Infrastructure

One of the aims of the project was to complement laboratory infrastructure. Purchases included broad band UV excluding plastic foils, UV screening cut-off glass filters, analytical balance, and a vacuum centrifuge with ice trap. In addition to increasing the laboratory infrastructure, various columns, eluents, consumables, and test compounds used in liquid chromatography and spectrophotometry were also purchased.

Research

At the beginning, in the first year of the start of the work, the project progresses according to the work plan, but SARS-CoV-2 pandemic negatively influenced on the continuation. Safety regulations at the University of Pécs and due to travel restrictions of worldwide, experiments including laboratory analyses and plants growing in chambers could not have been realized (neither at our own nor at the cooperation partner's laboratory). Nevertheless, new experiments were designed for work outdoors and new projects was discussed with the foreign partner as an alternative, which were not included in the original work plan.

As a pilot experiment in the first year, Arabidopsis thaliana plants were grown for flavonoid identification. The phenolic profile of these plants is more complex than tobacco or pepper, moreover Arabidopsis leaves contain several multiple glycosylated compounds, which are difficult to identify with a simple HPLC system available in our laboratory. Nevertheless, Arabidopsis thaliana experiments and their total antioxidant (TAC) and UV absorbing pigment capacity measurements also were finished at the University College Cork (UCC), but the analysis of the phenolic pattern is in progress in a collaborating laboratory's at Hungary. In this study we focused on carotenoids and their antioxidant effects. For this purpose, we used a wild type (Col-0) and different carotenoid synthesis altered mutant (aba1-6, npq1-2 and szl1-1npq1-2). The above mentioned Arabidopsis seeds were sown directly into Jiffy plugs, after a 72 hours cold treatment (4 °C). The seedlings were grown under around at 70 μmol m^{-2} s^{-1} PAR with a 16/8 hours light/dark regime at 21/18 °C and around at 60% relative humidity. After two and a half weeks, the plants were exposed to the outdoors conditions used to three different filters for treatment. Total UV blocking filter, UV-B blocking mylar foil and UV-B transparent cellulose acetate film which were placed over UV-B treated plants to attain uniformity. After the experiment is over, Arabidopsis leaves were harvested, and the materials were pulverized using liquid nitrogen and stored at deep frozen (-80 °C).

The following carotenoids were analyzed in the Arabidopsis plant during the experiment: violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, lutein, and β-carotene. As expected, plants which not exposed to UV radiation, clear differences occurred in the level of carotenoids among genotypes. Mutant szl1-1npq1-2 displayed reduced while npq1-2 showed higher carotenoids quantity than wild type and aba1-6. Furthermore, it was clear that the most significant change in UV-induced metabolites was found the UV-B irradiation. In case of aba1-6, carotenoid level was similar when used to all screening filters (exception of lutein which showed higher concentration without UV-B). All things considered, we can say that similar carotenoid levels occurred in case of npq1-2 Arabidopsis plants with and without UV. On the other hands we can say that higher carotenoid concentrations occurred in case of szl1-1npq1-2 Arabidopsis plants with UV-B radiation.

HPLC and spectrophotometry analysis was supplemented with photosynthesis measurements, where the results displayed that the Fv/Fm and Y(II) were different between the genotypes and the wild type being the healthiest. Also, Y(NPQ) level was the most increased in case of aba1-6 plants then in the wild type plants and finally in all other genotypes while Y(NO) was the most decreased in case of the wild type and aba1-6.

Antioxidant results displayed (Figure 1), that in the case of the wild type, each filter foils had significant effect for the compounds with antioxidant properties, most of which are special metabolites, flavonoids, carotenoids, and phenolic acids in both Folin-Ciocalteu (FC) and FRAP while in case of szl1-1npq1-2 the UV-B caused significant differences in both FC and FRAP.

