Workplan:

1st year:

Different ways of polyamine (PA) application: seed priming and adding to the hydroponic solution will be used in cereal species in order to determine the most effective way, compound and concentration against abiotic stress factor.

2nd year:

In order to reveal relationship and overlap between the signalling mechanisms, the changes induced by PA and abscisic acid (ABA) treatment in the endogenous PAs synthesis will be studied under control or stress condition.

3rd year:

As a continuation of the first year the effect of PA treatment will be studied under stress conditions including gene expression studies, microchip technology, to determine the common and the different points of stress resistance regulation processes.

4th year:

Microarray data analysis and control of the obtained genes of interest.

1st year:

The most abundant polyamines (PAs), putrescine (PUT), spermidine (SPD) and spermine (SPM) applications (seed priming or hydroponic treatment) were tested in wheat and maize plants in order to determine the most beneficial way, compound and concentration as a pre-treatment. Although seed priming in PA solutions for 16h did not influence the endogenous PA contents of the 3d wheat seedlings, the germination percentage and the shoot and root height of plants increased especially in the SPD-treated ones. In contrast in hydroponic treatment for 7d, PUT did not affected, but SPD or SPM caused significant decrease in the growth parameters both in wheat and maize, with higher sensitivity in the case of maize. Anthocyanin accumulation in SPD- or SPM-treated plants was observed also suggesting stress conditions. If 7d PA treatment was followed by 7d recovery, PUT-treated plants have higher biomass compared to the control. PUT treatment increased the root PUT level, SPD treatment increased leaf PUT level and root PUT and SPD content, while SPM treatment increased the accumulation of PUT and SPD in the leaves and PUT, SPD and SPM accumulation in the roots. According to these, besides uptake and translocation from root to shoot, higher PAs (SPD and SPM) can quickly interconvert in the PA cycle. These results showed that the PA pool is dynamic, changing over time, and PAs also undergo rapid interconvertion in the PA cycle (Fig. 1)**(Pál et al., 2015 *Plant Science*, review)**.

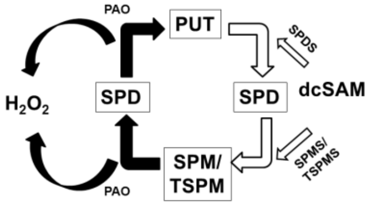

Figure 1. Polyamine cycle.

Endogenous PA accumulation was accompanied with high salicylic acid (SA) accumulation in SPD- or SPM-treated plants. Based on these, relationship between PAs and SA may exist **(Szalai et al., 2017 *Plant Physiology and Biochemistry*)**.

2^{nd} year:

In order to find connection between PAs and various protective effectors involved in the development of cold tolerance, different cereal genotypes were investigated during the acclimation phase to low temperature. Low temperature induced different changes in the levels of PAs, signalling molecules, such as SA and ABA, and of other protective compounds, namely flavonols, sugars and antioxidant enzyme activity, and in the lipid composition of certain membrane factions. Correlation analysis revealed that SPD shows strong positive relationship with flavonols, ABA and ascorbate peroxidase, while was in negative relationship with *trans*-Δ_3-hexadecanoic acid. These results suggest that SPD may have a crucial role in the cold acclimation signalling processes in cereals **(Gondor et al., 2016 *Journal of Agronomy and Crop Science*)**.

