Final report of K124430 project entitled “Investigation of salicylic acid-related stress acclimation processes in crop plants”

The major aims of the present proposal are the better understanding of the mode of action of SA, with a special regard of the phenylpropanoid pathway and fructan metabolism. These compounds are important in acclimation processes; and they also have important food quality aspects as antioxidants or dietary fibres. Part of these works will be carried out on rocket salad with an Israeli cooperation. Since the SA signalling is also connected with other hormonal actions; in order to better understand these cross-talk mechanisms, the stress tolerance of wheat genotypes carrying DELLA/gibberellic acid-related rht genes will also be tested. Due to its practical aspects the effects of different forms of SA and the different ways of applications will also be further investigated.

The following questions have been addressed:

Comparison of the mode of actions of SA and Na-SA during stresses in various crop plants

Investigation of the influence of different SA treatments on phenypropanoid metabolism during various stresses in crop plants.

Effects of SA treatment on fructan metabolism under stress conditions

Investigation how genetic and ecological variations can influence the acclimation processes to the changing environment

At the beginning of the work some stress-related processes related to the phenylpropanoid metabolism were studied.

It is known that light is a critical factor for the development of stress tolerance in plants. In the present work the effects of light during the cold acclimation period were studied in chilling-sensitive maize plants. Before exposure to chilling temperature at 5◦C, plants were cold acclimated at non-lethal temperature (15◦C) under different light conditions. Although exposure to relatively high light intensities during cold acclimation caused various stress symptoms, it also enhanced the effectiveness of acclimation processes to a subsequent severe cold stress. It seems that the photoinhibition induced by low temperature is a necessary evil for cold acclimation processes in plants. Greater accumulations of soluble sugars were also detected during hardening at relatively high light intensity. Certain stress responses were light-dependent not only in the leaves, but also in the roots. The differentially expressed genes with phenylpropanoid pathways in the focus were further investigated in relation to changes in certain phenolic compounds and other plant growth regulators. Phenylalanine ammonia lyase (PAL), one of the key enzyme of the phenylpropanoid biosynthesis, was mainly activated under limited light conditions. However, light-induced anthocyanin accumulation occurred both in the leaves and roots. Chilling stress induced the accumulation of salicylic acid (SA), but this accumulation was moderated in the cold-acclimated plants. Acclimation also reduced the accumulation of jasmonic acid (JA) in the leaves, which was rather induced in the roots. The level of abscisic acid (ABA) is mainly related to the level of the stress, and less indicated the level of the acclimation. The highest glutathione (GSH) amount was observed during the recovery period in the leaves of plants that were cold acclimated at growth light, while their precursors started to accumulate GSH even during the chilling. In conclusion, different light conditions during the cold acclimation period differentially affected certain stress-related mechanisms in young maize plants and changes were also light-dependent in the root, not only in the leaves.

These results were published in Frontiers in Plant Science (Front. Plant Sci., 2018, 9:850. doi: 10.3389/fpls.2018.00850) and IJMS (Int. J. Mol. Sci. 2020, 21, 1942; doi:10.3390/ijms21061942).

There are some results that aspirin (acetylsalicylic acid) had a positive effect on the treatment of certain types of cancer. However, the results cannot be generalized and it is not always clear whether it is a direct anticancer effect or a general health effect. Since plants produce different amounts of salicylic acid, we have sought a relationship between the salicylic acid content of some plant extracts and their anticancer activity. Growing of wheat and rice plants were carried out under controlled conditions. The salicylic acid content was determined by high-performance liquid chromatography. The viability and cell cycle assays were performed on HepG2 and Caco-2 cell lines. Despite the high content of salicylic acid, the extracts from rice plants did not show significant anticancer activity. In spite of the low salicylic acid content, the positive effect of wheat germ was confirmed in both tests. There is no direct relationship between the salicylic acid content of the plant extracts and their anticancer activity. However, it has been proven that young wheat germ is more effective than mature leaf. These results were published in Biologia Futura (2020, 71:265–271 https://doi.org/10.1007/s42977-020-00026-4).

Based on preliminary experiments Brachypodium experiments (where acid and salt forms of salicylic acid were used in Brachypodium plants) the applications of Na-salicylate were selected. During the work the effects of various NaSA treatments (soaking seeds in NaSA or spraying with NaSA solution) were investigated during mild heat stress in Brachypodium plants. The maximum efficiency of PSII was not affected by the heat but the Quantum Yield of PSII increased in the control and seed soaking plants at elevated temperature but did not changed in the sprayed plants. Seed soaking and spraying with NaSA affected the antioxidant systems in different ways. Changes in the endogenous SA level and flavonol content were also investigated using LC-MS/MS method. A decrease in the abscisic acid level was also observed during heat stress and it was more pronounced in NaSA treated plants especially after spraying. Results were published in PLoS ONE (2020, 15(1): e0227608. https://doi.org/10.1371/journal.pone.0227608).