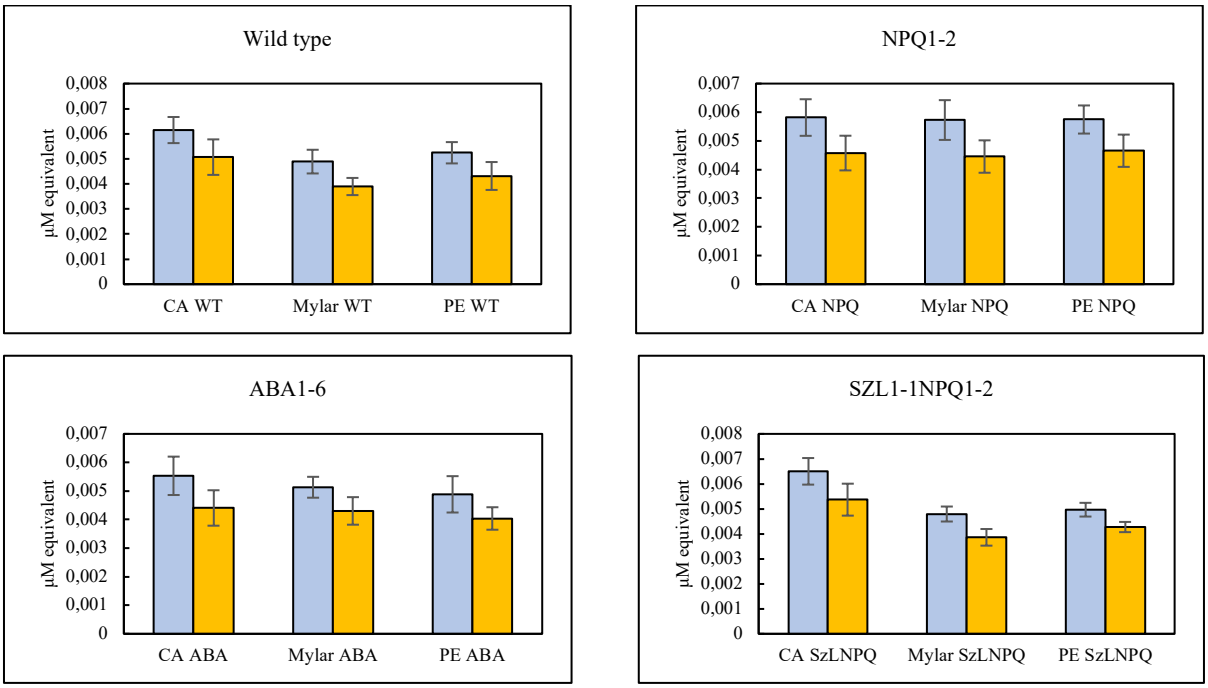

Figure 1: Folin-Ciocalteu (blue columns) and FRAP (orange column) antioxidant capacity of wildtype (WT) and carotenoid biosynthesis altered mutants (aba1-6, npq1-2 and szl1-1npq1-2) Arabidopsis thaliana (Col-0). The plants were grown under UV transparent cellulose acetate (CA), UV-B excluding (Mylar) and UV excluding (PE) filters. Bar plots represents average of 4 biological replicates and error bars the standard deviation.

Contrary to our expectations, we did not experience any changes in the other genotypes in case of antioxidant capacity and the situation was similar in the case of UV-absorbing pigment capacity results, where significant changes were not observed. The data were collected from this study and the results of which we plan to publish in an international journal (paper writing is in progress).

In the cooperating partner's laboratory, another experiment was carried out in which Arabidopsis plants were grown indoor conditions under various monochromatic LED lights. We used monochromatic LED illumination with the following wavelengths: 310 nm, 325 nm, 340 nm, 365 nm, and PAR (no UV). The growing conditions were the same as in the previous experiment, but in this case, we worked with wild type (Col-0) only. During the experiment, we investigated how wavelength-dependent the UV inducibility of phenolics in Arabidopsis thaliana leaves. The most important thing would be to know what kind of phenolics are in the Arabidopsis leaf, but in the absence of this, I can illustrate our results with a chromatogram (Figure 2) with unknown peaks. For the evaluation, the peaks were calibrated to quercetin-3-*O*-glucoside, because according to the literature, Arabidopsis leaves may contain various kaempferol and quercetin derivatives.