In order to reveal relationship or overlap between ABA and PA signalling, comparison of the effects of PUT and ABA treatments either alone or in combination with polyethylene glycol(PEG)-induced drought stress were investigated in young wheat plants ABA treatment had powerful effect on PA metabolism. The most characteristic changes were the increase in PUT and the decrease in SPD contents of ABA-treated plants. The degree of PUT accumulation was lower than the depletion in SPD content both in the leaves and roots of ABA-treated plants, but these changes were not resulted from decreased gene expression level of SPD synthase (*SPDS*) and S-adenosyl-methionine decarboxylase (*SAMDC*) indicating that the modification on the synthesis side is not responsible for the observed decrease of higher PAs. The accumulation of PUT in the wheat plants was in parallel with increased arginine decarboxylase (*ADC*) gene expression in the case of ABA and PUT treatments. These results suggest that the exogenously applied PUT was not only taken up by the wheat plants but also induced its *de novo* synthesis. Furthermore, both PUT biosynthesis pathways, catalysed by ornithine decarboxylase (ODC) or ADC were involved. In the present study, ABA or PUT treatments did not influence the activities of diamine oxidase (DAO) or polyamine oxidase (PAO) remarkably, which are localized in the apoplast and have a role in the terminal catabolism of PUT or SPD/SPM. However, the gene expression of the peroxisomal *PAO* was induced after ABA treatments indicating that the interconversion of SPD/SPM to PUT occurred in the leaves. These results suggest that ABA modulates PA metabolism in a complex way at transcriptional level and the result of the induced changes in back-conversion displayed in the observed PA pattern. Interestingly, ABA-induced PA pattern in the wheat leaves was similar to that of PEG-treated plants, as 15% PEG for 5 days increased PUT and decreased SPD content in the leaves. However, significant changes could not be detected in the roots. Drought stress alone or in the combined treatments (ABA+5dPEG and PUT+5dPEG) did not affect the apoplastic DAO or PAO activities, but it increased the gene expression level of the peroxisomal *PAO* **(manuscript has been prepared**.

Near isogenic durum wheat lines that differ at a major grain yield QTL were also investigated, in order to find connection between the PA content and antioxidant enzyme activity of the flag leaves and yield-related parameters, as well as drought tolerance. Yield components showed close, negative relationship with the antioxidant enzyme activities, which in turn may indicate that changes in these parameters more related the cause of the drought stress. In contrast, yield-related parameters were in close positive relationship with PA contents pointing out that the understanding of flag leaf physiology under drought, revealing the role of the hormonal balance in the flag leaf is important **(Bányai et al., 2017 *South African Journal of Botany*).**

3rd year:

As a continuation and complementation of the first year, further investigations were made in order to reveal the background of the positive effect of PUT. Although PUT pre-treatment protected both wheat and maize plants (proved by photosynthesis measurements) during PEG-induced drought stress, inhibited the growth of maize plants (proved by biomass parameters). The same treatment also induced different changes in the PA metabolism, SA and ABA contents of maize and wheat plants.

In wheat although PUT pre-treatment did not influence significantly the investigated endogenous PA contents in the leaves and roots, decreased the DAO activity in the leaves. The PEG treatment increased the PUT level, but decreased SPD and SPM contents in the leaves, and decreased the amount of SPD in the roots. According to these increased DAO and PAO activities were found in the roots of PEG treated plants. When PUT preceded the PEG treatment the increase in PUT and the decrease in SPM contents of the leaves could not be observed. Parallel with these, DAO activity decreased in the leaves. Compared to these in maize PUT pre-treatment increased the endogenous PUT content in the roots either applied alone or follow by PEG treatment, while in the leaves only the combined treatment could increase it. Increment of PUT content resulted in increased DAO activity in the leaves of maize plants treated with PUT+PEG, while PAO activity did not change.

As PA metabolism is linked with other hormones, SA content was also measured. The greater positive effect of PUT in wheat than in maize can be explained by differences in the PA metabolism under normal and osmotic stress conditions, and by relationship between PA and SA. Leaves and roots of the two plant species also responded differently in the case of ABA content. In wheat, although the leaf ABA content did not change, drought stress increased the root ABA level, which could be slightly mitigated by PUT pre-treatment. In maize PEG induced high ABA accumulation in the leaves and PUT pre-treatment could moderate it, while in the roots the combined treatment caused the highest ABA level. The results demonstrated that changes in the PA pool are important for fine tuning of PA signalling, which influences the hormonal balance required if PUT is to exert a protective effect under stress conditions **(Szalai et al., 2017 *Plant Physiology and Biochemistry*)**.

As more pronounced positive effects of PUT treatment were observed in wheat for further experiment under PEG-induced drought stress - microarray analysis - only the wheat genotype was used. The microarray analysis has been performed and the detailed data analysis has been finished in the 4th year.