The next part of the project the differences caused by SA or NaSA treatments were investigated. These experiments were made on *Eruca sativa* plants.

Seeds of *Eruca sativa* were soaked either in distilled water (DW), SA or NaSA for overnight then plants were grown in soil in a plant growth chamber for five weeks. After it half of the plants were exposed to UV stress for a week. The chlorophyll content and chlorophyll-a fluorescence parameters were not affected either by treatments or the UV. The NO level was higher in SA and NaSA plants but it decreased during UV stress and in the case of SA treatment it was under the detection limit. In DW plants the NO level increased under UV stress. The H2O2 content increased in DW and NaSA plants under UV but not in SA treated plants. Antioxidant enzyme activities were also measured. The catalase activity increased during UV and it was more pronounced in DW plants and smallest activity was detected in SA plants but there was no different between the SA or NaSA plants. The guaiacol peroxidase activity was higher in NaSA plants but not in the SA treated plants. The activity increased in DW plants during UV stress but did not change either in SA or NaSA treated plants. The ascorbate peroxidase activity of NaSA plants decreased compared to the control and it was more pronounced in SA plants. This activity increased during UV in DW and SA plants but not in NaSA ones.

Certain enzymes of phenylpropanoid pathway (phenylalanine ammonia-lyase [PAL]; dihydroflavonol-4-reductase [DFR]; flavanone 3-hydroxylase [F3H] were also studied. The PAL activity decreased

during UV stress but there was no differences between the treatments. The DFR activity did not change after NaSA treatment but it was not detectable in SA plants. This activity increased only in NaSA plants under UV. F3H activity increased in the SA plants but decreased under UV stress while in DW and NaSA plants an increase occurred which was more pronounced in DW plants. Further metabolomics analysis has been planned but because of the COVID these measurements were delayed so the results has not been published yet. The MS is under preparation.

Changes in the fructan metabolism after SA treatment was also investigated under control and stress conditions in various mono- and dicotyledonous plants.

Wheat, maize and pea plants were investigated after SA treatment under control and stress conditions. Unfortunately, there were no differences between the SA-treated and non-treated plants in the fructan metabolism. The expression of genes involved fructan biosynthesis (6(G)-fructosyltransferase, Sucrose:sucrose 1-fructosyltransferase, beta-fructofuranosidase) were also studied but no differences could be found.

This work about fructans has been planned for three years but at the beginning we had to realise that it is not worth to continue this part. Instead of it a salicylic acid derivative, S-methylmethionine salicylate (MMS). Studies on beneficial effects of certain biologically active substances, S-methylmethionine (SMM) and salicylic acid (SA) have provided a lot of valuable information regarding their role to counteract harmful effects of environmental stresses such as chilling. To obtain a more complex and stable defence compound with an extended range of stress-protective effect, the new derivative S-methylmethionine salicylate (MMS) was synthesised from the natural, biologically active substances SMM and SA. Since both original materials have complex stress-protective roles, the new compound was expected to combine the effects of original substances and to stabilise the unstable SMM in the new compound, thus providing an extended stress tolerance. Our data show how the MMS combines the efect of SMM and SA on molecular level, thus causing numerous changes in the gene expression pattern and metabolite content, which can be linked to either one or the other compound. SMM acts as a substrate or stimulates other metabolic pathways through its derivatives. Additionally, SA, as a signal molecule, helps to fine tune the stress response; moreover, also stimulates metabolic processes. The MMS pre-treatment induces priming mechanisms, through which it can alleviate the negative effects and prevent severe damages caused by chilling and salt stress in maize. This biologically active compound gives rise to a better physiological condition, thus it could provide an alternative, environmental friendly way to enhance the plants defence mechanisms against stressors. As MMS is more stable than SMM, it promises easier, more long-lasting and more cost-effective usage in agriculture, with a complementing effect of SA. The results have been recently published in Journal of Plant Growth Regulation (2022, 41:2073–2091).

In the first part of the Task 4 the influence of the dwarf genes (Rht1 and Rht3) on the cold tolerance of wheat plants were investigated.

The wheat semi-dwarfing genes Rht (Reduced height) are widely distributed among the contemporary wheat varieties. These genes also exert pleiotropic effects on plant tolerance towards various abiotic stressors. In this work, frost tolerance was studied in three near-isogenic lines of the facultative variety ‘April Bearded’ (AB), carrying the wild type allele Rht-B1a (tall phenotype), and the mutant alleles Rht-B1b (semi-dwarf) and Rht-B1c (dwarf), and was further compared with the tolerance of a typical winter type variety, ‘Mv Beres’. The level of freezing tolerance was decreasing in the order ‘Mv Beres’ > AB Rht-B1a > AB Rht-B1b > AB Rht-B1c. To explain the observed differences, cold acclimation-related processes were studied: the expression of six cold-related genes, the phenylpropanoid pathway, carbohydrates, amino acids, polyamines and compounds in the tricarboxylic acid cycle. To achieve this,