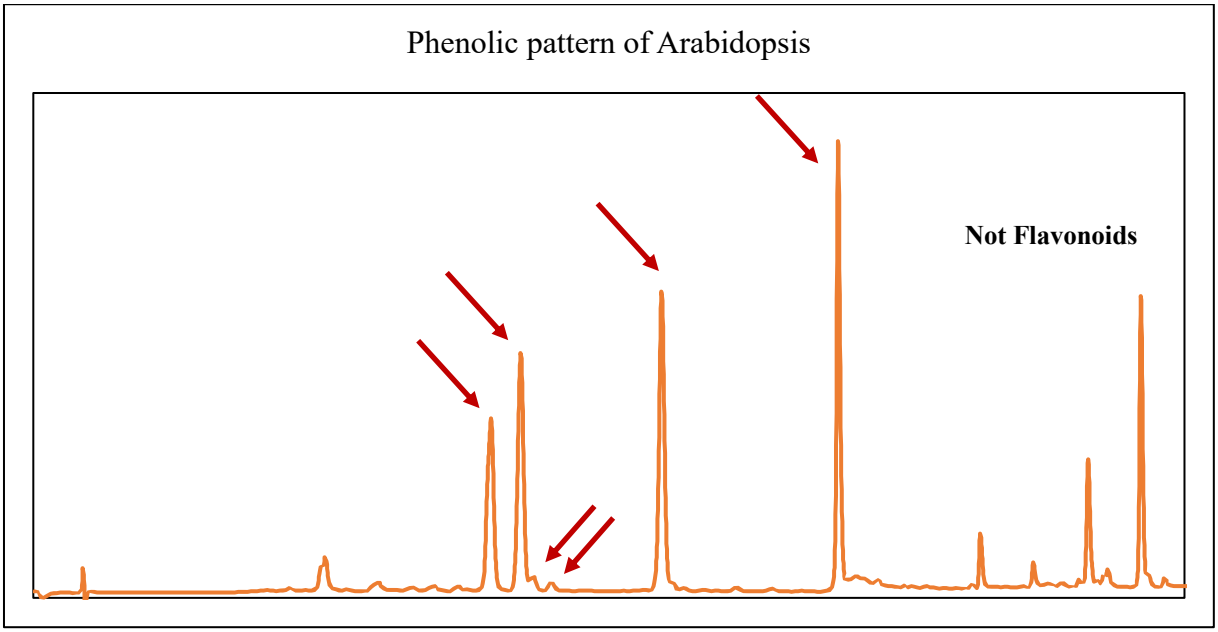

Figure 2: Chromatogram of phenolics (orange line) in Arabidopsis thaliana leaf with currently unknown peaks

The most striking result is that without UV radiation the plants were able to synthesize phenolics in very low amounts, their concentrations were small. However, different wavelengths of LED changed their concentration to varying degrees in which the 310 nm LED proved the most effective. The knowledge of phenolics from the leaves of Arabidopsis can filling a gap, after which we can learn which wavelengths induce which flavonoids and to what extent. The HPLC results were supplemented with antioxidant measurements (FC and FRAP), and the antioxidant data also confirmed the liquid chromatography results, according to which the 310 nm LED was the most effective. The experiment is currently being continued, in which the intensity of the monochromatic wavelengths is changed (increased). The size of the LED boxes in the UCC laboratory's is limited, that is why we can measure Arabidopsis plants. After results will be collecting and evaluating, we plan to write a publication from this experiment as well (paper writing is in progress).