As a continuation, PUT pre-treatment was further tested during heavy metal stress, applied as Cd treatment in rice. Among plant species rice can be characterised with a very high level of SA content. Thus, ideal plant species to reveal relationship between metabolism of PA and SA. One of the ways to increase the endogenous PA contents is the application of exogenous PA treatment. Due to the PA cycle, PAs are capable to interconvert to each other and the PA pool is thus dynamic, changing over time. On the other hand, investigation on decreased endogenous PA content can also give valuable information. If it is also taken into consideration that starting with cysteine (Cys), as a common precursor, the synthesis of glutathione (GSH), and thus phytochelatins (PCs) – compounds, which are reported to play role in heavy metal detoxification and stress tolerance - are in antagonistic relationship with the formation of S-adenosylmethionine (SAM), which is necessary for the synthesis of higher PAs (SPD and SPM) from PUT (Fig.2.).

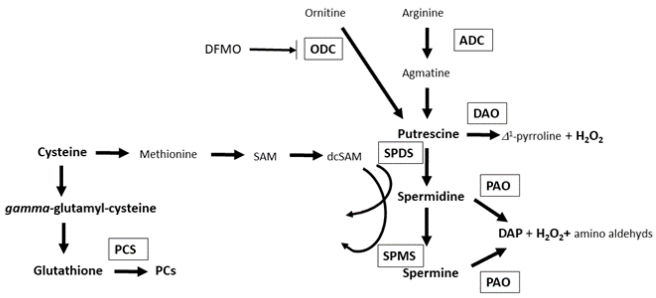

Figure 2. Relationship between polyamine and phytochelatin metabolism.

Difluoromethylornithine (DFMO) is capable to covalently bind to and thus irreversibly inhibit the PUT synthesis enzyme, ODC. In order to answer the question how changes in PA metabolism influence the thiol-origin heavy metal detoxification system, exogenous PUT pre-treatment followed by Cd stress or DFMO treatment combined with exposure to Cd were applied in rice plants. Our results indicate the higher decrease in all the biomass parameters was found in the case of combined PUTpre+Cd treatment compared to the Cd alone, while when DFMO was applied with Cd, higher root length and shoot FW were measured compared to the Cd-treated ones. In addition, lower Cd uptake was found in the roots of DFMO+Cd treated plant compared to the Cd-treated or PUTpre+Cd-treated ones. Similarly, changes in H_2O_2 content, glutathione reductase and guaiacol peroxidase enzyme activities showed that the highest oxidative stress was induced by the PUTpre+Cd treatment both in the leaves and roots as an additive effect of the PUT and Cd stresses. In addition, DFMO applied with Cd could alleviated the damage caused by Cd. Taken together, these results suggest that PUT pre-treatment enhance the adverse effect of Cd, while application of DFMO to a certain extent reduces it. However, Cd stress induced dramatic SA accumulation in the root of rice plants, which was decreased by both combinations of PUT pre-treatment or DFMO treatment with Cd, in the leaves the highest increase of SA content was observed in the case of PUTpre+Cd treatment. As expected, the PUT pre-treatment increased the polyamine content in rice, suggesting, that the exogenous PUT taken up, used up for further synthesis of higher PAs (SPD and SPM). Cd treatment alone also increased the endogenous PA contents, while the combined PUTpre+Cd treatment induced the highest PA accumulation, which was the most pronounced in the case of PUT level. In contrast to these, DFMO treatment resulted in only decreased PUT content in the leaves, which can be explained by the existence of compensatory mechanisms in the leaves of DFMO-treated plants, as RT-PCR measurements indicated increased gene expression level of both ADC and ODC. However, as a self-generating process the transcription of these genes and that of SPD/SPM synthase also increased in PUT pre-treated ones. Significant decrease in the gene expression level of phytochelatine synthase (PCS) was found in the leaves after PUT pre-treatment, which was also manifested in the leaf in vitro PCS activity, while at that time the thiol and PC contents were not influenced either in the leaves or roots of rice. However, when PUT pre-treatment was followed by Cd stress, depletion in both total thiol and PC contents were observed compared the Cd alone or DFMO+Cd treatments. In summary, according to the literature in certain cases PAs prove protection against heavy metals, it should be also taken into consideration that the synthesis of higher PAs and PCs are in antagonistic relationship. Nevertheless, higher level of SPD and SPM may convert back to PUT or SPD in the PA cycle, which lead to higher H_2O_2 accumulation and inhibition of root elongation. In addition, it is also possible, that PUT treatment inhibit PC synthesis not only by the synthesis antagonism, but also as PAs are metal chelators, the PA-Cd binding resulted in the lack of enough stimulus for the induction of the

