chloroplast development in *Arabidopsis* under blue light [20]. However, the involvement of Phys in blue light-induced processes is still less known.

PAs are small N-containing polycationic compounds with various roles in plant physiological and molecular processes, such as biosynthesis, structural maintenance, stabilization and function of proteins and nucleic acids, photosynthesis, plant growth and development, and stress signaling [21]. Thus, the maintenance of a proper level of PA pool is important for normal plant growth and development [22]. In plants, the diamine PUT can be synthesized mainly by ornithine decarboxylase (ODC) or arginine decarboxylase (ADC), with one well-known exception, as in *Arabidopsis* the ODC pathway is lacking, but this species displays two ADCs genes (*AtADC1* and *AtADC2*) [23]. Light and several stress factors have been shown to have more influence on *ADC2* promoter activity [24,25]. PUT can be converted to the triamine spermidine (SPD), which can be further converted to the tetramine spermine (SPM) by the repetitive addition of an aminopropyl moiety in reactions catalyzed by two closely related but specific enzymes, SPD synthase (SPDS) and SPM synthase (SPMS) [22]. The *Arabidopsis* genome carries two genes encoding SPDSs (*AtSPDS1* and *AtSPDS2*) and one encoding SPMS (*AtSPMS*) [26]. The expression of *AtSPDS1* and *AtSPDS2* have been reported to be constitutive in all organs [27], but *AtSPDS2* was found to be inducible by plant hormones, such as indoleacetic acid, gibberellin A3 and abscisic acid [28]. The fine-tuning of the PA metabolism is achieved by the balance between the synthesis and catabolism, uptake and conjugation of PAs, and it can also be assumed that light may affect not only the synthesis but also the catabolism of PAs [24]. Among the ten annotated Copper-Containing Amine Oxidase (CuAOs) genes in *Arabidopsis*, *AtCuAO1*, *AtCuAO2* and *AtCuAO3* have been well characterized. These genes encode functional CuAOs that use PUT and SPD as substrates and are responsible for terminal catabolism. *CuAO1* has extracellular, while *CuAO2* and *CuAO3* have peroxisomal localization. The three genes present different expression profiles [29]. *AtCuAO1* transcripts were abundant in rosette leaves, and the expression increased continuously during development. Similarly, the expression pattern of *AtCuAO3* increased during development, and its transcript level was high both in the leaves and in the stems. While the expression level of *AtCuAO2* was abundant rather in stems and was low in the leaves, in addition did not increase during the developmental stages. Plant hormone treatments, such as abscisic acid or salicylic acid, also induced the gene expression of *AtCuAO1* and *AtCuAO3* but not that of *AtCuAO2* [29]. In *Arabidopsis*, all the known polyamine oxidases (PAOs) are responsible for the back conversion of higher PAs. Characterization of five PAO isoforms in *Arabidopsis* revealed that *AtPAO1* has a specific role in flowers, *AtPAO2* was expressed in shoot and root meristems, and to a greater extent at later growth stage in the rosette, stem and in flowers. The expression of *AtPAO3* was constitutive in the whole plant body but was the highest in flowers. *AtPAO4* was expressed in young and adult plants, especially in the roots and floral organs, while *AtPAO5* expression was observed in all the organs throughout various growth stages [30]. *AtPAO1* and *AtPAO5* are located in the cytoplasm, while *AtPAO2*,

*AtPAO3*, and *AtPAO4* are in peroxisomes. *AtPAO1* and *AtPAO5* prefer thermospermine, whereas *AtPAO4* has higher substrate specificity for SPM and converts it to SPD, while *AtPAO2* and *AtPAO3* mainly convert SPD to PUT [31,32]. *AtPAO2* and *AtPAO5* have been reported to be inducible by, for example, salt stress and plant hormones or Pas [33–36].

It has also been demonstrated that the actual PA pool is influenced by light intensity and light composition [37–39]. In addition, it was found that far-red and blue lights induced opposite responses in PA metabolism-related gene expression in wheat plants [39]. Pas increasingly accumulated in tomato leaves from the beginning of illumination [40]. Furthermore, light-responsive elements in the promoter region of genes involved in PA synthesis, such as *SAMDC* encoding S-adenosylmethionine decarboxylase, have been identified in *Arabidopsis* [41].

Although there are some limited results on the changes in PA metabolism in *phy* mutant *Arabidopsis* plants, these studies focused on the modifying effect of the red/far-red ratio with the investigation of only dedicated parts of PA metabolism (just one polyamine compound, or only one of the synthesis/catabolite gene) (reviewed in [24]). Furthermore, these studies examined the effects of the mutation during alternating light conditions or pulse-like light treatments. However, the relationship between light perception and PA metabolism has still not been fully understood.

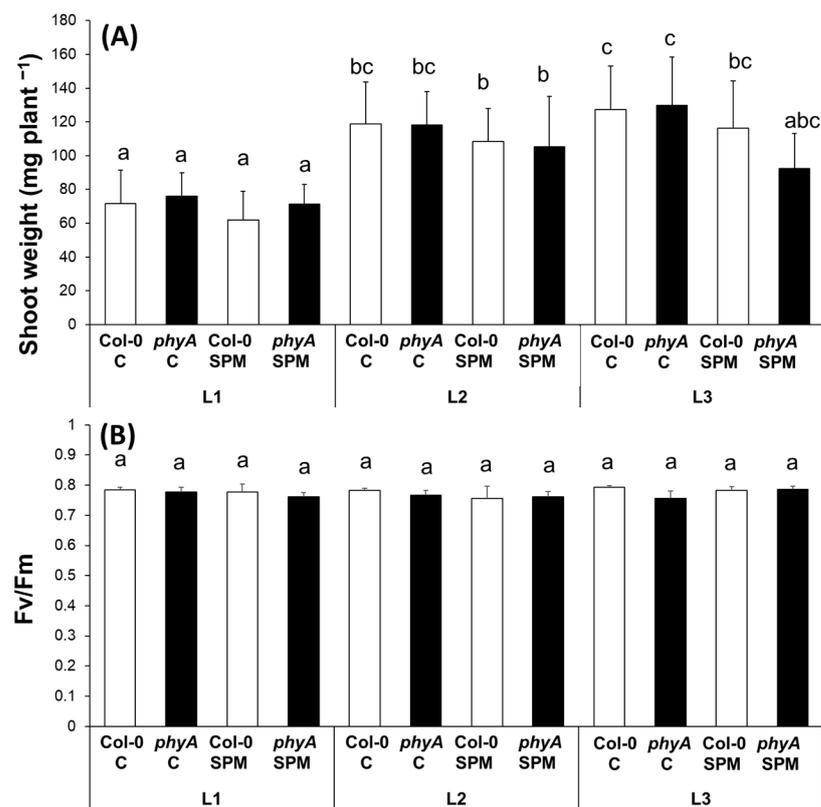
The present study aimed to reveal the influence of PhyA on PA synthesis and catabolism. Therefore, PA metabolism changes in *phyA* insertion mutant *Arabidopsis* plants were compared to the wild type. As PhyA responds to far-red, plants were exposed to white light, as well as white light with an enhanced far-red spectrum. Moreover, the effect of supplemental blue light also was investigated because some studies suggested that Phys play a role in special blue-light-mediated responses [16,17,19]. In order to estimate the direct impact of PhyA on PA catabolism, some plants were supplemented by exogenous SPM.

## 2. Results

### 2.1. Measurements of the Shoot Weight and Chlorophyll-A Fluorescence Induction Parameters

Wild type Col-0 and *phyA* mutant *Arabidopsis* plants were grown at different light spectral conditions (the light source for L1: “white light” was a continuous wide-spectrum LED, in the case of the L2 regimen: “red light”, elevated far-red component was applied, while under L3 light condition: “blue light” the blue light component was the most dominant), with or without exogenous SPM treatment. In order to characterize the physiological status of the plants, shoot weight and the maximum quantum efficiency ( $F_v/F_m$ ) chlorophyll-*a* fluorescence induction parameter representing the maximum quantum efficiency of Photosystem II (PSII) was measured first. The *phyA* mutation did not significantly influence the shoot weight under any of the light conditions (Figure 1A). However, the L2 and L3 light treatments increased it in both genotypes compared to the control, white light (L1), indicating that light quality has more influence on plant growth rate than the mutation. The one-day SPM treatment also did not significantly influence the shoot weight of the plants under different light conditions (Figure 1A). The  $F_v/F_m$  parameter was around 0.8, and neither the treatments nor the genotypes influenced it, indicating that the plants were under optimal growth conditions (Figure 1B).

Besides the  $F_v/F_m$ , the actual quantum yield  $[Y(II)]$  of PSII was also measured. Under L1 and L2 light conditions, no differences were detected between the wild type and the mutant plants in  $Y(II)$ . Under L3 light, slightly lower  $Y(II)$  was measured for the *phyA* mutants compared to the Col-0 (Supplementary Figure S1b). It has also been shown that the actual quantum yield was slightly more sensitive to the SPM treatment in the wild genotypes than in the *phyA* mutant plants under L1 and L3 light conditions (Supplementary Figure S1a,b). At L2 treatment, SPM application slightly increased the  $Y(II)$  in the *phyA* mutant plants. Despite a few statistically significant differences, these changes were not substantial.

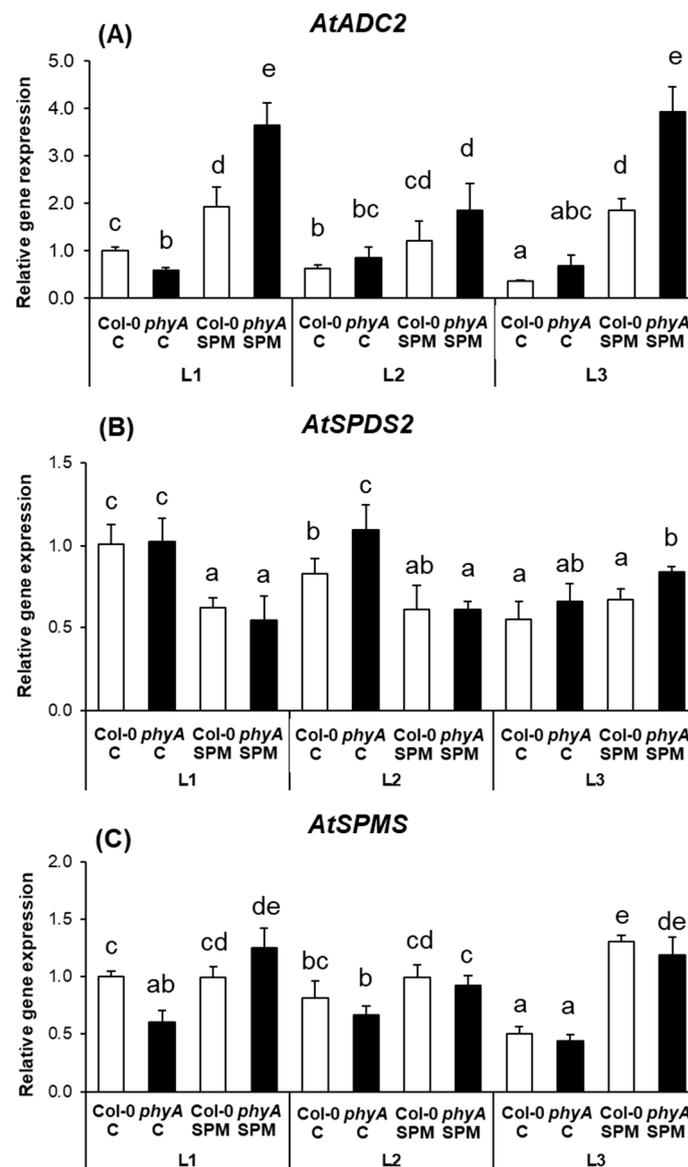


**Figure 1.** Shoot weight (A) and the maximum quantum efficiency of photosystem II (Fv/Fm) chlorophyll-a fluorescence induction parameter determined at the steady state level of photosynthesis of the leaves (B) of wild type (Col-0) and mutant (*phyA*) *Arabidopsis* plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD ( $n = 14$  for shoot weight and  $n = 5$  for Fv/Fm). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

## 2.2. Modulation of Polyamine Content and Polyamine Metabolism-Related Gene Expression by Light Spectra

As light and several stress factors have been shown to be important regulators of *ADC2* promoter activity, here, the *ADC2* gene was in focus. In accordance, we detected downregulated expression of *AtADC2* by L2 and L3 treatments in Col-0 plants (Figure 2A). In addition, the expression of *AtADC2* was lower in the mutant plants under L1 but the same under L2 and L3 light conditions. A much stronger effect was found in the case of SPM-treated plants (Figure 2A). After the SPM application, *AtADC2* expression increased in both genotypes, which suggested that the SPM treatment induced de novo synthesis of PUT. *AtADC2* expression increased, especially in the case of the mutant plants, with a higher degree under L1 and L3 light conditions (Figure 2A).

As the expression of *AtSPDS2* was found to be inducible by plant hormones, such as indoleacetic acid, gibberellin A3 and abscisic acid, here, we focused only on *AtSPDS2*. Without SPM treatment, the *AtSPDS2* transcription levels did not differ between the two genotypes under L1 and L3 conditions, and under L2, it was only slightly higher in *phyA* mutant than in Col-0 (Figure 2B). The lowest *AtSPDS2* expression level was found in both genotypes under the L3 light treatment. SPM treatment decreased the expression of *AtSPDS2* in both genotypes under L1 and L2 conditions, while under L3 conditions, where the expression level was initially lower, no pronounced differences were induced (Figure 2B).

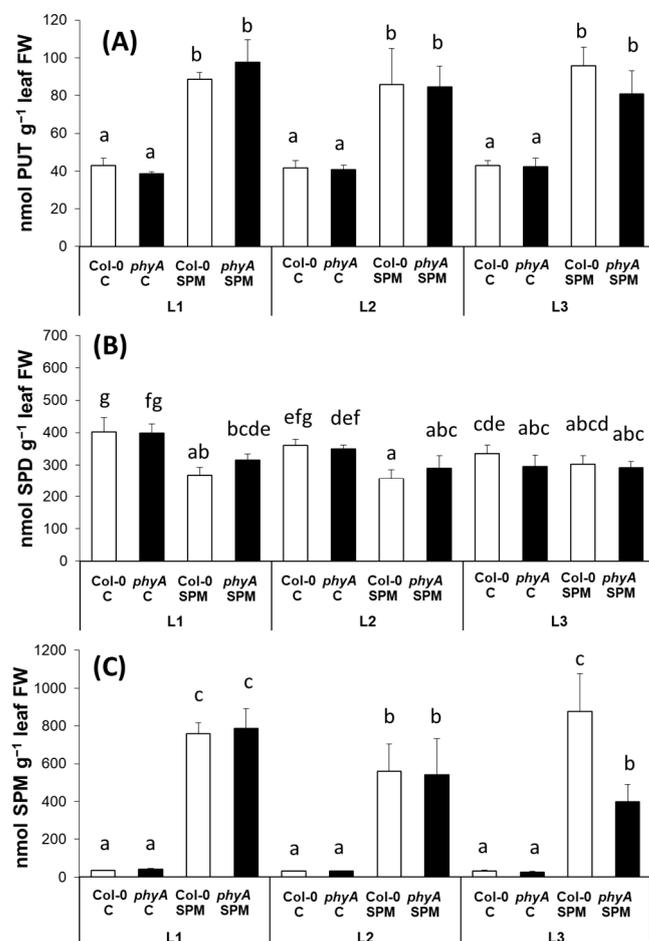


**Figure 2.** Changes in the expression levels of polyamine synthesis-related genes, namely arginine decarboxylase (*AtADC2*) (A), spermidine synthase (*AtSPD2*) (B) and spermine synthase (*AtSPMS*) (C) of wild type (Col-0) and mutant (*phyA*) Arabidopsis plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD (n = 3). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

The expression level of *AtSPMS* downregulated in *phyA* mutant compared to the wild type under L1 and L2 light treatments, but not under L3 light conditions, where the expression level was similar to the wild type and initially lower (Figure 2C). SPM treatment induced the highest increment under L3 in Col-0, a slight increase in both genotypes was induced under L2, while under L1, the transcript level of *AtSPMS* increased only in the mutant.

PUT content was similar in the wild type and the *phyA* mutant plants under all the light spectral conditions tested here (Figure 3A). A substantial increase was detected in the SPM-treated plants compared to their corresponding control. Despite the well-recognized differences between the expression of *AtADC2* in the two SPM-treated genotypes, as well

as under different light regimes, SPM treatment increased the PUT level similarly in both genotypes, which was independent of the light conditions.