Bell pepper (*Capsicum annuum* var. grossum) is an important economic crop that is widely grown in Hungary and in the world. The leaves contain large amounts of phenolics which are highly effective antioxidants and UV screeners. For these reasons, it was an excellent choice as a model plant however, many varieties are known. In this experiment, we investigated how different varieties (Darina, Édes Alma, Rekord) tolerate the cold stress. The seedlings were grown in a chamber under 110 μmol m^{-2} s^{-1} PAR with a 16/8 hours light/dark regime at 25/20 °C and around at 60% relative humidity until the leaves on the fourth node become a fully developed, then half of the plants received a cold treatment 15/10 °C for 5 days. In terms of examination methods, we used invasive (antioxidant capacity measurements) and non-invasive techniques (pigment estimation, photosynthesis parameter measurements).

Chlorophyll fluorescence data were shown that pepper varieties perceived the cold treatment as a stress effect. As an important variable of plants photosynthetic activity and damage of PSII, the Y(II) also decreased in every treated seedling. This change is common when plants need to defend themselves against low temperature. Low temperature as cold stress increased the Y(NO) parameter in all treated plants perhaps the plants needed to use non-regulated nonphotochemical quenching of PSII to avoid generation of reactive oxygen species (ROS). The adaptation to low temperature does not require NPQ improvement which were found in this study, but NPQ is negatively correlated with the estimated chlorophyll pigments (Figure 3) which is the result of freezing induce photoinhibition. The estimated chlorophyll pigments were displayed same pattern in all varieties, their contents decreased with the low temperature. Parallel to these changes, IRGA data in net photosynthetic activity were also

decreased with cold temperature, which is also supported by the results of the non-invasive measurements.

Untreated plants Cold treated plants

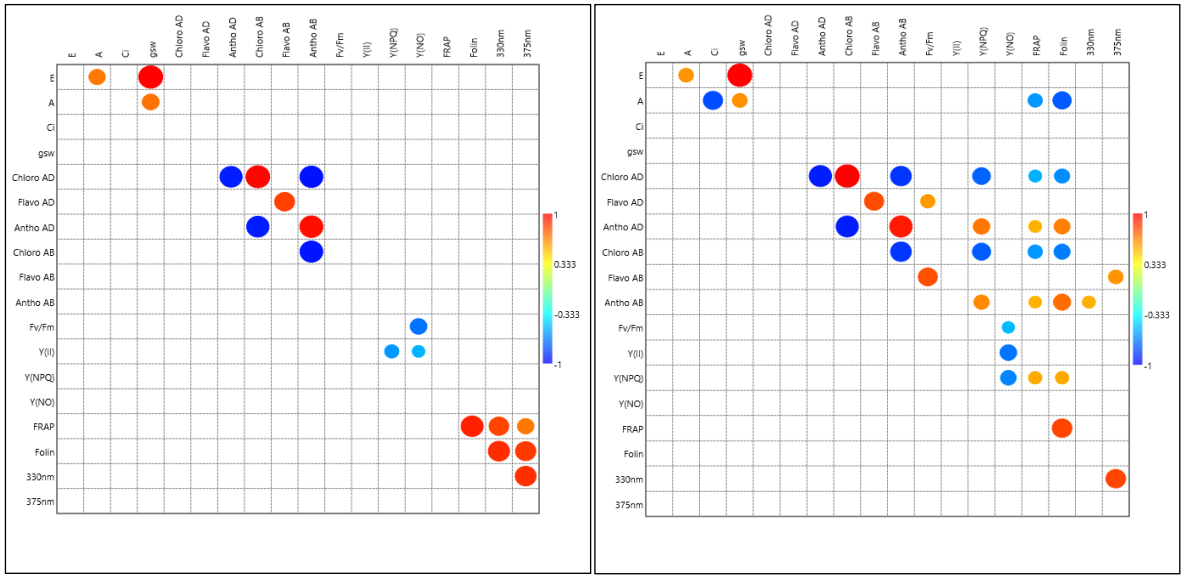

Figure 3: Correlation table of all measured variables in untreated (left) and in cold-treated (right) plants

The gas-exchange results were not surprised, stomatal guard cells quickly close the stomata under unfavourable temperatures (low, cold) to prevent the plants from dehydrating. Photosynthetic carbon assimilation, transpiration and stomatal conductance data showed decreasing change due to cold stress, and this reduced stomatal aperture was reflected by a lower transpiration rate and lower net CO_2 assimilation but not in all varieties. Interestingly, the results of Darina prove the non-stomatal regulation of these processes against cold. The decreasing stomatal conductance absence in case of Édes Alma suggest a stomata-independent strategy for acclimation to cold.