PC synthesis. However, the fact that PA-Cd complexes are as efficiently compartmentalised to vacuoles, as PC-Cd complexes, has not been demonstrated yet. These two inhibition mechanism may explain the changes in PCS activity in the leaves of rice: 1. PUT pre-treatment alone decreased PCS activity due to depletion in GSH content which resulted from the increased PA metabolism, while 2. in the presence of Cd, as an additive effect the lowest PCS activity was found in the case of PUTpre+Cd treatment, where not only the lack of enough GSH precursor but also the lack of adequate amount of free Cd2+ ion, which is necessary for the induction of PCS activity and PCS gene expression, due to the PA-Cd binding (**Pál et al., 2017 *Journal of Hazardous Materials***).

However, the individual polyamines may have different effects, which also vary depending on the type of treatment, and also on the plant species. PA pre-treatments (PUT and SPD) applied as seed soaking (ss) or added hydroponically (hyd), during Cd stress were investigated in wheat plants. The greatest increase in PA content induced by Cd was observed for PUT in the leaves and roots of wheat plants, in both the experiments, which can be explained by the increased gene expression level of PUT synthesis enzyme (ADC). Although the combined treatments, PUTss+Cd, SPDss+Cd and even PUThyd+Cd resulted in similar PA patterns, SPDhyd+Cd caused higher leaf PUT accumulation than the corresponding Cd treatment. The PA pool is dynamic, changing over time as PAs undergo rapid interconversion in the polyamine cycle. Plants try to maintain an optimum PA pool and PA ratio, as the accumulation of PUT in the present experiment resulted from the uptake of PUT (PUThyd+Cd) or the back-conversion of the SPD taken up to PUT (SPDhyd+Cd), leading to a high PUT/(SPD+SPM) ratio, which has been reported to result in plant injury under stress conditions. The resulting high PUT level in SPDhyd+Cd-treated plants may have negative effects, as shown by the decrease in biomass parameters and chlorophyll content and the highest Cd accumulation in both the leaves and roots. In the present experiments, Cd treatments in combination with SPD resulted in the highest Cd accumulation in the roots; however, the translocation of Cd into the leaves in SPDhyd+Cd-treated plants was not as intensive as in the case of PUThyd+Cd treatment. PAs were also found to have a metal-chelator function, indicating a relationship between metal uptake/translocation and the pattern of PA accumulation. Depending on the applied PA and on the type of treatment, different mechanisms may occur and result the observed changes in the PA pool (Fig.3.).

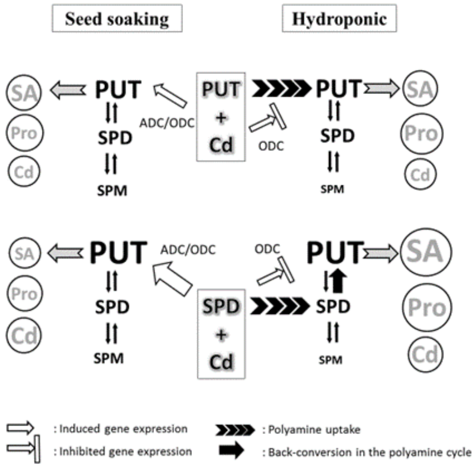

Figure 3. Comparison of the effects of PUT and SPD pre-treatments applied as seed-soaking or hydroponically under cadmium stress