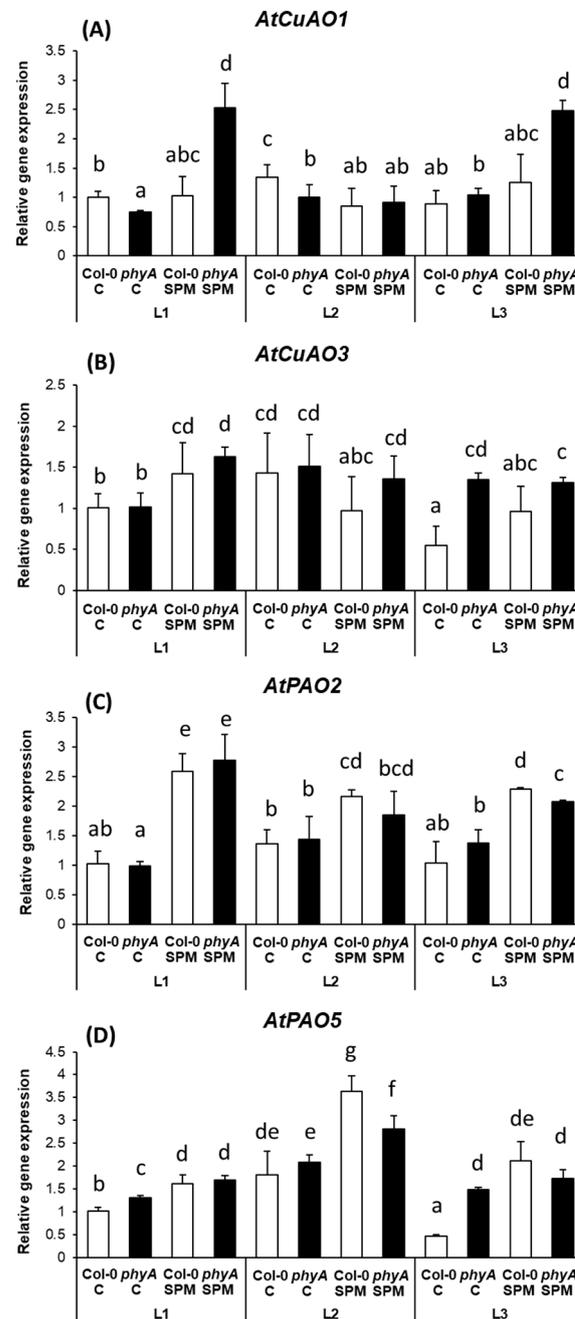
**Figure 3.** Polyamine contents, namely putrescine (PUT) (A), spermidine (SPD) (B) and spermine (SPM) (C) of wild type (Col-0) and mutant (*phyA*) *Arabidopsis* plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD (*n* = 3). Different letters indicate statistically significant differences at *p* < 0.05 level, using Duncan's post hoc test.

Under the present conditions, the most abundant PA in the leaves of *Arabidopsis* plants was SPD (Figure 3B), the amounts of PUT and SPM were about an order of magnitude lower compared to SPD, and no remarkable changes were observed in the SPD levels after the treatments compared to changes in PUT and SPM. In addition, SPM treatment only slightly decreased SPD levels, especially under white and far-red light conditions.

Despite the modifications of the enzyme expression, the pattern of SPM changes did not follow the tendency of *AtSPMS* expression levels. The SPM concentration was similar in both genotypes under the three applied light treatments (Figure 3C), and exogenous SPM increased equally in Col-0 and *phyA* under L1 and L2 light conditions, with a lower extent in the case of L2. Interestingly, under the L3 condition, the SPM accumulation in SPM-treated Col-0 was double that of the *phyA* mutant.

Based on the localization of the CuAO and PAO enzymes, organ-specific expression and plant hormone inducibility of their genes here, the changes in the transcript level of *AtCuAO1* and *AtCuAO3*, in addition to *AtPAO2* and *AtPAO5* were studied.

Under the present conditions, it was found that the *AtCuAO1* expression was independent of the light condition or the *phyA* mutation. However, in the presence of an excess SPM, *AtCuAO1* expression was induced in the mutant genotypes under L1 and L3 light conditions (Figure 4A).



**Figure 4.** Changes in the expression levels of polyamine metabolism-related genes, namely copper-containing amine oxidases (*AtCuAO1*: (A) and *AtCuAO3*: (B)), and polyamine oxidases (*AtPAO2*: (C) and *AtPAO5*: (D)) in the leaves of wild type (Col-0) and mutant (*phyA*) Arabidopsis plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD ( $n = 3$ ). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

The expression level of *AtCuAO3* did not show pronounced changes, except downregulation under L3 conditions in Col-0 (Figure 4B). The initially higher *AtCuAO3* expression in *phyA* mutant under the L3 regime might reflect the involvement of PhyA in this process, which is similar to the above-mentioned modifications in *AtCuAO1* expression under SPM treatment combined with L3 conditions. A slight increase of *AtCuAO3* expression was detected after SPM application in both genotypes under L1 light and in Col-0 under L3 (Figure 4B), which also indicates that this process is dependent on the actual level of PAs.

In the present study, the increased SPM content in the leaves, due to the excess of SPM in the nutrition solution, induced the expression of *AtPAO2* under all the light conditions regardless of the genotypes (Figure 4C). Compared to this, the *AtPAO5* levels showed more differences. Its transcript level was initially the lowest under L3 conditions in Col-0 plants (Figure 4D), and its expression was mostly elevated after SPM treatment, especially under L2 conditions in both genotypes, while under L3 conditions only in Col-0 plants. The transcript level of *AtPAO5* was significantly higher in the mutant plant compared to the wild type under L3 conditions, and excess SPM could not increase further it. It should also be noticed that under the L2 condition in both genotypes, the initial level of *AtPAO5* expression was higher than that was detected under L1 conditions in Col-0 or *phyA* mutant plants.

### 2.3. Correlation Analyses and Principal Components Analysis (PCA) of the Measured Parameters

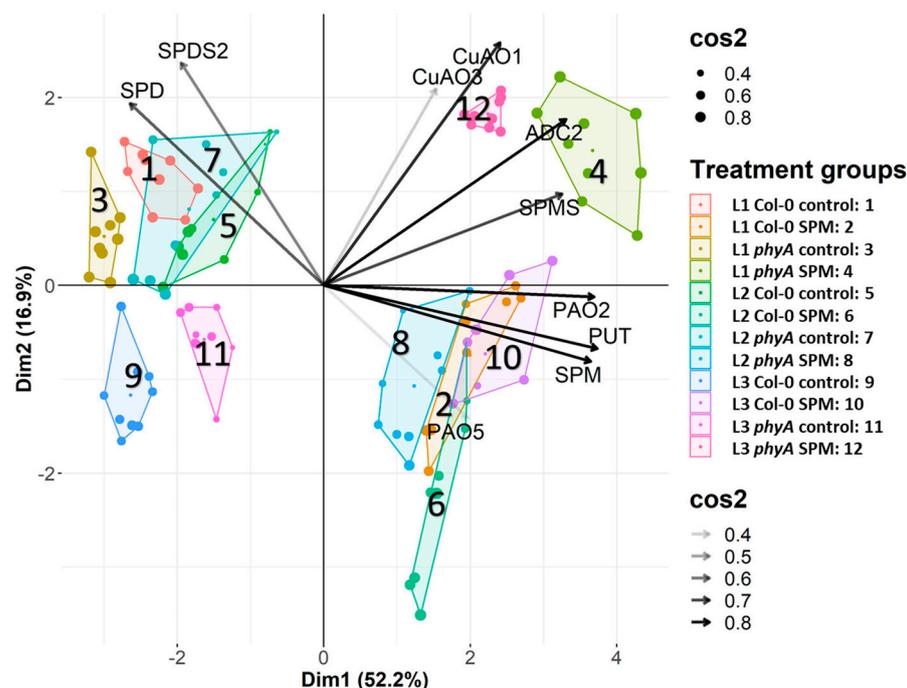
The correlation analyses (Table 1) showed that PUT content was in a close, positive relationship with the SPM content, and both PUT and SPM contents were in a close, positive relationship with the *ADC2*, *SPMS* and *PAO2* expression levels. Additionally, high and positive correlations were detected between the expression levels of *ADC2*, *SPMS*, *CuAO1* and *PAO2*. However, close, negative relations were found between PUT or SPM levels and SPD content, and *SPDS2* transcript level. Furthermore, SPD content also showed a strong, negative correlation with *PAO2* and *PAO5* expression levels.

**Table 1.** Correlation analysis on the polyamine metabolism-related parameters. Strong significant correlations at 0.05 are in bold; a positive correlation is highlighted in green, and a negative correlation is highlighted in red. PUT: putrescine content, SPD: spermidine content, SPM: spermine content, *ADC2*: expression level of arginine decarboxylase 2, *SPDS2*: expression level of spermidine synthase 2, *SPMS*: expression level of spermine synthase, *CuAO1* and *CuAO3*: expression level of copper amine-oxidase 1 and 3, *PAO2* and *PAO5*: polyamine oxidase 2 and 5 genes.

	PUT	SPD	SPM	<i>ADC2</i>	<i>SPDS2</i>	<i>SPMS</i>	<i>CuAO1</i>	<i>CuAO3</i>	<i>PAO2</i>	<i>PAO5</i>
PUT	1									
SPD	<b>−0.555</b>	1								
SPM	<b>0.915</b>	<b>−0.572</b>	1							
<i>ADC2</i>	<b>0.688</b>	<b>−0.379</b>	<b>0.617</b>	1						
<i>SPDS2</i>	<b>−0.524</b>	<b>0.559</b>	<b>−0.502</b>	−0.188	1					
<i>SPMS</i>	<b>0.737</b>	<b>−0.293</b>	<b>0.753</b>	<b>0.710</b>	−0.139	1				
<i>CuAO1</i>	<b>0.414</b>	−0.147	<b>0.345</b>	<b>0.820</b>	−0.095	<b>0.553</b>	1			
<i>CuAO3</i>	0.169	−0.124	0.159	<b>0.413</b>	0.130	<b>0.261</b>	<b>0.410</b>	1		
<i>PAO2</i>	<b>0.834</b>	<b>−0.593</b>	<b>0.829</b>	<b>0.688</b>	<b>−0.431</b>	<b>0.666</b>	<b>0.470</b>	<b>0.399</b>	1	
<i>PAO5</i>	<b>0.469</b>	<b>−0.500</b>	<b>0.423</b>	0.157	−0.135	<b>0.334</b>	−0.050	<b>0.260</b>	<b>0.423</b>	1

The principal component analysis (PCA) analysis of the PA metabolism-related parameters (Figure 5) showed great separation in some of the treatment groups based on their measured genetic and biochemical variables. This multivariate analysis reflected that L3 light conditions had a specific effect on PA metabolism. On the other hand, SPM treatment was a more significant factor in the distinction of treated plants. Three variable groups, SPD—*SPDS2*, *ADC2*—*CuAO1*—*SPMS*, and *PAO2*—PUT—SPM, were responsible for the differences among the twelve treated populations. The changes in the first two variables were responsible for the altered PA metabolism in the Col-0 and *phyA* populations without

SPM treatment under L1 and L2 and, to a lesser extent, under L3 light treatment. The second group of three genes led to the discrimination of SPM-treated *phyA* mutant under L1 and L3, and the third group differentiated the SPM-treated Col-0 genotype at each of the lights and the mutant one under the L2 condition.



**Figure 5.** The biplot illustration of PCA analysis was carried out on the standardized concentration values of polyamines and the relative expression values of polyamine metabolism-related genes. Squared cosine ( $\cos^2$ ) shows how accurate the representation of our variables or individuals on the PC plane is. Dim1 and Dim2 are equivalent to principal components 1 and 2 (PC1 and PC2). Investigated parameters: PUT: putrescine content, SPD: spermidine content, SPM: spermine content, *ADC2*: expression level of arginine decarboxylase 2, *SPDS2*: expression level of spermidine synthase 2, *SPMS*: expression level of spermine synthase, *CuAO1* and *CuAO3*: expression level of cooper amine-oxidase 1 and 3, *PAO2* and *PAO5*: polyamine oxidase 2 and 5 genes. (Col-0 and *phyA Arabidopsis* plants grown under L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Different colors and numbers indicate the 12 treatment combinations.

### 3. Discussion

PA biosynthesis is controlled by light (light quality and quantity) [38,39], and on the other hand, PAs are able to influence photosynthesis in several ways (reviewed in [24]). PhyA is a unique photoreceptor responsible for the high far-red light irradiance response of seedlings, and PhyA regulates various light-dependent responses at metabolite and gene expression levels; however, PhyA may regulate genes other than light-responsive ones through the interaction with corresponding transcription factors [23]. In addition, some results suggest the involvement of PhyA in mediating the blue light photoresponses, such as chloroplast biogenesis, phototropism, anthocyanin synthesis or stem elongation and shade-avoidance responses [16–19]. Although some limited information suggests a relationship between PhyA and PA metabolism under white and far-red light conditions [12], until now, no information has been available on the involvement of PhyA in blue light-influenced regulation of the PA metabolism in plants. Therefore, the present study attempts to uncover the impact of far-red and blue light on PA production through the investigation of *phyA* mutant plants, as well as changed light spectra and supplementation by exogenous PA.

The *phyA* mutation did not influence the shoot weight under the different light conditions compared to the adequate wild-type controls. At the same time, the light quality has more influence on the shoot weight than the mutation, as L2 and L3 light treatments increased it in both genotypes compared to the control white light (L1) condition. Similarly to these results, the biomass of individual monogenic *phyA* or *phyB* mutant plants was the same as that of the wild type, suggesting that the action of either *phyA* or *phyB* is sufficient for normal biomass production [42]. *Arabidopsis* leaf area and petiole length also showed a correlation with blue light irradiance levels [43], and a decrease in the R/FR ratio has also been reported to significantly increase petiole elongation and leaf area expansion of the Col-0 line [44]. In addition, the biomass parameters of the *phyA* mutant were similar to that of the wild type, even under far-red light supplementation [42].

Although the effects of exogenous Pas have been tested in *Arabidopsis* mutants, especially in PA metabolism mutants [35,45], no information is about the effect of exogenous SPM on *phyA* mutants. Previously we also demonstrated that one-day treatment with 0.5 mM SPM was enough to induce changes in PA metabolism in *Arabidopsis* Col-0 and salicylic acid-related mutants [36]. In the present study, the SPM treatment did not influence remarkable changes in the shoot weight or chlorophyll-*a* induction parameters of the plants, indicating that the plants were under optimal growth conditions. In order to better understand the biological effect of PA metabolism and its relationship with the PhyA, PA metabolism was investigated at metabolite and gene expression levels.

The expression of *AtADC2* was lower in the mutant plants under L1 but the same under L2 and L3 light conditions; in addition, it was downregulated by L2 and L3 treatments in Col-0 plants. This indicates that the expression of *AtADC2* is dependent on far-red, as well as blue light signaling and that this signaling pathway goes through PhyA. SPM treatment increased *AtADC2* expression in both genotypes, suggesting that exogenous SPM induced de novo synthesis of PUT. As *AtADC2* expression increased, especially in the case of the mutant plants, with a higher degree under L1 and L3 light conditions, these results support that PhyA is involved in the regulation of PUT synthesis, but also another pathway regulated by far-red light is probably included, for example, PhyB might play a role in the process [5]. As it has been demonstrated, PUT and SPD contents only decreased in *phyB* mutants under far-red light in contrast to the white light conditions [14]. It was also reported that the stimulating effect of red light on the activities of ADC and S-adenosylmethionine decarboxylase enzymes could be reversed with the application of far-red light, implicating Phy again in the regulation of PA biosynthesis. While blue light-induced changes in PA synthesis enzyme activity could not be reversed by far-red light, thus the influence of PA synthesis via different signal transduction pathways suggests the involvement of a separate blue-light receptor [46,47]. Despite the well-recognized differences between the expression of *AtADC2* in the two SPM-treated genotypes, as well as under different light regimes, SPM treatment increased the PUT level similarly in both genotypes, which was independent of the light conditions, suggesting that other processes than the synthesis are involved.

The *AtSPDS2* transcription levels did not differ pronouncedly between the two genotypes under L1, L2 and L3 conditions indicating less influence of PhyA and, in turn, far-red light on the regulation of SPD synthesis than on PUT synthesis. In addition, the lowest *AtSPDS2* expression level was found in both genotypes under the L3 light treatment, which suggests the negative impact of blue light on this PA biosynthetic enzyme. Nevertheless, no remarkable changes were observed in the SPD levels after the treatments. SPM treatment decreased the expression of *AtSPDS2* in both genotypes; in addition, it slightly decreased SPD levels under L1 and L2 conditions.

The expression level of *AtSPMS* downregulated in *phyA* mutant compared to the wild type under L1 and L2 light treatments, while under L3 light conditions, it was initially the lowest. These results further support the hypothesis that PhyA has a role in the synthesis of PAs (in this case, that of SPM), and blue light suppresses it. However, SPM treatment induced the highest increment under L3 in Col-0; a slight increase in both genotypes

was induced under L2, while under L1, the transcript level of *AtSPMS* increased only in the mutant. This is in contradiction with the response of SPM non-treated plants, which means that the high concentration of SPM could influence PhyA signaling in opposite feedback, at least under white light conditions. Moreover, *AtSPMS* upregulation under L3 conditions after SPM treatment indicates that the high SPM level stimulated the production of SPM biosynthetic enzyme by a blue light signal. Despite the modifications of the enzyme expression, the pattern of SPM changes did not follow the tendency of *AtSPMS* expression levels, as the SPM concentration was very similar in both genotypes under the three applied light treatments. Exogenous SPM increased the SPM level in Col-0 and *phyA* under L1 and L2 light conditions equally, suggesting that SPM was transported from the roots to the leaves. Moreover, lower SPM accumulation under higher far-red light irradiance may have resulted from the conversion of SPM, but this process was not driven by PhyA. The SPM accumulation in SPM-treated Col-0 was double that of the *phyA* mutant under the L3 condition, which is clear evidence that PhyA-induced accumulation of SPM under higher blue light irradiance and high SPM concentrations and that PhyA is influenced by blue light. This process was probably regulated at the level of PA degradation and SPM back-conversion and not by PA synthesis. However, modification in the PA uptake transport mechanisms cannot be excluded too.

The *AtCuAO1* expression was independent of the light condition or the *phyA* mutation. However, SPM induced *AtCuAO1* expression in the mutant genotypes under L1 and L3 light conditions. Therefore, the gene expression pattern of *AtCuAO1* was partly similar to that of *AtADC2* under L1 and L3, suggesting that the higher SPM concentration-induced de novo synthesis of PUT was compensated by its higher degradation in the mutant plants. In turn, no differences were observed in the actual PUT contents. The expression level of *AtCuAO3* did not show pronounced changes but was downregulated under L3 conditions in Col-0. It indicates that higher blue light irradiance diminished the degradation of PUT under the control of PhyA. The initially higher *AtCuAO3* expression in *phyA* mutant under the L3 regime might reflect the involvement of PhyA in this process, which is similar to the above-mentioned modifications in *AtCuAO1* expression under SPM treatment combined with L3 conditions. Slight increases in *AtCuAO3* expression after SPM application in both genotypes under L1 light and in Col-0 under L3 indicate that this process is dependent on the actual level of PAs. It is possible that PAs might affect the phosphorylation of light receptors, probably not only PhyA [48] and enhance their role in gene transcription [49], or that PAs directly stimulate the translation of PhyA and other genes [50].