a comprehensive approach was applied, involving targeted analyses and untargeted metabolomics screening with the help of gas chromatography/liquid chromatography—mass spectrometry setups. Present results show that mutant alleles Rht-B1b and Rht-B1c may negatively affect the freezing tolerance in 'April Bearded' facultative wheat plants. However, several coldrelated processes exhibited similar changes during the cold hardening period in these genotypes. High resolution MS and MS/MS based untargeted metabolomics revealed a series of differentiating compounds, especially coumaroylated and feruloylated poliamines and apigenine derivatives, that showed a remarkably different time profile compared to the stress-induced fructans. The mechanisms of frost tolerance might be different for a typical winter variety (Mv Béres) than for a facultative variety. Although oligosaccharides are able to differentiate between them, it is unlikely that they play an important role in Mv Béres, which may use other strategies. Compared to flavonoids, the polyamine derivatives exhibited very diverse patterns, and the accumulation of these compounds correlated with the level of freezing tolerance of the 'April Bearded' lines. However, in Mv Béres, which had the highest level of tolerance, the accumulation of these compounds was much less pronounced. Obviously, the interaction between the major developmental genes, frost tolerance genes and the Rht genes merits further deep exploration. Results were published in IJMS (Int. J. Mol. Sci. 2022, 23, 7969. https://doi.org/ 10.3390/ijms23147969).

In the second part of Task 4. the investigation how ecological variations can influence the acclimation processes to the changing environment was planned.

Seeds of *Eruca sativa* from the Mediterranean and arid regions of Israel were purchased, the optimum plant growth parameters in the phytotron in Martonvásár was set.

We has started the phytotron experiments with two selected ecotypes in Martonvásár. P2 is collected from the Negev desert (desert type), P8 is from the Golan Heights (Mediterranean type). Plants were grown in plant growth chamber at a short day light periodicity (8/16 hours light/dark period) for 5 weeks then photosynthetic parameters and salicylic acid (SA) content of the ecotypes were measured. The Fv/FM parameter and Quantum Yield of PSII (YII) were measure on the old and middle leaves of the plants. There was no difference between the old and middle leaves in the Fv/Fm parameter but it was a little smaller in P2 plants. YII was higher in younger leaves but there was no difference between the genotypes. The free SA content was a little higher in P8 plants but P2 has much higher amount of bound SA form. A biochemical analysis were carried out after 7 days of moderate UV-B stress on phenylpropanoid compounds and plant hormones. Quercetin and its glucosides were in a very low amount or below the detection limit in both ecotypes but an increase was found during UV stress which was more pronounced in the P2 desert ecotype. Same tendencies were found in the contents of other flavonoids (isorhamnetin and its glucoside, kaempferol-glucoside, esculin, caffeic acid, phlorizin and phloratin). In contrast to these results, the amount of sinapic acid decreased during UV stress.

Plant hormones: P2 desert plants contained less abscisic acid than the Mediterranean P8 plants but no changes were found during the UV stress. The jasmonic acid level showed similar tendency to the flavonoids; increased during the stress, but the level in P8 plants were lower. No significant changes were found in the level of salicylic acid and its precursor, benzoic acid.

Investigation of different ecotypes collected from various habitats of Israel: measurements of activity of antioxidant enzymes, anthocyanins, flavonols and other stress markers.

The collection of *Eruca* plants from different regions of Israel was not possible because during the springs there were strict rules in Israel because of the pandemia so the travel in the country, even for citizens, was forbidden during the natural growing season of *Eruca*.

Instead of it exogenous applied plant growth regulators were investigated in vegetables in different regions of Hanoi in the frame of a cooperation with the CRETECH, Hanoi, Vietnam.

Plant growth regulators (PGRs) play a pivotal role in increasing vegetable productivity, but they have many drawbacks that can include health and safe concerns of consumers. In this study, the plant growth regulators (PGRs) contents of 111 *Brassica juncea* (L.) Czern. samples collected from six main suburban areas of Hanoi, Vietnam, were determined by liquid chromatography—electrospray tandem mass spectrometry. Results showed the presence of PGRs residues in 83 analyzed samples (74.77%). Eight PGRs, including Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Indole-3-carboxylic acid (ICA), 3-Indolepropionic acid (IPA), Gibberellin A3 (GA3), Gibberellin A4 (GA4), Gibberellin A7 (GA7) and Trans-zeatin (tZ), were found in *B. juncea* with the average residues dropped in the range of 0.04 and 0.65 mg kg−1. GA4 was considered as the most popular PGR applying in vegetable production in the investigated areas. Notably, the concentrations of gibberellins were higher than the regulations in vegetables and fruits in Europe, USA and Japan. This might be the potential health risks to consumers and environmental pollution, which necessary to be controlled with consideration of residue regulations. Results were published in Biologia Futura (2020, 71:323–331).