In both of the present experiments Cd treatment led to SA accumulation especially in the leaves. However, when PUTss or SPDss preceded Cd treatment a less pronounced increase in SA content was found in the leaves compared to plants treated with Cd alone, which may explain the observed protective effect. In contrast to the seed soaking pre-treatment, only hydroponic pre-treatment with PUT was able to mitigate the rise in SA content caused by Cd in the leaves, while the SPDhyd+Cd treatment had an additive effect on SA content in the roots. The highest SA and PUT accumulation was found in SPDhyd+Cd-treated plants. This is not surprising, as previously we found a close correlation between the endogenous PA and SA contents, which may be responsible for the negative effect of greater concentrations of higher PAs (SPD and spermine: SPM). Proline acts as a major reservoir of energy and nitrogen, which can be utilised to resume growth under stress conditions, and it has been observed to accumulate in response to heavy metals in several plant species, suggesting that it plays a role in heavy metal tolerance. In the leaves all the combined treatments increased the proline content, with the most dramatic increase in the leaves of SPDhyd+Cd-treated plants **(Tajti et al., 2018 *Ecotoxicology and Environmental Safety*).**

4th year:

Microarray analysis of the 3rd year revealed that PUT pre-treatment upregulated several similar genes as it was found in the case of PEG treatment. However, PUT induced the unique expression of various general stress-related genes, such as arogenate dehydratase 3, electron transfer flavoprotein ubiquinone oxide reductase, metallothionein 3 and 6-phosphogluconate dehydrogenase decarboxylating 1, suggests that PUT pre-treatment activates among others the pentose phosphate and shikimate-chorismate pathways in the chloroplasts as part of the acclimation process (Fig. 4).

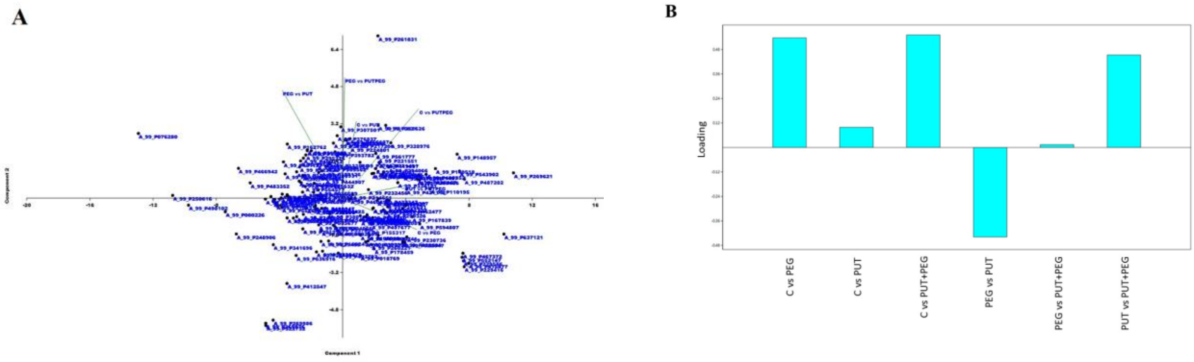

Figure 4. A: Scatter plot diagram of the expression datasets derived from principal component analysis based on the logFC values of the genes. B The loadings of the original variables along the PC1.

As a complementation of the microarray analysis, the changes in the fatty acid composition of certain membrane fractions together with the antioxidant enzyme activities were also investigated. It was found that although PUT treatment had a few specific effects, these differences were less substantial than the effects for osmotic stress (resulted in higher double bond index in phosphatidylethanolamine, but lower double bond index in monogalactosyldiacylglycerol fractions) and they probably do not explain the previously observed protective effects of PUT. Although, early upregulation of genes encoding peroxidase superfamily proteins was also found in PUT pre-treated plants, at the end of the experiment similar activity of the antioxidant enzymes, and similar pattern of guaiacol peroxidase isoenzymes (detected by gel electrophoresis) was found in PUT-pre-treated plant as in the controls. After PUT pre-treated+PEG treatment increased peroxidase expression was also found; and the POD isoenzyme analysis showed that in the presence of PEG there is a great possibility that PUT pre-treatment has a role in keeping the POD activity around the control level. In conclusion, although there were obvious differences between the effects of PUT and PEG treatments, there was also a remarkable overlap between the effects of putrescine and osmotic stress responses in wheat plants, suggesting that putrescine has already induced acclimation processes under control conditions **(Pál et al., 2018 *Plant Science*)**.

Cumulative IF of the project: 23,727.