Excess SPM in the nutrition solution increased SPM content in the leaves, which in turn induced the expression of *AtPAO2* under all the light conditions regardless of the genotypes. At the same time, *AtPAO5* levels were initially the lowest under L3 conditions in Col-0 plants indicating suppressed back-conversion and suggesting again the influence of PhyA under intensive blue light irradiance (similarly to genes of biosynthetic: *AtADC2*, *AtSPDS* and *AtSPMS*, and degradation enzymes: *AtCuAO3*). SPM treatment mostly induced the *AtPAO5* expression, especially under L2 conditions, but under L3 conditions only in Col-0 plants. Under the L2 condition in both genotypes, the initial level of *AtPAO5* expression was higher than that was detected under L1 conditions in Col-0 or *phyA* mutant plants. This higher expression already without SPM treatment under far-red light suggests a higher inducibility of the back-conversion mechanism, which in turn can be responsible for the lower SPM accumulation after SPM treatment under L2 light condition. The initially higher transcript level of *AtPAO5* in the mutant plant compared to the wild type under L3 conditions can be again in relation to the observed lower SPM content in SPM-treated mutants under L3 light conditions.

A close, positive relation between PUT the SPM content, and both PUT and SPM contents with *ADC2*, *SPMS* and *PAO2* expression levels showed that the increase in SPM content not only resulted from the uptake of SPM from the hydroponic solution but the in vivo synthesis of SPM was also induced. SPM treatment also increased the PUT content due to the activation of the in vivo PUT synthesis, and parallel with it, the more intensive

SPM-SPD-PUT back-conversion pathway could be responsible, too. Close, negative relations between PUT or SPM levels and SPD content and *SPDS2* transcript level indicate that the accumulation of PUT and SPM occurred at the expense of SPD content. Furthermore, a strong, negative correlation between SPD content and *PAO2* and *PAO5* expression levels confirmed that lower SPD content has resulted from not only the decreased *SPDS* expression but also the induced back conversion. The PCA analysis reflected that L3 light conditions had a specific effect on the PA metabolism, and SPM treatment was a more significant factor for the distinction of treated plants. Without SPM treatment, the treatments groups of L1 and L2 light conditions, both of the wild type and mutant genotypes, were separated from that of the L3 light, while after SPM treatment, the treatment group of mutant plants under L1 and L3 were similar to each other and partly different from the others (mutant under L2 light and wild type under all light conditions).

Previously, we have demonstrated that among PAs applied hydroponically, especially SPM-induced rapid PA responses in the leaves of Col-0 *Arabidopsis* plants under white light conditions. The SPM application induced the PUT accumulation due to the increased expression level of *AtADC2*; the level of SPD did not change; however, the transcript levels of *AtSPDS1* and *two* slightly decreased, while the expression of *AtSPMS* increased [36]. These results indicate that SPM could influence the de novo synthesis of PAs. Partly similar results were found in the present experiment, as SPM induced the synthesis of PUT and activated the expression of *AtADC2* and *AtSPMS* but decreased that of the *AtSPDS2*. In wheat, it was also demonstrated that the effect of supplementary blue and far-red light was opposite on the PA metabolism-related gene expression in the leaves; in addition, PA treatment could partly reverse these differences [39]. Nevertheless, in the present study, we first demonstrated that the responses of wild-type and *phyA* mutant plants are partly different and depend on the light spectral conditions. Our results on changes induced by SPM treatment reflected that PhyA under blue light might primarily inhibit SPM back-conversion and subsequently downregulate PUT synthesis.

## 4. Materials and Methods

### 4.1. Plant Material, Plant Growth Conditions and Treatments

In the present experiment, the *phyA*-T mutant *Arabidopsis* (SALK\_014575C), T-DNA insertion line in Col-0 background [51] was investigated, where Col-0 ecotype was used as control. Seeds were obtained from the European Arabidopsis Stock Centre (NASC, Sutton Bonington Campus, Loughborough, LE125RD, United Kingdom). The plants were self-pollinated for two generations, and the presence of the mutation was confirmed by genotyping (Supplementary Figure S2). The *phyA* mutant is a T-DNA insertion line created by SALK Institute/SAIL, so the genotyping primers (Supplementary Table S1) were designed with the help of [signal.salk.edu/tdnaprimers.2.html](http://signal.salk.edu/tdnaprimers.2.html) website.

Plants were cultivated hydroponically using an Araponics system (Arapronics, Liège, Belgium). For hydroponic solution, 25% Murashige and Skoog Medium (Duchefa) were used. Plants were grown in a Conviron GB-48 phytochamber (Controlled Environments, Winnipeg, MB, Canada) under control conditions at 22/20 °C with 8/16 h light/dark period and 75% humidity for 28 days.

Plants were grown under different spectral conditions at the same light intensity from germination ( $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Three different light regimens were established using modules equipped with a continuous wide spectrum LED (Philips Lumileds, LXZ2-5790-y) and three narrow bands of LEDs with the dominant wavelengths of 448 nm (Philips Lumileds, LXZ1-PR01); 655 nm (Philips Lumileds, LXZ1-PA01); 750 nm (Edison Edixeon, 2ER101FX0000001). All light source modules were equipped with these LEDs, and each type of LED could be independently controlled within the module. The spectral composition of the three applied light treatments used in the experiments is described in Table 2. The spectral composition was chosen with some modifications based on our previous study, where the effects of supplementary blue and far-red light and their combination was investigated on PA metabolism, and blue light caused a drastic decrease in the gene

expression level of PA metabolism-related gene expression, while the far-red light-induced slight increase of them in the leaves of wheat plants [39].

**Table 2.** Characteristics of the applied three light regimes. Light treatments are colored according to their typical characteristic; the light source for (L1: white color) was a continuous wide-spectrum LED; in the case of the L2 regimen (red color) elevated far-red component was applied, while under L3 light condition (blue color) the blue light component was the most dominant.

Treatment	Intensity PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	Blue $\mu\text{W}/\text{cm}^2$ (400–500 nm)	Green $\mu\text{W}/\text{cm}^2$ (500–600 nm)	Red $\mu\text{W}/\text{cm}^2$ (600–700 nm)	Far-red $\mu\text{W}/\text{cm}^2$ (700–800 nm)	Blue/Red	Red/Far-Red	Blue%	Green%	Red%	Far-Red%
L1	100	400	640	1020	30	0.39	34	19.14	30.62	48.8	1.44
L2	100	450	680	930	240	0.48	3.88	19.57	29.57	40.43	10.43
L3	100	890	650	690	30	1.29	23	39.38	28.76	30.53	1.33

Four-week-old plants were treated with 0.5 mM spermine (SPM) for one day, which was added directly to the nutrient solution. The concentration and the duration of SPM treatment were chosen based on our previous study, where 1 day 0.5 mM SPM treatment was sufficient and induced the most pronounced changes in polyamine metabolism compared to putrescine or spermidine application [36]. Thereafter shoots were collected, measured for shoot weight parameter, and fully developed leaves were frozen immediately in liquid nitrogen. Samples were stored at  $-80\text{ }^{\circ}\text{C}$  until further analysis.

#### 4.2. Chlorophyll-A Fluorescence Induction (FI) Analysis

The FI analysis was carried out using pulse amplitude modulated fluorometer (PAM) with a blue LED-Array Illumination Unit IMAG-MAX/L ( $\lambda = 450\text{ nm}$ ) (Imaging-PAM M-Series, Walz, Effeltrich, Germany) on the fully expanded leaves of Arabidopsis plants, which were exposed to dark for 15 min in order to reach the open state of the acceptor side of the electron transport chain. The determination of the maximum quantum efficiency ( $F_v/F_m$ ) and the actual quantum yield [ $Y(II)$ ] of photosystem 2 was carried out as it was described in [39].

#### 4.3. Polyamine Analysis

Leaf samples were homogenized in 2 mL 0.2 N  $\text{HClO}_4$ , and the homogenates were centrifuged at  $4\text{ }^{\circ}\text{C}$  for 10 min, with  $10,000\times g$ . The supernatant was used for the pre-column derivatization with dansyl chloride. PUT, SPD, SPM, and one of the products of terminal catabolism of SPD and SPM, 1,3-diaminopropane (DAP), were analyzed on a reverse phase Kinetex column (C18,  $100\times 2.1\text{ mm}$ ,  $5\text{ }\mu\text{m}$ , Phenomenex, Inc., Torrance, CA, USA) by HPLC consists of a W2690 separation module and a W474 scanning fluorescence detector with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA) according to [39].  $2\text{ }\mu\text{L}$  of the derivatized sample injected onto the column, two types of solvents were used during the measurement (A: 44%ACN C: ACN: MeOH = 7:3). Gradient program was used for separation and during the analysis the flow rate was  $0.5\text{ mL min}^{-1}$ , and the column temperature was  $40\text{ }^{\circ}\text{C}$ . Data evaluation was performed using the Millennium32 program (WATERS, Milford, MA, USA).

#### 4.4. Gene Expression Analysis

For gene expression studies, fully developed leaves of the wild type and the phyA mutant were taken at the end of the treatments and immediately stored in liquid nitrogen. Total RNA extraction and cDNA synthesis was performed as described in [36] using TRI Reagent, Direct-zol<sup>TM</sup> RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), including on-column Dnase I treatment for RNA purification and with M-MLV Reverse Transcriptase

(Promega Corporation, Madison, WI, USA) and oligo (dT)18 (Thermo Fisher Scientific Inc., Wilmington, MA, USA) for cDNA synthesis according to the manufacturer's instructions. For RT-qPCR measurements, a BioRad CFX96 Touch Real-Time Detection System was used with 1  $\mu$ L 4-fold diluted cDNA, 200 nM forward and reverse primers (primer sequences are available in Table 3), 2.5  $\mu$ L PCR BIO Mastermix (PCR Biosystem Ltd., London, United Kingdom) and 2.5  $\mu$ L molecular grade water. Relative transcript levels were determined with the  $2^{-\Delta\Delta C_t}$  method [52], with AtActin8 as the internal control gene, and values were compared to the control Col-0 genotype grown under L1 light condition.

**Table 3.** Primer sequences.

Gene Name		Primer Sequences (5' → 3')	Amplicon Size (bp)	References			
AtActin8	forward	TTACCCGACGGACAAGTGATC	73				
	reverse	ATGATGGCTGGAAAAGGACTTC					
AtADC2	forward	GCGATGGACCACACAGCTTT	64		[53]		
	reverse	AGAACATCCGCTGAGGACTGA					
AtSPDS2	forward	TTGCCCGTGAAGAGACCTAGA	72				
	reverse	TCCACCGTTCTCTGTTTCCAT					
AtSPMS	forward	TGGCTCCATACTCATCTTATGAA	72				
	reverse	CGCATAGTGAACACTTTTGAATG					
AtPAO2	forward	GGAATGCCGGAAGATCTTCCGTGATTGTGATCGG	142				[54]
	reverse	CGATTCCAACACCGAGATTGCATACTCCATGCAGC					
AtPAO5	forward	GTTGGGATGAACCAGAAGGA	132	[55]			
	reverse	GAGGAGCCTCGTAAGAAGA					
AtCuAO1	forward	AGCTGGCGACATTCTGAGAT	238	[29]			
	reverse	GTCCAGCATCATCTCCCTA					
AtCuAO3	forward	GTAAGTTTGTCCTACTCCCCC	153				
	reverse	GCCACTCGACAAAGTACCCCC					

#### 4.5. Statistical Analysis

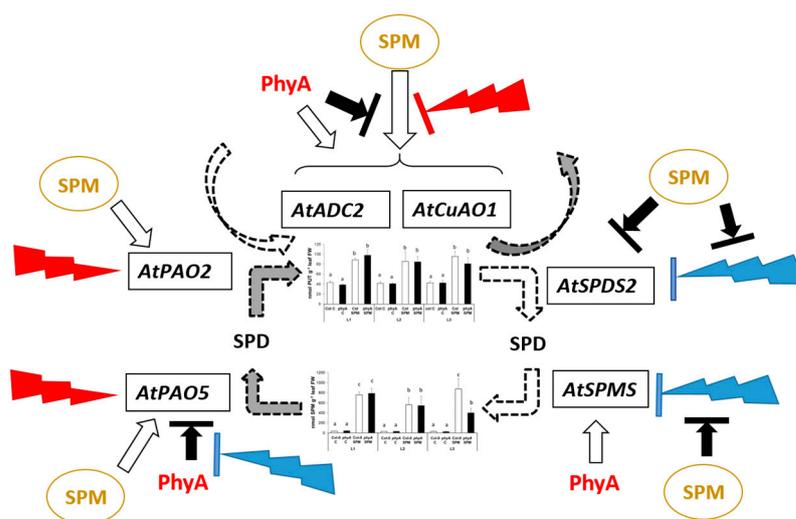
The results are the means of 14 biological replicates for biomass parameters, five biological replicates for chlorophyll-a fluorescence induction measurement, and at least three biological replicates for chromatographic determinations. All reactions for gene expression analyses were performed in triplicate using 3 biological and 3 technical repetitions. The data were statistically evaluated using the standard deviation in Microsoft Excel (STDEV.S function) with  $n \geq 3$ . Different letters indicate statistically significant differences ( $p < 0.05$ ) between multiple groups (one-way ANOVA with Duncan's post hoc test was performed using SPSS 16.0). Pearson's correlation coefficients were calculated using the SPSS 16.0 version. The principal component analysis (PCA) was carried out in the R environment (ver. 4.0.3) using the packages FactoMineR, factoextra and ggplot2.

#### 5. Conclusions

Light spectrum significantly affects PA metabolism-related genes. Under the used experimental conditions, the blue light influences the synthesis side, while the far-red light affects on the catabolism and back-conversion of the PAs. The observed responses to elevated far-red light were found to be less dependent on PhyA, as the mutation alone induced no remarkable differences. Contrarily, the blue light response seems to be highly dependent on PhyA signaling. PhyA under higher blue light irradiance downregulated

the transcript levels of genes involved in PA synthesis, back-conversion and degradation compared to the white light.

Our results demonstrated that *phyA* mutation has some influence on PA metabolism. The PA metabolism-related gene expression of the wild type and the *phyA* mutant plants responded more similarly under white and far-red light conditions than under blue light conditions. The highest differences between the two genotypes were observed under the blue light treatment. Nevertheless, the PA contents were similar under all the light conditions in the two genotypes without SPM application, suggesting that a stable PA pool is important for normal plant growth conditions even under different light spectral conditions. The proper shift in PA levels required a well-maintained dynamic balance of PAs through fine-tuning of PA metabolism. However, SPM treatment brought out more differences in metabolite and gene expression levels. After SPM treatment, the blue dominant spectral regime has more similar effects on PUT synthesis/catabolism and SPM to PUT canalization to the white light than the far-red light conditions. The additive effect of differences observed on the synthesis, back-conversion and catabolism side of PUT metabolism may be responsible for the similar PUT content pattern under all light conditions, even in the presence of an excess SPM. However, the regulatory system was well fine-tuned, and no alterations were detected at the level of PAs; only the SPM content differed significantly between the two genotypes after SPM treatment under blue light conditions. Figure 6. presents the hypothesized mode of action of SPM treatment on PA metabolism under different light spectral compositions and the influence of PhyA on it.



**Figure 6.** Schematic mode of action of spermine (SPM) treatment on polyamine metabolism under different light spectral compositions and influence of PhyA on it. PUT: putrescine, SPD: spermidine, SPM: spermine content, *AtADC2*: arginine decarboxylase 2, *AtSPDS2*: spermidine synthase 2, *AtSPMS*: spermine synthase, *AtCuAO3*: cooper amine-oxidase 3, *AtPAO2* and *AtPAO5*: polyamine oxidase 2 and 5. Blue lightning arrows indicate blue light treatment, red lightning arrows indicate far-red light treatment, SPM indicates spermine treatment, and PhyA means the presence of the wild type of phytochrome A. Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test.

Nevertheless, as PhyA may not only serve as a photoreceptor but regulates genes other than light-responsive ones (including members of morphogenesis, hormone, stress, and defense signaling pathways) through the interaction with corresponding transcription factors, further research is necessary to uncover the proper mechanisms of interaction between PAs and light-regulated processes.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants12081689/s1>, Supplementary Figure S1. (a) The actual quantum efficiency of photosystem II [Y(II)] chlorophyll-a fluorescence induction parameter determined at the steady state level of photosynthesis of the leaves (b) of wild type (Col-0) and mutant (*phyA*) *Arabidopsis* plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD (n = 5 for Y(II)). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test. (b) The chlorophyll fluorescence imaging screens of Y(II) in the leaves under different treatments and light conditions created by the Imaging PAM instrument. The colored bar shows the range of pixel intensity values. Supplementary Figure S2. Confirmation of T-DNA insertion in *phyA* mutant. PCR of the genomic DNA of Col-0 and *phyA* mutant plants. PCR fragments were obtained using the primer pairs LP and RP and/or LBb1.3 (T-DNA specific primer) and RP, respectively. Primers are listed in Supplemental Table S1. Supplementary Table S1. Primer sequences for genotyping of *phyA* mutation.

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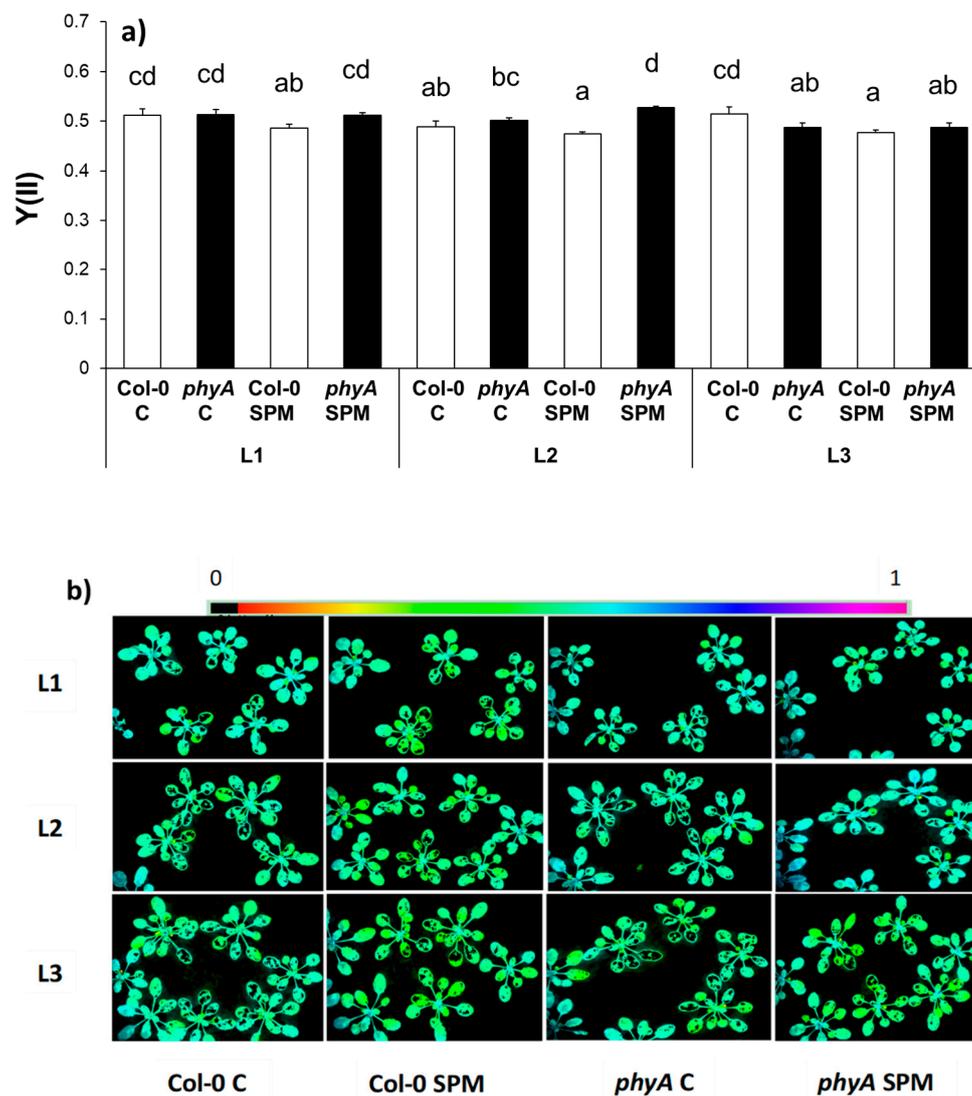
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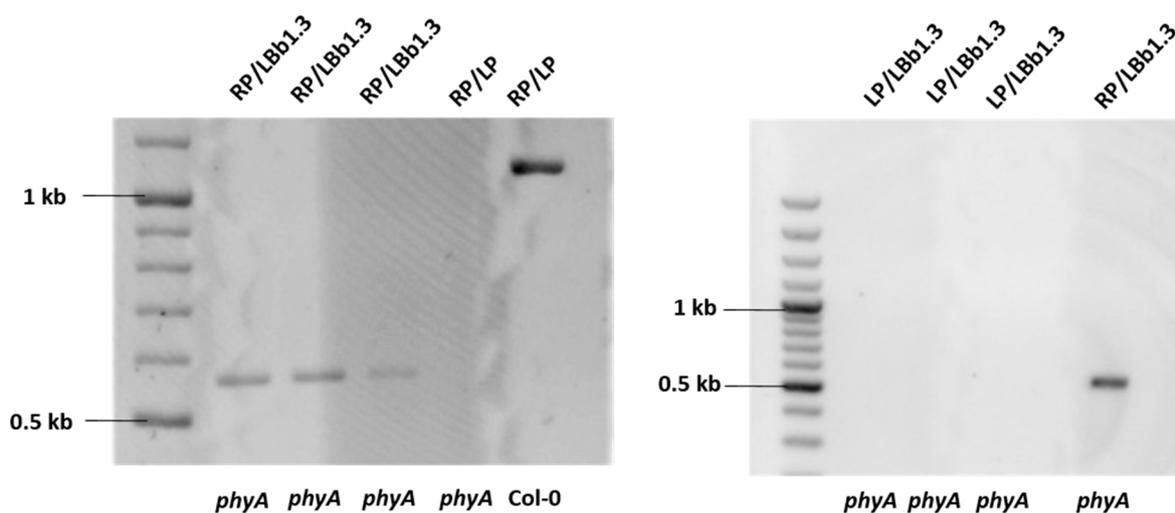
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**Suppl. Figure S1.** (a) The actual quantum efficiency of photosystem II [Y(II)] chlorophyll-*a* fluorescence induction parameter determined at the steady state level of photosynthesis of the leaves (b) of wild type (Col-0) and mutant (*phyA*) *Arabidopsis* plants grown under three different light regimes (L1: blue%: 19.139, green%: 30.62, red%: 48.8 and far-red%: 1.44; L2: blue%: 19.57, green%: 29.57, red%: 40.43 and far-red%: 10.43; L3: blue%: 39.38, green%: 28.76, red%: 30.53 and far-red%: 1.33) treated with or without 0.5 mM spermine (control: C and spermine: SPM). Values are means  $\pm$  SD (n=for Y(II)). Different letters indicate statistically significant differences at  $p < 0.05$  level, using Duncan's post hoc test. (b) The chlorophyll fluorescence imaging screens of Y(II) in the leaves under different treatments and light conditions created by Imaging PAM instrument. Coloured bar shows the range of pixel intensity values.



**Suppl. Figure S2.** Confirmation of T-DNA insertion in *phyA* mutant. PCR of the genomic DNA of Col-0 and *phyA* mutant plants. PCR fragments were obtained using the primer pairs, LP and RP and/or LBb1.3 (T-DNA specific primer) and RP, respectively. Primers are listed in Supplemental Table 1.



**Suppl. Table S1.** Primer sequences for genotyping of *phyA* mutation.

Gene name	Primer sequences (5' → 3')	
<i>phyA</i> mutant (NASC code: 66049)	phyA_LP	CCAGTCAGCTCAGCAATTTTC
	phyA_RP	AATGCAAAACATGCTAGGGTG
	phyA_LBb1.3	ATTTTGCCGATTCGGAAC

# **Appendix E**



## Research Paper

## Cadmium stress responses under white or blue light are influenced by putrescine pre-treatment in wheat

Altafur Rahman, Katalin Nagy, Kamirán Áron Hamow, Magda Pál\*, Tibor Janda, Mihály Dernovics, Csaba Szóke, Gabriella Szalai

Hungarian Research Network (HUN-REN), Centre for Agricultural Research, Agricultural Institute, Hungary Martonvásár, Brunszvik 2, H-2462, Hungary



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## ABSTRACT

Blue light plays an important role in most plant functions: it influences plant morphology, photosynthesis, primary and secondary metabolism. Although certain studies have already revealed that blue light may have positive effects under certain stress conditions, its exact mechanism is largely unknown. The importance of polyamines in stress tolerance has been widely investigated; however, their interaction with the dependent processes is still poorly understood. In addition, the quality of light can also affect the polyamine metabolism, thus it can influence their roles and relationships with other protective compounds. According to these, the main question of the present work was that whether blue light induces different responses during Cd stress, especially which are related to polyamine metabolism, and whether it may be able to modify the protective effect of exogenous putrescine compared to white light conditions in wheat. It has been demonstrated that less pronounced Cd stress was detected under blue light than at white light conditions. Blue light had its own effect at metabolite and gene expression levels and the lower Cd uptake was accompanied by lower phytochelatin but higher conjugated polyamine accumulation at the same time. Putrescine pre-treatment had protective effect especially under white light conditions, and it highlighted certain differences observed under Cd stress between blue light and white light conditions, especially in phytochelatin synthesis, polyamine metabolism, and accumulation of phenolic compounds and plant hormones. Our data demonstrated that blue light regulated Cd tolerance in wheat and modified defence strategy when excess putrescine was present.

## 1. Introduction

One of the major toxic metal pollutants with high penetration ability, is cadmium (Cd). Cd induces a number of physiological, biochemical and transcriptional changes in plants. Besides the visual symptoms, the disturbance of photosynthesis, disruption of nutrient uptake/transport and formation of free radicals, several defence mechanisms are also activated to reduce the toxic effects and accumulation of Cd in plants (Li et al., 2023). The activation of nitrate and sulphate assimilation

pathways and the chelation of Cd with phytochelatin (PCs) followed by the compartmentalisation of PC-Cd complexes into the vacuoles is usually suggested to be the most important defence mechanism against Cd toxicity. Parallel with the thiol origin chelator compounds (PCs and their precursor, glutathione: GSH), other non-thiol origin molecules, such as proline, polyamines (PAs) and organic acids are also involved in the metal-chelation. GSH participates not only in metal detoxification – either by itself or as the precursor of PCs – but it is involved in both the direct and indirect controls of ROS levels (Anjum et al., 2015). Parallel

**Abbreviations:** ABA, abscisic acid; Cys, cysteine; CG, cysteinyl-glycine; DAP, 1,3- diaminopropane; GA1, gibberellin A1;  $\gamma$ -EC, gamma-glutamyl-cysteine; GSH, glutathione; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; IAA, indole-3-acetic acid; JA, jasmonic acid; MDA, malondialdehyde; *p*CA, *para*-coumaric acid; Met-*p*CAGM, methyl-*para*-coumaroyl-*agmatine*; N-FPUT, N-feruloyl-putrescine; N-pCPUT, N-*para*-coumaroyl-putrescine; ohGSH, hydroxy-methyl-glutathione; *p*HBA, *para*-hydroxy-benzoic acid; PA, polyamines; *p*CHAGM, *para*-coumaroyl-hydroxyagmatine; PC, phytochelatin; PCS, phytochelatin synthase; PUT, putrescine; SPD, spermidine; SPM, spermine; SA, salicylic acid, *TaICS*, isochorismate synthase gene; *TaNCED*, 9-cis-epoxycarotenoid dioxygenase gene; *TaPAL*, phenylalanine-ammonia-lyase gene; *TaPCS*, phytochelatin synthase gene; *TaPUT*, polyamine uptake transporter gene; *TapxPAO*, peroxisomal polyamine oxidase gene; *TaSPDS*, spermidine synthase gene.

\* Corresponding author.

**E-mail addresses:** [altafur.rahman@atk.hun-ren.hu](mailto:altafur.rahman@atk.hun-ren.hu) (A. Rahman), [nagy.katalin@atk.hun-ren.hu](mailto:nagy.katalin@atk.hun-ren.hu) (K. Nagy), [hamow.kamiran@atk.hun-ren.hu](mailto:hamow.kamiran@atk.hun-ren.hu) (K.Á. Hamow), [pal.magda@atk.hun-ren.hu](mailto:pal.magda@atk.hun-ren.hu) (M. Pál), [janda.tibor@atk.hun-ren.hu](mailto:janda.tibor@atk.hun-ren.hu) (T. Janda), [dernovics.mihaly@atk.hun-ren.hu](mailto:dernovics.mihaly@atk.hun-ren.hu) (M. Dernovics), [szoke.csaba@atk.hun-ren.hu](mailto:szoke.csaba@atk.hun-ren.hu) (C. Szóke), [szalai.gabriella@atk.hun-ren.hu](mailto:szalai.gabriella@atk.hun-ren.hu) (G. Szalai).

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changes in PAs and GSH and/or PC levels under heavy metal stress have been well studied in plants (Sharma and Dietz, 2006), however, only a few studies take into account that the synthesis of PCs and higher PAs (spermidine: SPD and spermine: SPM) partly overlap due to a common precursor, cysteine (Cys) (Pál et al., 2018).

PAs are ubiquitously distributed in all cells at relatively high concentrations and regulate several processes during normal plant development at cellular, physiological and biochemical levels (membrane and protein stabilisation, maintaining the osmotic balance, transcription, translation, photosynthesis, etc.) (Tyagi et al., 2023). Apart from these, they can act as direct scavengers of ROS or activate the antioxidant enzymes, and they are also supposed to be important metal chelators. In addition, it is also evident that PAs play role in plant signalling under stress conditions (Hasanuzzaman et al., 2019; Tyagi et al., 2023). Both the overexpression of PA synthesis-related genes and the exogenous PA applications have been reported to have protective effects during various abiotic stresses. It has been recently reviewed, that different PA treatments (putrescine: PUT, SPD or SPM) applied e.g. as seed soaking, foliar spray or in hydroponic culture, induced Cd tolerance in several plant species, such as wheat, and the protective effects were coupled with for example increased antioxidant activities and GSH content and modulated plant hormone levels (Hasanuzzaman et al., 2019). It has also been demonstrated that during Cd stress in wheat plants, only PUT had a positive effect in a hydroponic solution, while as seed soaking both PUT and SPD treatments were beneficial. The positive effect of PUT/SPD seed priming and PUT hydroponic treatment was associated to lower salicylic acid (SA) and proline accumulation but higher gene expression level of PC synthase (PCS) compared to the Cd treatment alone. While the damaging effect of the hydroponic SPD treatment before Cd application resulted from the excessive accumulation of endogenous PAs (Tajti et al., 2018). Nevertheless, when PUT pre-treatment preceded Cd application in rice, it enhanced Cd-induced oxidative stress and the accumulation of PAs, while it decreased the PC synthesis (Pál et al., 2017). Thus, the positive effect depends on the type of the applied PA, the mode of application, the plant species, and the given PA metabolism intensity in the plant.

It was clarified that PAs are involved both in direct interactions with a number of metabolic routes and in hormonal cross-talk (Tyagi et al., 2023). According to these, the exact regulation of the PA metabolism is very important. Although, the relationship between PAs and photosynthesis has been known for a long time, the exact regulatory role of light quality in PA metabolism has only now started to be outlined (Pál et al., 2021). Light spectra influence primary and secondary metabolism, including groups of several protective compounds like amino acids, flavonoids, ascorbate or thiol compounds, too (Toldi et al., 2019; Gyugos et al., 2021). A detailed comparison of the effect of dominant red+far-red and dominant blue light conditions on PA metabolism in wheat revealed that despite the similar PA contents, completely opposite gene expression profiles of PA metabolism-related genes were found in plants grown under red+far-red or blue lights, and light quality may modify plant growth by the influence of PA metabolism via changes in gene expression. In addition, hydroponic PA (PUT, SPD and SPM) treatments under these conditions could reverse the antagonistic effects of red+far-red and blue light on PA metabolism in the leaves at gene expression level (Pál et al., 2022).

Light also has a crucial role in stress tolerance, and light spectrum plays an important role in the adjustment of metabolism, for example during drought stress in wheat plants (Gyugos et al., 2021). Manipulating the supplemental light spectrum has also been reported to reduce the negative effects of frost stress (Ahres et al., 2023). Generally, plants grown with supplementary blue light are shorter and have smaller, thicker and darker green leaves compared to plants grown without it (Vitale et al., 2020). Nevertheless, blue light is essential during the early vegetative stage for healthy plant development and it establishes more compact morphology (Kalaitzoglou et al., 2021), while it can increase the photosynthetic activity as well (Yang et al., 2018). Blue light also

induced Cd tolerance in cucumber (Guo et al., 2022) and rice (Sebastian and Prasad, 2014), and the tolerance was associated to the lower Cd uptake or translocation, higher GSH accumulation and antioxidant enzyme activities.

Based on these listed results, it is evident that PCs and PAs have important role in Cd stress responses and even protection, and their relationship at synthesis level is a special crucial investigation point of view. As light spectrum composition can modify plant metabolism, so the PA metabolism, it is also important to reveal the function of light quality in the responses of plants to heavy metals, especially in PA metabolism and PC synthesis, which is largely unknown. Therefore, in the present study, a hydroponic experiment was conducted to investigate the effects of dominant blue light on Cd-induced responses in wheat, and the influence of blue light on the putative protective effect of PUT against Cd stress compared to the white light conditions. Similarities and differences were analysed at metabolite and gene expression levels, with special regards on how the observed alterations are connected to the PA metabolism, and how changes in PA metabolism and PCs synthesis relate to each other.

## 2. Materials and methods

### 2.1. Plant material, growth conditions, and treatments

Winter wheat (*Triticum aestivum* L.) variety 'Mv Béres' was used in the present experiment. After germination (3 days at 26 °C in the dark), seedlings were planted on stainless steel net into plastic pots (15 plants/plastic pot) covered with black paper disk and grown on modified Hoagland solution (250 ml/pot) (Pál et al., 2005) in Conviron PGR-15 plant growth chambers (Controlled Environments Ltd., Winnipeg, Canada) under the following conditions: 22/20 °C day/night temperature with 16/8-h light/dark periodicity and 75% relative humidity. Pots were placed in the growth chamber in a fully randomized manner. The nutrient solution was renewed every 2 days in case of all the treatments.

The plants, from the beginning, were grown under two different spectral light conditions, referred as white (W) and blue (B) light treatments. Under W light, 31.6% blue, 47% green, 20.4% red and 1% far-red composition, while under B light, 82.1% blue, 1% green, 16.7% red and 0.2% far-red composition was applied at 250  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD). The detailed characteristics of the light regimes are presented in Table A.1. Light spectral conditions were established using modules equipped with LEDs as previously described by Monostori et al. (2018).

7-day old seedlings were divided into two groups, and one part of the plants was further grown in hydroponic solution without any treatment, while the other part of the plants was treated hydroponically with 250 ml of 0.5 mM PUT. After 7 days, the roots were washed in distilled water twice, then half of both groups of plants was exposed to 50  $\mu\text{M}$  Cd ( $\text{NO}_3$ )<sub>2</sub> for 7 days. According to these, four groups were formed under both light regimes, namely control (C), PUT-pretreated (Put), Cd-treated (Cd) and PUT- treated followed by Cd treatment (Put+Cd).

In order to clearly distinguish the PUT treatment from the endogenous PUT content, the treatment was referred to as Put, while the content as PUT. At the end of the treatments the leaves and the roots of the plants were sampled for further analysis. Plants from six containers were used for the detailed analyses for every treatment.

### 2.2. Determination of Cd and hydrogen peroxide contents and the level of lipid peroxidation

For the determination of Cd contents, leaves and roots were fried, and after the microwave digestion method with a  $\text{HNO}_3/\text{H}_2\text{O}_2$  mixture, the inductively coupled plasma optical emission spectrometry method was applied. The measurement was performed by Eurofins Minerág Ltd. (Szekszárd, Hungary). The ferrous ammonium sulphate/xylene orange (FOX-1) method was performed to determine  $\text{H}_2\text{O}_2$  contents of the

samples according to Gay et al. (1999) spectrophotometrically at 560 nm, using a H<sub>2</sub>O<sub>2</sub> calibration curve. Malondialdehyde (MDA) level was estimated spectrophotometrically according to Pál et al. (2005) at 532 nm with the subtraction of non-specific absorption at 600 nm (extinction coefficient of 155 mM<sup>-1</sup>cm<sup>-1</sup>). For all the spectrophotometric measurements a UV-visible spectrophotometer (160 A, Shimadzu, Kyoto, Japan) was used.

### 2.3. Antioxidant enzyme assays

The extraction and the measurement of antioxidant enzyme activities, namely glutathione reductase (GR) (EC 1.6.4.2.), glutathione-S-transferase (GST) (EC 2.5.1.18.) and ascorbate peroxidase (APX) (EC 1.11.1.11.) were performed according to the detailed description of Pál et al. (2005).

### 2.4. Determination of thiol contents

The contents of cysteine (Cys), *gamma*-glutamyl-cysteine (*γ*-EC), hydroxy-methyl-glutathione (ohGSH), glutathione (GSH), and cysteinyl-glycine (CG) a metabolite of GSH catabolism, were measured as described by Pál et al. (2017). For HPLC separation and analysis an Alliance 2690 system (Waters, Milford, MA, USA) with a Hyperprep HS C18 column (250 × 4.6 mm, 8 μm) (Thermo Fisher Scientific Inc. Waltham, MA, USA) was used equipped with a W474 fluorescence detector (Waters, Milford, MA, USA) (excitation wavelength: 380 nm, emission wavelength: 480 nm).

### 2.5. Determination of in vivo phytochelatin (PC) content and in vitro phytochelatin synthase (PCS) activity

The PCs levels and PCS enzymes activity were measured by HPLC analysis according to Pál et al. (2017) on a reverse phase column (Hypersil ODS, 100 × 2.1 mm, 5 μm, Thermo Fisher Scientific) using an Alliance 2690 system and UV W996 photodiode array detector (Waters).

### 2.6. Analysis of PUT, SPD, SPM, and 1,3-diaminopropane (DAP)

The analysis of dansylated derivatives of PAs was carried out according to Pál et al. (2022) via HPLC using a W2690 separation module on a reverse phase column (Kinetex C18, 5 μm, 100 × 4.6 mm, Phenomenex, Inc. Torrance, CA, USA) and a W474 scanning fluorescence detector (Waters) (excitation wavelength: 340 nm; emission wavelength: 515 nm).

### 2.7. Analytical procedure of conjugated polyamines

Estimation of certain polyamine derivatives (*para*-coumaroyl-hydroxyagmatine: *p*CHAGM, methyl-*para*-coumaroyl-agmatine: Met-*p*CAGM, *N*-feruloyl-putrescine: *N*-FPUT and *N*-*para*-coumaroyl-putrescine: *N*-*p*CPUT) was performed using a Vion ion mobility quadrupole time-of-flight mass spectrometer (Waters) equipped with a UniSpray (Waters) ion spray source (UPLC-ESI-MS-QTOF-MS) as described in details by Szalai et al. (2022). The standards and identification of conjugated PAs can be found in supplementary information (Table A.2).

### 2.8. Analytical determination of plant hormones

Measurement of plant hormones was carried out according to Pál et al. (2019). The parameters of target compounds (indole-3-acetic acid: IAA, *para*-hydroxy-benzoic acid: *p*HBA, SA, paracoumaric acid: *p*CA, jasmonic acid: JA, abscisic acid: ABA and gibberellic acid A1: GA1) were presented in Table A.3. The UPLC-MS/MS analysis was performed on a Acquity I class UPLC system coupled to a Xevo TQ-XS (Waters, Milford, MA, USA), with a UniSpray ion source (US) in timed MRM mode.

### 2.9. Gene expression measurement

Total RNA was extracted and cleaned using TRI Reagent® and Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). Total RNA was reverse transcribed by using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) and oligo(dT)18 (Thermo Fisher Scientific). PCR BIO SyGreen Mix (PCR Biosystems, London, UK) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) were used for the quantitative real-time PCR reaction according to Tajti et al. (2021), Paolacci et al. (2009), Xiong et al. (2017), Pál et al. (2019, 2022), Gondor et al. (2021), Kovács et al. (2014), and Gallé et al. (2013). For primer sequences see Table A.4.

### 2.10. Statistical analysis

Three independent experiments were performed, and the most representative data are presented here. The results represented at least 3 biological replicates. The data were statistically analysed by means of multifactorial analysis of variance, using the Agronomix Inc. (Winnipeg, Canada) software and the Microsoft Windows® Excel program. Duncan post-hoc test was performed using SPSS 16.0 (IBM, Armonk, NY, USA). Principal component analysis (PCA) of the investigated parameters was visualised by Origin 2021 (OriginLab, Northampton, MA, USA).

## 3. Results

### 3.1. Cadmium content, lipid peroxidation, hydrogen peroxide content, and antioxidant enzyme activities

During Cd stress, Cd was taken up by the roots, and also transported to the leaves, but the majority of the Cd was retained in the roots. Under the present conditions, neither the light conditions nor the Put pre-treatment influenced the leaf Cd content after Cd treatment, and similar levels were detected in all the Cd-treated ones (Table 1). However, less Cd was detected in the roots of the B light-treated plants compared to those grown under W light. In addition, Put pre-treatment inhibited the Cd uptake under both light conditions, thus the lowest Cd content was detected in the roots of plants treated with Put+Cd under B light conditions (Table 1). The lower Cd uptake could be partly responsible for the lower level of lipid peroxidation in the leaves, and lower H<sub>2</sub>O<sub>2</sub> content in the roots of plants grown under B light after Cd stress compared to the values detected under W light (Table 1). But, the combined Put+Cd treatment can also decrease the Cd-induced stress under W light growth conditions, which was manifested in the lower leaf MDA and root H<sub>2</sub>O<sub>2</sub> contents, while it could not influence them under B light compared to the Cd application alone (Table 1).

Under B light higher initial enzyme activities were detected in the case of GR both in the leaves and roots, and in addition in the case of root APX (Table 1). In the leaves, Cd decreased the GST and APX activities under W light, but could not influence either of the investigated antioxidant enzymes under B light. Put pre-treatment increased the GR and GST activities under W light, and the GR and APX activities under B light. The additive effect of the Put+Cd treatment was found in the case of GR activity under both light conditions. In the roots, more pronounced effects of the Cd stress could be detected, as the GR activity increased after Cd application under W light, in addition to the GST and APX activities under both light conditions. Put pre-treatment alone caused only slight modifications, namely increased the GST and decreased the APX activities under B light. The additive effect of the combination of Put pre-treatment and Cd stress under B light resulted in the highest GR and GST activities, while the lowest APX activity among the treatments (Table 1).

### 3.2. Thiol content and phytochelatin synthesis

The total levels of Cys, *γ*-EC, ohGSH, GSH and the catabolite product

**Table 1**

Cadmium (Cd), malondialdehyde (MDA) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) contents and activities of glutathione reductase (GR), glutathione-S-transferase (GST) and ascorbate peroxidase (APX) enzymes in the leaves and roots of wheat plants grown under control (C) conditions or treated with 50 μM Cd for 7 days with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions. “nd” means under the detection limit. Data are mean values ± SD. Statistically significant differences indicated with different letters at  $p < 0.05$  level.

		W				B			
		C	Cd	Put	Put+Cd	C	Cd	Put	Put+Cd
Cd content (mg kg <sup>-1</sup> DW)	leaf	nd	36.6±2 a	nd	46.7±12.5 a	nd	31.3±4.7 a	nd	39.4±9.8 a
	root	nd	653±31.4 c	nd	572.7±51.7 b	nd	607±13 b	nd	460.6±47.6 a
MDA content (nmol g <sup>-1</sup> FW)	leaf	17.7±0.5 cd	21.1±1.6 e	16.2±0.4 a	18.0±0.6 d	12.8±1.5 a	15.6±1.5 b	13.2±0.5 a	16.0±0.5 bc
	root	3.4±0.3 ab	4.2±0.05 c	3.1±0.7 ab	3.8±0.7 bc	2.8±0.1 a	4.4±0.6 c	3.3±0.5 ab	3.7±0.4 bc
H <sub>2</sub> O <sub>2</sub> content (μM g <sup>-1</sup> FW)	leaf	88.7±1.8 ab	97.2±8.0 bc	88.2±3.0 ab	97.2±4.7 bc	80.2±7.0 a	89.5±8.8 ab	87.5±4.9 ab	100.4±6.6 c
	root	37.9±0.7 a	76.2±7.8 c	39.7±4.3 a	45.3±4.8 a	42.0±2.7 a	61.1±8.6 b	41.4±5.6 a	57.3±8.2 b
GR (nkatal g <sup>-1</sup> FW)	leaf	14.1±0.2 a	13.6±0.4 a	16±0.8 b	17.7±1.2 c	19.2±0.9 cd	18.8±1.4 c	20.8±0.6 de	21±1.2 e
	root	2.5±0.3 a	4.7±0.3 c	2.8±0.4 ab	4.7±0.3 c	3.3±0.3 b	3.3±0.2 bc	3±0.4 ab	6.7±0.5 d
GST (nkatal g <sup>-1</sup> FW)	leaf	9.4±0.6 bc	7.1±0.6 a	14.2±0.4 d	8.4±1.3 ab	10.8±0.4 c	10.3±0.8 bc	17.1±1.4 e	14.3±2.1 d
	root	8.2±0.3 b	13.1±1 d	6.8±0.4 ab	13.3±0.8 d	5.6±0.7 a	10.5±0.8 c	7.6±0.7 b	13.8±1.6 d
APX (nkatal g <sup>-1</sup> FW)	leaf	28.6±0.8 c	22.2±1.5 ab	25.4±2.3 bc	18.8±1.3 a	25.9±1.5 bc	26.3±3.1 bc	35.3±5.6 d	28.6±5.1 c
	root	27.2±0.8 b	50.4±4.1 d	27.2±3.4 b	50±6.3 d	38.8±2.3 c	54±2 d	28.1±2.3 b	21±2 a

of GSH, CG were detected in the leaves and roots (Table 2). The initial levels of them were similar under W and B light conditions, except for GSH, which was higher under B light treatment both in the leaves and roots.

In the leaves, no remarkable changes were found in the Cys content under either light condition. Under W light, the γ-EC level could be influenced only by the combined Put+Cd treatment, as decreased, while under B light Put pre-treatment alone or when it was followed by Cd stress increased its level (Table 1). The GSH content decreased by Cd treatment under W and B light conditions, but after Put+Cd treatment this could be reversed. The most characteristic changes in the leaves after Cd stress were observed in the ohGSH levels, as Cd treatment increased it with a higher level under W light, however, the combined Put+Cd treatment had no further effect on it. The CG content showed only slight changes, which were partly similar to those described for γ-EC and GSH (Table 1).

In the roots, almost the same patterns were detected under W or B light conditions. The Cys content increased after Cd stress, and the Put pre-treatment could not influence it. A similar tendency was observed for the γ-EC content. The GSH content decreased by the Cd exposure, Put pre-treatment and the combination of them under W light, with the highest decrease after Cd treatment alone. While the level of ohGSH

showed similar, but less statistically significant changes as in the case of γ-EC content. Changes in CG content showed an opposite tendency compared to Cys, γ-EC or GSH, as Cd treatment decreased it under both light conditions, and Put pre-treatment alone could increase it under W light, thus the combined Put+Cd treatment partly reversed the Cd-induced decrease (Table 1).

As GSH itself is not only an antioxidant and metal chelator, but the precursor of the PCs, the synthesis of PCs at metabolite and gene expression levels were also investigated. Neither of the treatments could influence the PC content in the leaves (data not shown), but Cd and Put+Cd treatments induced different changes in the root PC composition under W and B light conditions (Table 1). Cd alone induced the synthesis of PC2, PC3 and PC5 under W light, and induced only the accumulation of PC3 and PC5 under B light (Table 1). However, it is an important difference, that the total PC content after Cd stress under W light treatment was almost 3-fold higher than under B light conditions. Put pre-treatment did not influence the PC synthesis, but the combined Put+Cd resulted in altered PC compositions compared to the adequate Cd treatments. Under W light, in the Put+Cd-treated plant roots no PC2 was detected, lower levels of PC3 and PC4 were found, but accumulation of the longer chain length PC6 was detected (Table 1). Compared to these, under B light conditions, the combined Put+Cd treatment

**Table 2**

The effect of 7 days of 50 μM Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on total cysteine (Cys), gamma-glutamyl-cysteine (γ-EC), glutathione (GSH), hydroxy-methyl-glutathione (ohGSH) and cysteinyl-glycine (CG) contents in the leaves and roots of wheat plants. Data are mean values ± SD. Statistically significant differences indicated with different letters at  $p < 0.05$  level.

		W				B			
		C	Cd	Put	Put+Cd	C	Cd	Put	Put+Cd
Cys	leaf	5.4±0.3 ab	5.3±0.5 a	6.5±1.1 bc	6.6±0.6 c	5.8±0.2 abc	6.3±0.5 abc	6.0±0.2 abc	6.6±0.9 bc
	root	2.2±0.1 b	1.8±0.2 b	2.2±0.1 b	1.2±0.1 a	2.2±0.1 b	1.8±0.2 b	3.6±0.3 d	3.2±0.3 c
γ-EC	leaf	94.6±5.9 b	75.4±6.4 a	129.0±4.9 c	102.9±5.0 b	147.3±12.5 d	127.5±12.7 c	161.0±5.7 d	148.6±18.5 d
	root	18.9±2.1 ab	56.1±2.8 d	25.8±4.6 bc	64.3.6±10.4 d	11.5±0.6 a	29.0±6.8 c	16.0±1.4 a	33.2±3.8 c
GSH	leaf	1.7±0.2 bc	1.1±0.05 a	1.9±0.03 c	1.2±1.1 a	1.7±0.1 bc	1.6±0.2 b	2.2±0.2 d	1.6±0.1 b
	root	4.1±2.5 a	17.3±3.3 c	4.5±3.0 a	16.6±2.9 c	1.7±0.1 a	8.7±5.8 ab	3.6±0.11 a	8.8±1.5 b
ohGSH	leaf	0.3±0.01 a	0.5±0.1 bc	0.6±0.4 ab	0.6±0.1 c	0.3±0.04 a	0.5±0.1 bc	0.2±0.1 a	0.4±0.5 ab
	root	56.5±3.5 c	34.5±5.1 ab	42.0±7.4 b	45.5±7.2 b	27.9±7.1 a	29.7±9.1 a	29.5±5.5 a	35.7±3.5 a
CG	leaf	10.9±3.5 ab	16.5±2.9 cd	12.3±2.5 bcd	17.1±1.8 d	6.7±0.6 a	11.4±2.2 abc	8.3±5.0 a	11.9±1.6 abcd
	root	2.0±0.4 bc	1.1±0.1 a	2.5±0.03 d	1.7±0.1 b	1.8±0.2 bc	0.9±0.5 a	2.2±0.4 cd	1.0±0.1 a
PC content (nmol g <sup>-1</sup> FW)	Cd	C	Put	Put+Cd	C	Put	Put+Cd	Put+Cd	
	leaf	nd	11.3±5.5 a	nd	nd	nd	nd	37.8±11.6 a	
PC2	leaf	nd	13.1±1.8 b	nd	7.7±4 a	nd	15±3.5 b	nd	
	root	nd	nd	nd	nd	nd	nd	nd	
PC3	leaf	nd	132.2±20.9 b	nd	44.6±20.6 a	nd	40.7±13.6 a	nd	
	root	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
PC4	leaf	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
	root	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
PC5	leaf	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
	root	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
PC6	leaf	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
	root	nd	nd	nd	47.3±6.4 b	nd	nd	nd	
Total PC PCS activity (pkatal g <sup>-1</sup> FW)	leaf	138.5±25.1	98.4±32.8	55±20.4	71.5±25.1	4.6±1.3 ab	4.5±1.5 ab	4.5±1 b	4.9±0.7 b
	root	35.6±3.3 b	131.8±12.5 e	21.3±16.7 ab	92.8±9.1 d	33.9±8.3 ab	100.8±28.4 de	22.5±9 a	52.8±9.3 c

induced PC2 synthesis, decreased the PC3 accumulation, did not influence the level of PC5, but induced the synthesis of PC6. Overall, when Put pre-treatment preceded the Cd stress longer PC compounds (PC6) appeared under both light conditions, but the total PC synthesis decreased under W light, and increased under B light conditions (Table 1).

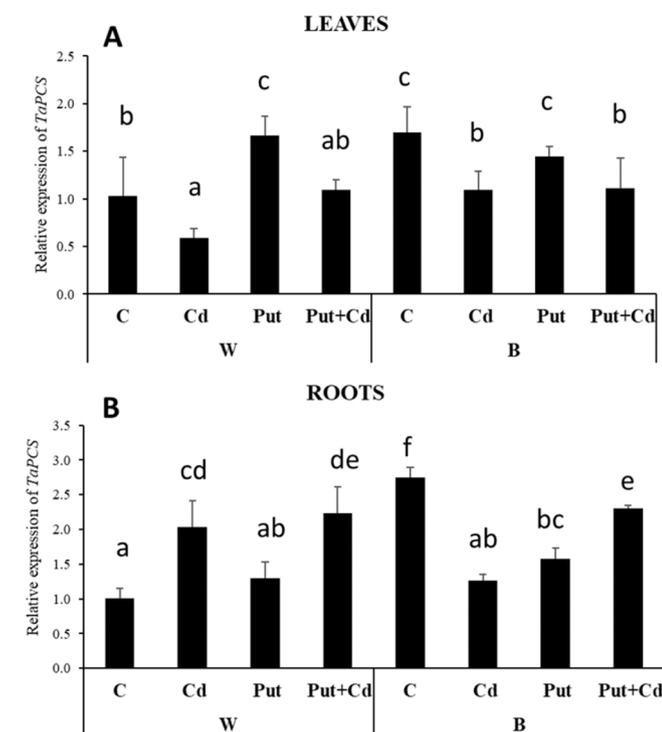
The applied treatments could hardly affect the initially low *in vitro* PCS activity in the leaves (Table 1), but in the roots characteristic changes were observed. Cd induced the *in vitro* PCS enzyme activity both under W and B light conditions, but in the case of the combined Put+Cd treatment it was less pronounced compared to the Cd exposure alone under either light condition (Table 1).

Cd treatment inhibited the gene expression of *TaPCS* in the leaves under both light conditions (Fig. 1), and although Put pre-treatment alone slightly induced it under W light, it could not alleviate the Cd decreasing effect in the combined Put+Cd treatment under either light condition (Fig. 4).

In contrast, in the roots, Cd increased the *TaPCS* transcript level under W light, but decreased it under B light conditions. While under W light the Put pre-treatment could not further influence the effect of Cd, but under B light treatment the expression level of *TaPCS* was higher after the combined Put+Cd treatment than in the only Cd-treated plants. However, it should be mentioned, that in the roots the initial *TaPCS* expression level under B light was more than double that measured under W light.

### 3.3. Polyamine metabolism

PUT, SPD and SPM contents were determined together with the level of DAP, which is one of the side-products of the terminal oxidation of higher PAs (SPD and SPM). The most abundant PA was the SPD both in the leaves and roots (Fig. 2). The initial levels of the individual PAs were almost the same under W or B light conditions, except for leaf SPM



**Fig. 1.** The effect of 7 days of 50  $\mu$ M Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on the gene expression level of phytochelatins synthase gene (*TaPCS*) in the leaves (A) and roots (B) of wheat plants. Values are means  $\pm$  SD. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

content, which was higher, and root PUT level, which was lower under B light conditions (Fig. 2).

In the leaves, Cd stress-induced PUT accumulation under both light conditions, significantly increased the level of SPD only under B light, while decreased the SPM content under either light condition; furthermore, parallel with these, the level of catabolite product, DAP increased after Cd application under both light regimes (Fig. 2a-d). Put pre-treatment did not influence the endogenous PA levels in the leaves. However, in some cases, it could modify the effect of Cd on the PA pool. The combined Put+Cd treatment caused a higher accumulation of PUT than Cd alone, but only under W light. Similar additive effect of the combined treatment was observed in the case of SPD content again only under W light (Fig. 2b). However, the effect of Cd was so dominant on the SPM content, that the Put pre-treatment could hardly further influence it (Fig. 2c). While in the case of DAP, when Put pre-treatment preceded the Cd treatment, less accumulation was found compared to that of the Cd-treated plants under both light conditions (Fig. 2d).

In the roots, the tendency of changes in PUT content were similar to those described for leaves, and here the combined Put+Cd treatment caused the highest PUT accumulation under both light regimes (Fig. 2e). The changes in SPD content under W light was similar to those observed in the leaves, as Put+Cd induced the highest accumulation, but under B light the opposite tendency was found, namely the lowest SPD content detected after the combined treatment (Fig. 2f). A remarkable increase in SPM level was only observed in the Put+Cd-treated plants under W light (Fig. 2g). The root DAP concentration showed only slight and non-characteristic changes (Fig. 2h).

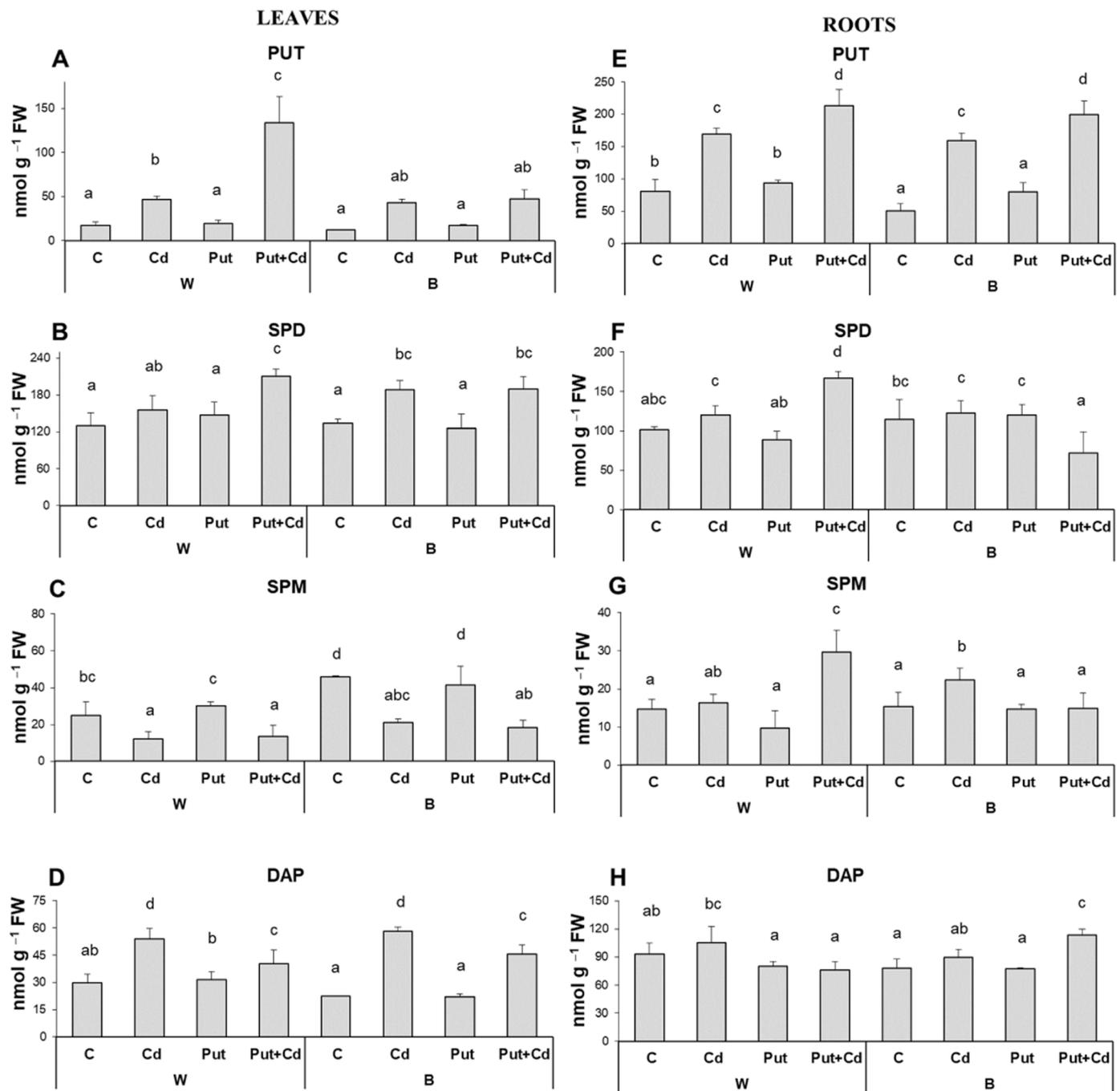
The expression pattern of certain genes related to PA metabolism was also investigated. These genes were chosen based on results of Pál et al. (2022), where significant differences in their transcript level were observed under blue light conditions compared to white or red+ far-red light treatments in wheat plant; in addition, they represent different sub-processes of the PA metabolism. The *TaPUT* encodes a PA uptake transporter, the *TapxPAO* encodes a peroxisomal polyamine oxidase, which is responsible for the back-conversion of SPM/SPD to PUT, while *TaSPDS* encodes a spermidine synthase enzyme.

The *TaPUT* transcript level showed slight fluctuation in the leaves, but was not remarkably influenced by either of the treatments (Fig. 3a), indicating that the increased amount of PA transport protein (PUTs) was not responsible for the changes in the endogenous PUT content after Put pre-treatments. A slight, but significant increase in the expression level of *TaSPDS* was detected only after Put+Cd treatment under W light (Fig. 3b), which was parallel with the above-described SPD accumulation after the same treatment. Interestingly, the *TapxPAO* expression was induced by Put pre-treatment and Put+Cd treatments under both light conditions, with a greater extent under W light, suggesting the increased role of back-conversion in the excess of exogenous PUT (Fig. 3c).

In the roots, compared to the control, under W light the *TaPUT* expression slightly increased after all the treatments (Fig. 4d). The *TaSPDS* transcript level under W light increased after Cd and Put+Cd treatments, similarly to the SPD content in the root, but under B light conditions, no pronounced changes were detected (Fig. 4e). The expression level of *TapxPAO* was also induced by Cd and Put+Cd treatments under W light, but under B light all the treatments decreased it compared to the control, indicating the negative regulation of the back-conversion under B light regime (Fig. 4f).

The actual PA pool is influenced not only by the uptake, the synthesis, the catabolism and/or the back-conversion but also by the conjugation, too. In the present work, the levels of certain conjugated PAs (pCHAGM, Met-pCAGM, N-FPUT and N-pCPUT) were also determined (Table 3).

In the leaves, Cd stress induced the accumulation of the investigated conjugated PAs under both light regimes, but to a greater extent in the case of B light treatment. Put pre-treatment alone did not cause significant changes in the levels of conjugated PAs, but the combined Put+Cd



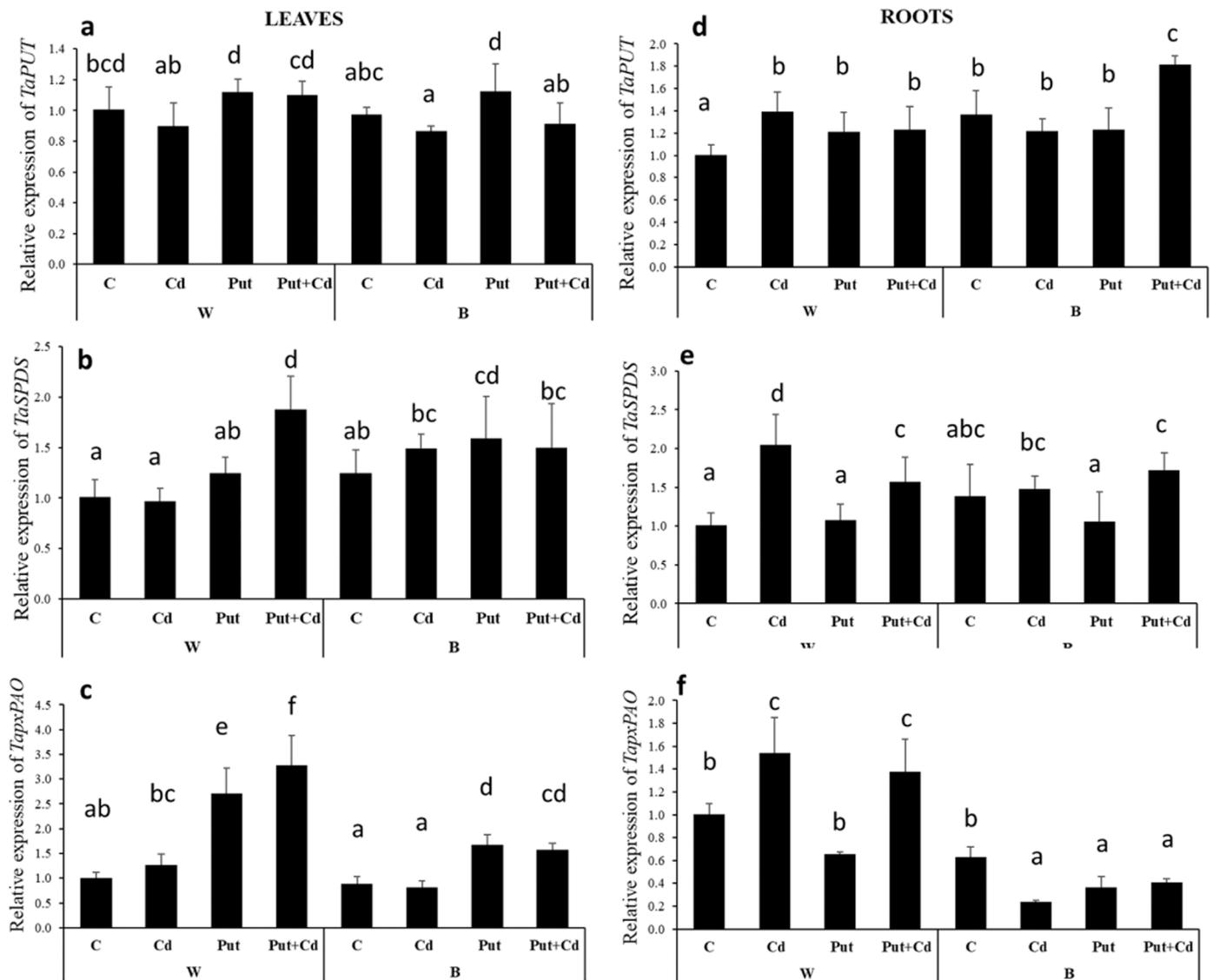
**Fig. 2.** The effect of 7 days of 50  $\mu$ M Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on putrescine (PUT) (a, e), spermidine (SPD) (b, f), spermine (SPM) (c, g) and 1,3- diaminopropane (DAP) (d, h) contents in the leaves (a-d) and roots (e-h) of wheat plants. Values are means  $\pm$  SD. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

treatment induced higher PA conjugation compared to the Cd alone, and this effect was higher under B light (Table 3). Nevertheless, after Cd stress in the roots both alone or in combination with Put pre-treatment, the *p*CHAGM and *N-p*CPUT could not be detected anymore under either light condition (Table 3). Put pre-treatment increased the *p*CAGM and *N-p*CPUT content only under B light regime. The Put+Cd combined treatment caused similar *N*-FPUT accumulation as Cd treatment alone, while the level of Met-*p*CAGM was approximately half of that was detected after Cd treatment alone, under both light conditions. These results showed that the leaves and roots responded differently to the applied treatments. Cd in the leaves induced, while in the roots decreased the degree of PA conjugation. In addition, in the leaves higher

PA conjugation (*N*-FPUT and *N-p*CPUT) was found under B light conditions, with the highest level in Put+Cd-treated plants, which may be responsible for the lack of great accumulation of PUT compared to the white light conditions.

#### 3.4. Contents of plant hormones and certain phenolic compounds and expression level of certain plant hormone synthesis-related genes

Among major types of phytohormones, indole-3-acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA), gibberellin A1 (GA1) and abscisic acid (ABA) were identified in the samples along with *para*-hydroxybenzoic acid (*p*HBA) and *para*-coumaric acid (*p*CA). Both SA, *p*HBA and



**Fig. 3.** The effect of 7 days of 50  $\mu\text{M}$  Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on gene expression level of polyamine uptake transporter (*TaPUT*) (a, d), spermidine synthase (*TaSPDS*) (b, e) and peroxisomal polyamine oxidase (*TapxPAO*) (c, f) genes in the leaves (a-c) and roots (d-f) of wheat plants. Values are means  $\pm$  SD. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

*pCA* are phenolic acids, which can be classified as hydroxybenzoic and hydroxycinnamic acids. SA and *pHBA* belong to the former, while *pCA* belongs to the latter group.

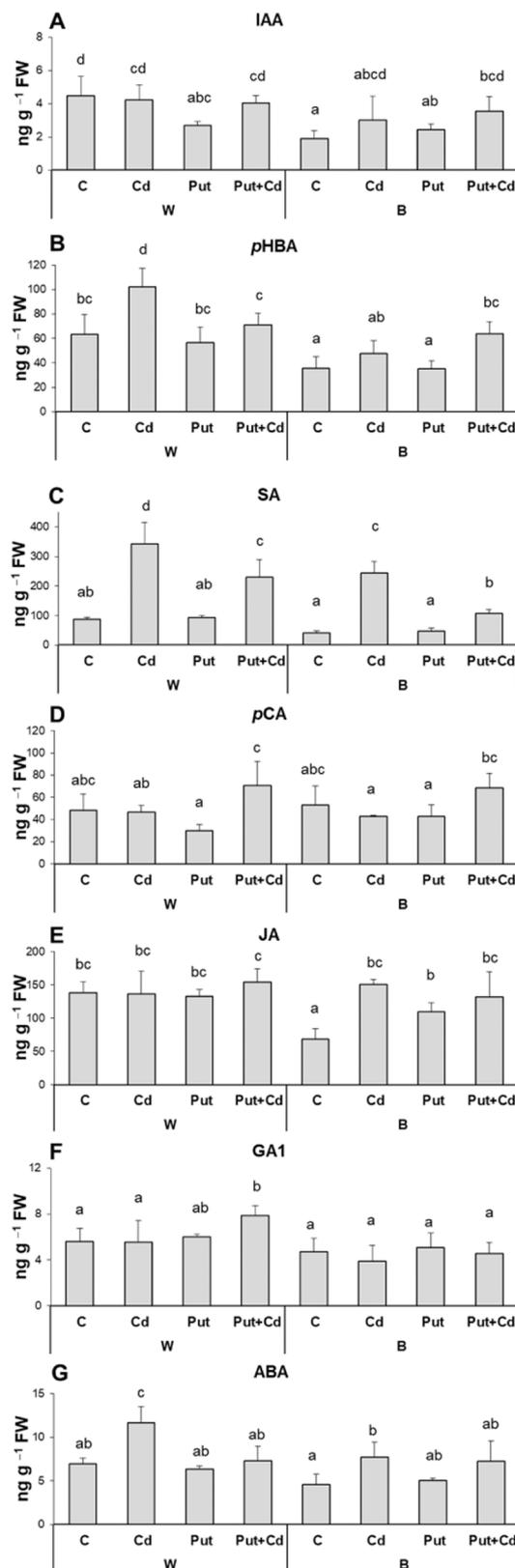
Under control conditions, significantly lower levels of IAA, *pHBA* and JA were detected under B light than under W light conditions in the leaves (Fig. 4a-g). Cd stress under W light increased the levels of *pHBA*, SA and ABA, while under B light induced the accumulation of SA, JA and ABA (Fig. 4b, c, e, g). Put pre-treatment could hardly influenced the level of the investigated compounds under either light condition, except IAA under W light, and JA under B light conditions (Fig. 4a, e). However, Put pre-treatment could modify the effect of Cd treatment in some cases. Compared to the Cd treatment alone, in the Put+Cd-treated plants the levels of *pHBA*, SA and ABA decreased, which may reflect on the protective effect of the Put pre-treatment under W light condition (Fig. 4b, c). A similar tendency was observed under B light only in the case of SA. The level of *pCA* and GA1 increased only after the combined Put+Cd treatment under W light (Fig. 4d, e). Similarly, under B light the levels of *pHBA* and *pCA* increased after Put+Cd treatment. Thus, the same tendency was observed under both light conditions in case of SA and *pCA*. Under W light the changes in *pHBA*, SA, *pCA*, GA1 and ABA levels were

similar. While under B light, the tendency of changes in *pHBA* and *pCA* was the same.

In the roots, the ABA level was below the detection limit and the initial levels of the other investigated compounds were similar under both W and B light regimes (Fig. 5a-f). Remarkable changes were found in the cases of *pHBA*, SA, *pCA* and GA1 (Fig. 5b, c, f). Cd treatment alone increased their levels under both light conditions, but Put pre-treatment did not influence them. When Put pre-treatment preceded the Cd stress, further accumulation of *pHBA* under W light, higher level of *pCA*, but decreased amount of SA were found under both light conditions, while similar accumulation of GA1 was found as in the roots of Cd-stressed plants under either light regime. In summary, the changes observed in the *pHBA*, SA, *pCA* and GA1 were similar under both light conditions.

The expression level of SA synthesis-related genes, namely phenylalanine-ammonia-lyase (*TaPAL*) and isochorismate synthase (*TaICS*), in addition the transcript level of the ABA synthesis key enzyme, 9-cis-epoxycarotenoid dioxygenase (*TaNCED*) were determined after different treatments in the leaves (Fig. 6a-c) and roots (Fig. 6d-f).

In the leaves, despite the similar SA content under W and B light conditions, the initial *TaPAL* expression was more than double under B



**Fig. 4.** The effect of 7 days of 50  $\mu$ M Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on plant hormone contents in the leaves (a-g) of wheat plants. IAA: indole-3-acetic acid, pHBA: *para*-hydroxy-benzoic acid, SA: salicylic acid, pCA: *para*-coumaric acid, JA: jasmonic acid and GA1: gibberellin A1, ABA: abscisic acid. Values are means  $\pm$  SD ( $n=3$ ). Statistically significant differences indicated with different letters at  $p < 0.05$  level.

light than under W light (Fig. 6a). According to this, while under W light only the Put+Cd treatment could decrease its expression level, under B light all the treatments decreased it compared to the adequate control. The transcript level of *TaICS* decreased by the Cd treatment under both light conditions, which can be a result of the feedback mechanism induced by the increased SA levels (Fig. 6b). Put pre-treatment caused only slight induction in the expression of *TaICS*, and only under W light. Different changes were found in the roots, where under W light, Cd stress induced the expression of *TaPAL*, but the other treatments did not influence them, while under B light condition its expression was dramatically inhibited after Cd or Put+Cd treatments (Fig. 6d). The transcript pattern of *TaICS* was similar under W and B light, as especially the combined Put+Cd treatment decreased it (Fig. 6e), which can be connected to the detected lower root SA content of the Put+Cd-treated plants.

The transcript level of *TaNCED* in the leaves decreased by all the Cd treatments, applied alone or in combination with Put pre-treatment under both light conditions (Fig. 6c), which can again be a feedback response to Cd treatment in the leaves. Although ABA level in the roots was lower than the detection limit, the transcript level of *TaNCED* increased after Cd stress under W and B light conditions (Fig. 6f). Put pre-treatment inhibited its expression level, and this decreasing effect was also observed in the case of the combined Put+Cd treatment, but only under W light. Under B light, the expression level of *TaNCED* was similar to that detected under Cd stress alone.

### 3.5. Multivariate analyses (MVA)

MVA were performed in order to highlight the most dominant effects and interactions of different treatments (light, Cd or Put pre-treatment and their combinations: light x Put pre-treatment, light x Cd, Put pre-treatment x Cd and light x Put pre-treatment x Cd) (Table 5 A). Results showed that Cd stress-induced reprogramming occurred at metabolite level (levels of plant hormones, PAs and thiol compounds) both in the leaves and roots. In the leaves, within the investigated metabolite groups, in most cases Cd treatment had the highest effect, followed by B light, indicating the influence of B light under Cd stress. While the effect of Put pre-treatment was statistically significant only in case of dedicated compounds (SA, GR, GST, ohGSH, GSH, N-FPUT and N-pCPUT). The most characteristic influence of the treatments was detected in the leaf SA content, where the effects of the individual treatments and their interactions were both pronounced (Cd > B light > Put pre-treatment x Cd > Put pre-treatment > B light x Cd), supporting the protective effect of Put pre-treatment under both light conditions. The effect of B light was only dominant over the other treatments (Cd or Put pre-treatment) in case of GSH both in the leaves and roots, suggesting that the B light have important role in the regulation of GSH synthesis. Not surprisingly, in the roots the light had very slight influence. The Cd treatments were the most dominant and this dominance was also manifested in the interactions of the treatments. Put pre-treatment in roots had modifying effects on the level of PUT and phenolic compounds (pHBA, SA pCA).

### 3.6. Principal component analyses (PCA)

PCA biplot analysis of the measured metabolite contents and enzyme activities in the leaves and roots (Fig. A1) showed remarkable separation in case of certain treatments. This analysis also showed that in the leaves B light had a specific effect, and it was higher than that of the Put pre-treatment alone under either light condition, without Cd treatment. While a more significant factor was the Cd stress in the distinction of treated plants. In addition, Cd treatment under W light conditions clustered separately. Interestingly, the antioxidant enzyme activities were in close correlation with the GSH and  $\gamma$ -EC contents and SPM level, while the PUT, SPD and pCA contents with the conjugated PA levels. The amounts of plant hormones also showed a close relationship. Some

**Table 3**

Comparison of changes in certain conjugated polyamine (*para*-coumaroyl-hydroxyagmatine: *p*CHAGM, methyl-*para*-coumaroyl-agmatine: Met-*p*CAGM, *N*-feruloyl-putrescine: *N*-FPUT and *N*-*para*-coumaroyl-putrescine: *N*-*p*CPUT) contents in the leaves and roots of wheat plants grown under control (C) conditions or treated with 50  $\mu$ M Cd for 7 days with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions. Data are mean values  $\pm$  SD. "nd" means under the detection limit. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

Conjugated polyamines	nmol g <sup>-1</sup> FW	W				B			
		C	Cd	Put	Put+Cd	C	Cd	Put	Put+Cd
<i>p</i> CHAGM	leaf	4.93 $\pm$ 1.42 ab	10.41 $\pm$ 5.26 c	3.02 $\pm$ 0.48 a	18.35 $\pm$ 1 d	8.28 $\pm$ 2.25 bc	18.08 $\pm$ 2.61 d	5.85 $\pm$ 1.29 ab	19.41 $\pm$ 1.74 d
Met- <i>p</i> CAGM		0.01 $\pm$ 0.002 a	0.03 $\pm$ 0.008 b	0.01 $\pm$ 0.004 a	0.08 $\pm$ 0.03 c	0.02 $\pm$ 0.004 ab	0.08 $\pm$ 0.007 c	0.01 $\pm$ 0.009 a	0.13 $\pm$ 0.016 d
<i>N</i> -FPUT		0.12 $\pm$ 0.04 a	3.32 $\pm$ 1.41 b	0.18 $\pm$ 0.11 a	6.57 $\pm$ 0.67 c	0.58 $\pm$ 0.09 a	6.39 $\pm$ 0.1 c	0.47 $\pm$ 0.18 a	9.74 $\pm$ 1.11 d
<i>N</i> - <i>p</i> CPUT		0.57 $\pm$ 0.31 a	24.8 $\pm$ 4.76 b	1.66 $\pm$ 0.71 a	38.95 $\pm$ 5.48 d	1.01 $\pm$ 0.3 a	32.57 $\pm$ 3.6 c	0.63 $\pm$ 0.1 a	51.36 $\pm$ 3.58 e
<i>p</i> CHAGM	root	9.22 $\pm$ 3.37 a	nd	9.75 $\pm$ 5.8 a	nd	8.61 $\pm$ 3.92 a	nd	15.38 $\pm$ 6.08 a	nd
Met- <i>p</i> CAGM		0.12 $\pm$ 0.11 a	3.18 $\pm$ 0.85 c	0.11 $\pm$ 0.03 a	2.03 $\pm$ 0.5 b	0.07 $\pm$ 0.02 a	4.95 $\pm$ 0.82 d	0.18 $\pm$ 0.07 a	2.63 $\pm$ 0.5 bc
<i>N</i> -FPUT		0.13 $\pm$ 0.16 a	9.37 $\pm$ 0.95 b	0.03 $\pm$ 0.03 a	10.03 $\pm$ 2.58 b	0.07 $\pm$ 1.11 a	8.88 $\pm$ 1.39 b	0.72 $\pm$ 0.87 a	10.3 $\pm$ 1.66 b
<i>N</i> - <i>p</i> CPUT		29.53 $\pm$ 2.65 ab	nd	28.63 $\pm$ 5.32 ab	nd	23.8 $\pm$ 1.77 a	nd	33.42 $\pm$ 5.4 b	nd

negative correlations were also found between PUT, SPD levels and the SPM content, antioxidant enzyme activities, and MDA contents.

In the roots, control and Put pre-treatments under W or B light conditions clustered more closely than in the leaves. While besides the Cd treatment under W light conditions, Put+Cd combined treatment under B light treatment also clustered separately from the Cd alone under B light or the Put+Cd under W light treatments. The correlation between PUT and conjugated PAs (except for two of them, which cannot be detected under Cd treatments), PCS activity and  $\gamma$ -EC content, in addition, SA, pHBA and pCA levels were close both between each other in these three groups, and even between the three groups. In the roots only a few negative correlations were found.

#### 4. Discussion

##### 4.1. Put pre-treatment showed different degrees of protection against Cd stress under W and B light conditions

In response to Cd, plants have evolved several defence strategies to overcome and reduce the toxic effects of Cd (Li et al., 2023). The application of naturally occurring compounds, which act at several levels, can be a useful support for the already existing defence system of plants in order to decrease the Cd-induced damages. There is a lot of evidence that PAs can increase stress tolerance in different plant species under various abiotic stresses (Parrotta et al., 2023). The metabolism of PAs is dynamic due to the PA cycle, the conjugation and catabolism, thus the actual free PA pool is well regulated, which is important during normal plant development and under stress conditions (Pál et al., 2015). However, not enough information is available about the effect of light on PA metabolism under stress conditions. To the best of our knowledge, this is the first study, where the influence of light quality was investigated on the putative protective effect of Put pre-treatment after Cd stress in wheat, with particular regard to the role of PA metabolism.

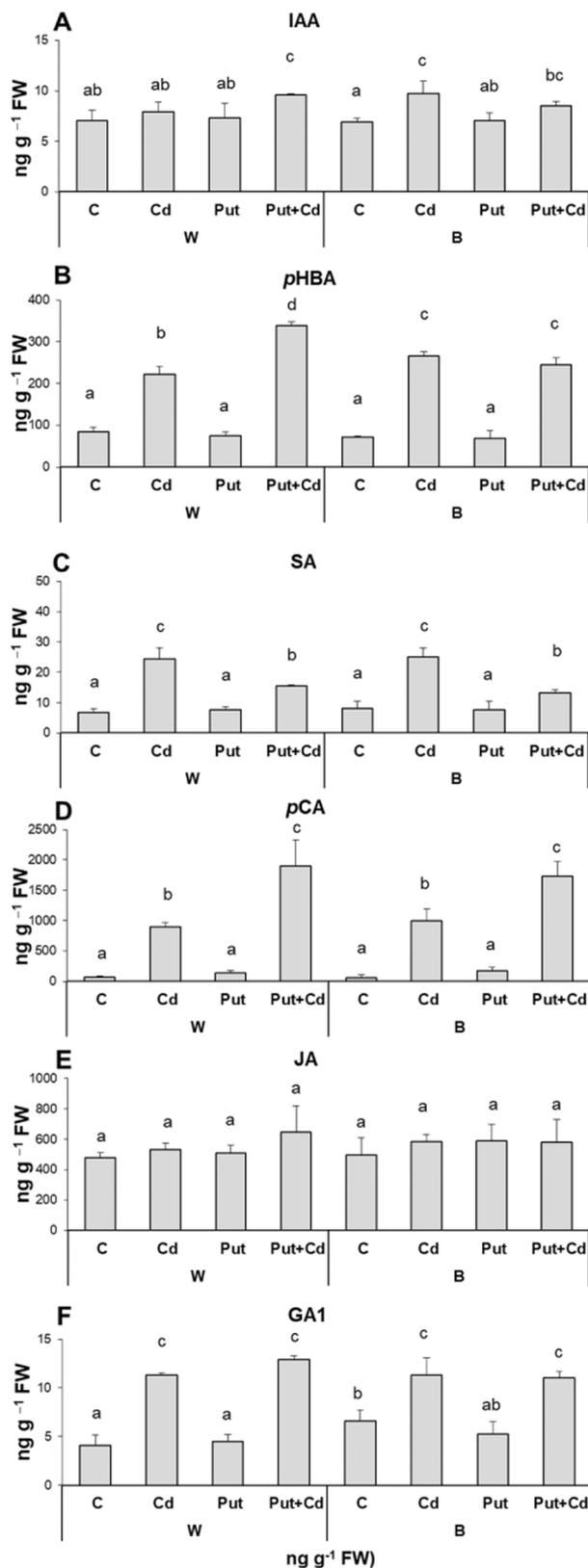
Earlier we demonstrated that 0.5 mM PUT applied in hydroponic solution or as seed-soaking had a protective effect on biomass parameters during Cd stress in wheat under white light conditions, namely shoot length and fresh weight, which were higher compared to the only Cd-treated plants, however could not decrease the root Cd uptake (Tajti et al., 2018). In the present experiment, lower root Cd accumulation was detected after Put pre-treatment after Cd stress, under W or B light conditions. However, in plants grown under B light already lower root Cd uptake was detected compared to the W light, in addition Put pre-treatment further decreased it. Similarly, in cucumber, under blue light, also lower level of Cd uptake was found - in both shoots and roots - compared to the white light conditions, which was accompanied by the down-regulation of the expression levels of *CsIRT1*, *CsNRAMP1* and *CsHMA3* genes involved in Cd uptake and transport (Guo et al., 2022).

Lower level of lipid peroxidation in the leaves, and lower H<sub>2</sub>O<sub>2</sub> content in the roots of Cd-treated plants grown under B light compared to W light conditions can be partly explained by the lower Cd uptake

under B light under the present conditions. According to these, Put pre-treatment could not further decrease them under B light. However, the combined Put+Cd treatment under W light growth conditions resulted in the lower leaf MDA and root H<sub>2</sub>O<sub>2</sub> contents. Likewise, lower H<sub>2</sub>O<sub>2</sub> content was detected in Cd-treated cucumbers under blue light conditions compared to the white light conditions (Guo et al., 2022). Previously in rice it was found that SPD and SPM treatments were effective against Cd stress, and were capable of decreasing Cd uptake into the detached leaves and alleviating Cd-induced increase in H<sub>2</sub>O<sub>2</sub> and MDA contents (Hsu and Kao, 2007). In *Brassica juncea*, PUT and SPD applied as foliar spray had protective effects during Cd stress, but only SPD could influence the leaf, stem or root Cd uptake (Aoun et al., 2008). In leaf segments of wheat plants, SPM could also decrease the lipid peroxidation level and H<sub>2</sub>O<sub>2</sub> formation (Groppa et al., 2007), while spraying with PUT or SPD decreased the MDA content in *Brassica juncea* (Aoun et al., 2008).

The positive effect of Put pre-treatment under the present conditions was also suggested by the changes in antioxidant enzyme activities, as for example in the case of leaf GR the highest values were detected after Put+Cd treatment under both light conditions, despite of the fact that under B light higher initial GR activity was detected. Similarly, additive effect of the combination of Put pre-treatment and Cd stress resulted in the highest root GR and GST activities under both light conditions. Higher drought tolerance in faba bean under blue light conditions was also resulted from the induced antioxidant enzyme activities (Huang et al., 2020). In rice, higher GR and APX activities were detected under blue light conditions compared to white light conditions. In addition, Cd stress under blue light conditions resulted in a much larger induction of GR and APX activities in the leaves than under white light treatment (Sebastian and Prasad, 2014). In cucumber, blue light during Cd stress also markedly increased the activities and even the transcript levels of APX, catalase, superoxide dismutase and GR enzymes compared to the white light conditions (Guo et al., 2022). Also, in Cd-treated wheat under white light conditions, SPD treatment induced a further increase in the root GR, GST and APX activities compared to the Cd treatment alone (Tajti et al., 2018). SPM treatment again in wheat plants could not reverse the Cd-induced decrease in APX activity, while it was efficient in elevating of GR activity back to the control value (Groppa et al., 2007). In contrast to our results, PA treatments decreased GR activity in the leaves of spinach, and the decrease was concentration-dependent (Erat et al., 2008).

The presented stress marker results showed that Cd stress had a higher effect on the roots than on the leaves, and the stress was more pronounced under W light, maybe partly due to the higher Cd accumulation. Put pre-treatment had some protective effects, especially under W light conditions, which may be explained by the Put-inhibited Cd uptake and/or induced antioxidant system.



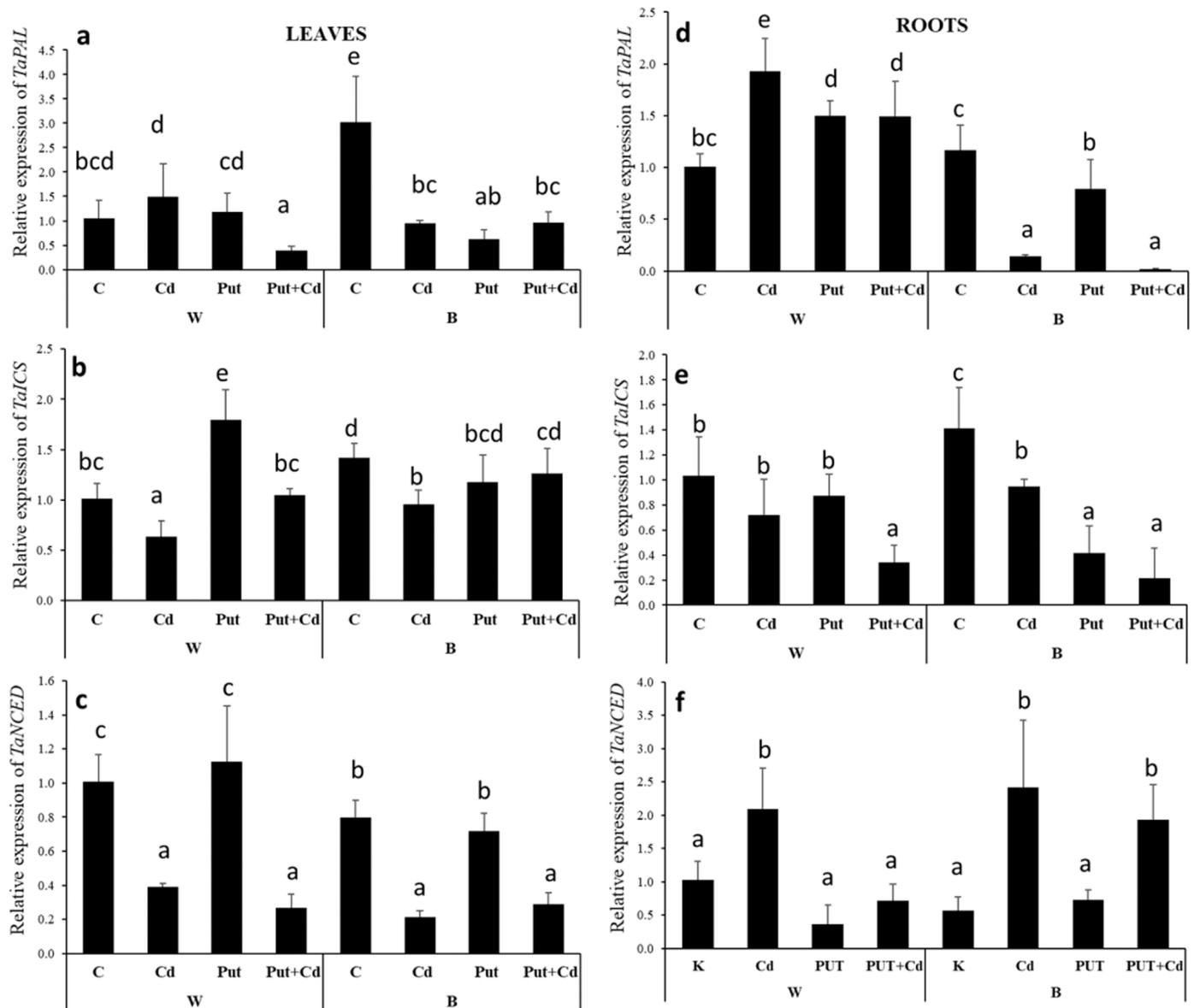
**Fig. 5.** The effect of 7 days of 50  $\mu$ M Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on plant hormone contents in the roots (A-F) of wheat plants. IAA: indole-3-acetic acid, pHBA: *para*-hydroxy-benzoic acid, SA: salicylic acid, pCA: *para*-coumaric acid, JA: jasmonic acid and GA1: gibberellin A1. Values are means  $\pm$  SD (n=3). Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

#### 4.2. Blue light modulates the effect of Put pre-treatment on PC synthesis under Cd stress

The most remarkable changes after the different treatments in leaf thiol content were found in the case of GSH. GSH level was decreased by Cd treatment under W and B light, but after Put+Cd treatment this could be reversed, reaching the control levels, which was initially lower under W than under B light conditions. In the roots, Cd also decreased the GSH accumulation under W light, and Put pre-treatment could enhance it as it was described in the leaves. Under B light the initial root GSH level was lower than under W light, and no significant changes were detected either after Cd or Put pre-treatment alone or the combined treatment. Parallel with these, in the roots Cd induced the accumulation of total PC, the amount and composition of which was different under W and B light condition. Overall, it was lower under B light; in addition, when Put pre-treatment preceded the Cd stress longer PC compounds (PC6) appeared under both light conditions, but the total PC synthesis decreased under W light, and increased under B light conditions. Despite of these differences, the Cd-induced root PCS activity was similarly lower after the Put+Cd under both light conditions, but it was more highly activated under W light than under B light. Cd also increased the *TaPCS* transcript level in the roots under W light, but decreased it under B light conditions, and while under W light the Put pre-treatment could not further influence it, under B light induced its expression level compared to the Cd treatment alone.

The increased levels of Cys,  $\gamma$ -EC and ohGSH after Cd treatment under both light conditions showed that Cd induced the thiol-related protective mechanism, especially in the roots. However, the decrease in GSH and CG after Cd application reflected that the GSH was used up in antioxidant enzyme reactions and/or for PC synthesis, which latter was in accordance with the accumulation of PCs, and induction of PCS activity in the roots, especially under W light conditions. Despite the observed protective effects of Put pre-treatments under both light conditions, partly different defence responses were observed under W and B light conditions, and Put pre-treatment had also some specific modifying effects. Opposite effect of Put pre-treatment was found under W and B light treatment during Cd stress on the PC accumulations, namely despite the lower Cd level in the roots of Put+Cd-treated plants grown under B light conditions, similar PCs accumulations were found as in those grown under W light conditions.

Higher GSH level was also detected in cucumber leaves under blue light conditions (Guo et al., 2022), while lower total GSH level was found in wheat (Monostori et al., 2018; Toldi et al., 2019) compared to the white light conditions. It was also demonstrated, that although PUT treatment did not influence the thiol content in rice, the combination of PUT and Cd treatments resulted in lower thiol content in the roots compared to the Cd treatment, which was in relation to the observed negative effect of the PUT under Cd stress (Pál et al., 2017). PUT treatment in mung bean increased Cd tolerance, and in parallel with lower Cd uptake, increased APX, GST and GR activities, and a higher level of GSH were detected compared to the Cd treatment alone (Nahar et al., 2016). In mung beans PUT treatment further increased PC accumulation during Cd exposure (Nahar et al., 2016). It has been also demonstrated in wheat that PUT had a protective role against Cd stress, but the PUT treatment either alone or in combination with Cd did not modify the *TaPCS* expression level in the leaves (Tajti et al., 2018). While, the negative effect of PUT under Cd stress conditions in rice is manifested in the inhibition of the PCS activity and gene expression of *TaPCS* both in the leaves and roots (Pál et al., 2017). These findings together with the here presented findings results suggest that the accumulation of PCs is not the only, but essential factor of Cd tolerance in wheat plants, and Put pre-treatment modulates this process depending on the light spectral composition, due to the synthesis starting from GSH.



**Fig. 6.** The effect of 7 days of 50  $\mu\text{M}$  Cd treatment with or without 7 days of 0.5 mM putrescine (Put) pre-treatment under white (W) or blue (B) light conditions on the gene expression level of phenylalanine-ammonia-lyase (*TaPAL*) (a, d) and isochorismate synthase (*TaICS*) (b, e), in addition, the transcript level of 9-cis-epoxycarotenoid dioxygenase (*TaNCED*) (c, f) genes in the leaves (a-c) and roots (d-f) of wheat plants. Values are means  $\pm$  SD. Statistically significant differences are indicated with different letters at  $p < 0.05$  level.

#### 4.3. Polyamine synthesis, catabolism and conjugation were differently affected after combined Put+Cd treatment under W and B light conditions

The Put+Cd treatment resulted in a higher amount of total PA both in the leaves and roots of plants under W light conditions, which was parallel with the observed protective effect. Under B light, the combined treatment caused a further increase only in the PUT level, and the amount of SPD+SPM together was the lowest in the roots. These results together with the gene expression data suggest that induction of SPD synthesis and the back-conversion process together responsible for the observed higher accumulation of PUT and SPD, but a lower level of SPM accompanied by lower catabolism of PAs to DAP in Put+Cd-treated leaf samples under W light conditions. Lower *TapxPAO* expression in the roots under B light can be related to the lower SPD and SPM contents, but the highest DAP and PUT levels after Put+Cd treatment. So, despite the similar pattern of the changes in the PA pool, Put pre-treatment could modulate the PA metabolism partly differently during Cd stress under the two light conditions. Also in wheat, it has been reported that

Cd treatment increased the PUT content, it did not change the SPD level, but decreased SPM content, which changes were in relation to increased activity of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), the enzymes involved in PUT synthesis (Groppa et al., 2007). The protective effect of PUT treatment was also found in wheat under Cd stress, however, the combination of PUT and Cd treatments did not cause further changes in the PA pool, but decreased the gene expression level of *TaADC* and *TaODC* (Tajti et al., 2018).

The actual PA pool is also influenced by the conjugation processes. Although conjugated PAs have been detected in a wide range of plants, their role especially during stress conditions is still ambiguous (Pál et al., 2021). Only a few studies are available, which are focused on abiotic stress-induced accumulation of conjugated PAs. For example, conjugated PAs were closely correlated with plum seedling growth, and might be involved in the tolerance of plum seedlings to osmotic stress (Du et al., 2022). Furthermore, during Cd stress in *Hydrocharis dubia*, it was found that the conversion of free PAs to conjugated forms could alleviate Cd stress. In the leaves of *H. dubia*, Cd treatment resulted in increased

levels of conjugated PAs, in addition exogenous SPD together with Cd, could greatly enhance the conjugation process (Yang et al., 2013). Our results showed that regarding of the levels of certain conjugated PAs, the leaves and roots responded differently to the applied treatments. The fact that the *N-pCAGM* and *N-pCPUT* could not be detected after Cd treatments in the roots, but the content of other two conjugated PAs increased, nevertheless the level of the former two showed high accumulation in the leaves, can be in relation with a shift in the synthesis/conjugation/translocation balance of the PA pool. In addition, in the leaves higher PA conjugation (*N-FPUT* and *N-pCPUT*) was found under B light conditions, with the highest level in Put+Cd-treated plants, which may be responsible for the lack of great accumulation of PUT compared to the W light conditions. At the same time, free *pCA* in the root after Cd and Put+Cd treatments also increased indicating the inhibition of conjugation, due to the need of *pCA* as antioxidant or lignin synthesis precursor, which in turn can reduce Cd uptake.

Taken together, while the PUT accumulation was induced after Cd stress both in the leaves and roots, the catabolism and/or back-conversion of the higher PAs were also induced, resulting in lower levels of SPM, but higher DAP content. In certain cases, the combined treatment caused further increases in the investigated parameters even at the gene expression level, especially under W light. Put pre-treatment further increased Cd-induced conjugation of PAs in the leaves, to a greater level under B light.

#### 4.4. Protective effect of Put pre-treatment during Cd stress is related to synthesis of plant hormones and the level of certain phenolic compounds

The induction of the synthesis and the role, even the crosstalk of plant hormones, such as SA, ABA, IAA, GA and JA are well-reviewed under heavy metal stress. In most cases increased levels of them have been reported in various plant species, in addition exogenous application of them has also been proven to be effective against the toxic effects of heavy metals (Bücker-Neto et al., 2017; Rahman et al., 2023).

In the present experiment, Cd stress induced the synthesis of almost all the measured plant hormones and phenolic compounds, but in most cases their levels in the leaves were slightly lower under B light conditions than under W light. However, blue light increased the synthesis of phenolic compounds and proved chilling tolerance in basil (Larsen et al., 2022), or induced ABA accumulation and in turn resulted in higher drought tolerance in lettuce and *Eruca* plants (Ginzburg et al., 2020). Cd-induced increments in SA content were accompanied by the upregulation of the gene expression of *TaPAL* gene, but only under W light conditions. Under B light both *TaPAL* and *TaICS* were inhibited by Cd in the leaves and roots. Cd alone resulted in higher SA accumulation, than Put+Cd under both light conditions.

The lower accumulation of ABA in leaves of Put+Cd-treated plants under W light also indicated the protective effect of Put pre-treatment. Plant hormones can exert their roles for example by activating of the antioxidative defence system, upregulating of the synthesis of thiol and other heavy metal chelator or osmolyte compounds, and by inducing complex metabolite remodelling. Previously, it has been reported that Cd stress increased the levels of SA and/or ABA in the leaves of wheat or maize plants, in addition their lower levels were in relation to higher Cd tolerance, or with lower degree of Cd stress caused by certain protective compound, such as exogenous PUT (Pál et al., 2005; Kovács et al., 2014; Tajti et al., 2019). Partly in a similar way, lower SA content and expression level of *TaICS* in the roots, in addition to lower expression level of *TaNCED* in the leaves and roots of dwarf wheat genotypes were relation to lower level of stress symptoms, and the degree of Cd tolerance (Szalai et al., 2020).

*pCA* belongs to the hydroxycinnamic acids, and is metabolically important as it is linked to the synthesis of other hydroxycinnamic acids such as caffeic-, ferulic-, and sinapic acids, which can be conjugated with PAs and form hydroxycinnamic acid amides (Liu et al., 2022). ROS scavenging properties of *pCA* is well-studied in human diseases,

however, it has only recently been suggested that exogenous application can be beneficial in plants (Nkomo et al., 2019). Although, *pHBA* is considered a widespread phenolic acid released into the soil by root exudates with allelopathic effect (Hussain et al., 2021), exogenous application of it also improved drought tolerance of winter wheat and freezing tolerance of spring wheat genotypes (Horváth et al., 2007). In the present experiment the great accumulation of *pCA* and *pHBA* in the roots during Cd stress under both light conditions, suggesting their antioxidant activity under these conditions. Their higher the accumulation in case of the combined Put+Cd treatment then after Cd treatment alone, indicating that as in the roots the PA conjugation were not further induced by Put pre-treatment, phenolic compounds may have role rather as antioxidant compounds. On the other hand, as Cd stress induces lignification in plants, and increased lignin decreases Cd uptake (Riaz et al., 2023), in addition *pCA* is also important during PA conjugation, the role of *pCA* in these directions cannot be ruled out either.

## 5. Conclusion

The presented results indicated that Cd stress was lower under B light conditions, due to the lower Cd uptake, which resulted in lower induction of certain protective compounds (GSH, PCs, SA and ABA contents) compared to those detected under W light. The Put pre-treatment could alleviate Cd-induced stress under both light conditions. It could decrease the Cd uptake under both light conditions, but increased PC accumulation only occurred under B light. The protective effect of Put pre-treatment against Cd stress might also be related to enhanced antioxidant enzyme activities and modulated PA metabolism. The alleviated Cd stress resulted in lower SA, ABA and *pHBA* contents under W light conditions, especially in the leaves, in addition, lower accumulation of PCs. Although B light had its own effect during Cd stress, it had some modifying effects on Put pre-treatment. The most remarkable differences between W and B light conditions were observed in the cases of PCs and conjugated PAs. Cd induced PCs synthesis in the roots, but B light inhibited it, however, Put pre-treatment could reverse this decrease. Parallel with these, the PA content also increased after Cd treatment together with the conjugated PA level in the leaves, and both B light and Put pre-treatment could induce further increases, which as an additive effect resulted in the highest level of conjugated PAs in the leaves of Put+Cd and B light-treated plants.

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## Author statement

The present work has not been published previously. The present work do not involve the use of human subjects or animal experiments.

## CRediT authorship contribution statement

**Gabriella Szalai:** Writing – review & editing, Methodology, Formal analysis. **Magda Pál:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Tibor Janda:** Writing – review & editing, Writing – original draft. **Mihály Dernovics:** Methodology, Formal analysis. **Csaba Szóke:** Writing – review & editing, Data curation. **Altafur Rahman:** Writing – original draft, Investigation, Formal analysis. **Katalin Nagy:** Writing – review & editing, Methodology, Formal analysis. **Kamirán Aron Hamow:** Writing – review & editing, Methodology, Formal analysis.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Pal reports financial support was provided by National Research Development and Innovation Office. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

All the data can be found in the manuscript.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.envexpbot.2024.105746](https://doi.org/10.1016/j.envexpbot.2024.105746).

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