Flavonoid estimation data were not shown significant differences in any varieties during the cold treatment. Presumably, the Dualex instrument detect only the absorbing pigment compounds in the upper epidermal layer meanwhile, the cold induced phenolic compounds probably concentrate in the mesophyll layer. Nonetheless, the estimated pigment parameters showed a positive correlation between the abaxial and adaxial epidermal layer in case of untreated plant leaves (Figure 3). Antioxidant capacity measurement results are positively correlated with the estimated anthocyanin content (Figure 3) because these colouring pigments are founding in the upper epidermal layer. Leaf extractions were measured at 375 nm because this is a well-known invasive flavonoid measuring technique and found a positive correlation with the estimated flavonoid.

Total antioxidant capacity results were positive correlated with the two types of UV wavelengths evaluated results (Figure 3) in case of the untreated plants however, because of the pepper varieties displayed a changed correlation pattern both for the antioxidant capacity data and the given UV wavelengths (Figure 3) in case of the cold-treated plants.

Even though the varieties of pepper examined were different (sweet, spicy), we found no difference in the response to low temperatures. It is conceivable that the low temperature responses are unified, which are not or only less affected by differences between pepper varieties. Recommended parameters for rapid testing of low temperature effects are the Y(II), the non-photochemical quenching of Y(NO), TAC, and the non-invasively estimated chlorophyll index. These mentioned parameters and methods are perfectly suitable for laboratory examination of the effects of cold stress, but the Dualex instrument could be useful in agricultural monitoring during the growing season.

Results of this experiment with cold stressed various pepper plants are publishing to an international journal with the following title “The responses of the fight against the cold: photosynthetic and antioxidant responses of different bell pepper cultivars (*Capsicum annuum* L.) to cold stress” (under review).

The bell pepper was one of the central model plants of the project, along with Arabidopsis. For the next experiment we chosen Amy because this pepper variety containing UV inducible phenolics. Therefore, it was obvious to examine the part of the UV range what reaches the surface of the earth and leaves of the plants to a large extent, almost entirely. The aim of the present study was to explore whether UV-A (320-400 nm) like UV-B (280-320 nm) is also capable change the flavonoid pattern in pepper leaves and to what extent. The rearing conditions were the same as in the above mentioned experiment until the seedling reached the fourth weeks. Then the peppers were exposed UV-A radiation for 5 days, meanwhile the plants were also exposed to PAR. Total antioxidant and UV absorbing capacities were measured with various spectrophotometric methods while phenolic compounds were analyzed with HPLC system and estimated with Dualex instrument.

The most significant phenolic compounds of the pepper leaf are the following, chlorogenic acid, luteolin-7-*O*-glucoside, luteolin-7-(apiosyl)glucoside, apigenin-7-(apiosyl)glucoside, apigenin-7-(6-acetylglucoside) which were analysed with HPLC-MS system (Figure 4). Estimated pigment parameters data showed that the flavonoid level in the upper epidermal layer was increased and interestingly the antioxidant capacities and UV absorbing capacities also were decreased from the whole leaf extract because of UV-A treatment. Presumably, Dualex device (non-destructive) can detect the presence of phenoloids

in the lower and upper epidermis of the leaf, while antioxidant measurement (destructive) can detect from the whole leaf extract therefore, the amount of phenolics in the epidermal layer increased at the expense of amount the phenolic molecules in the mesophyll layer. This assumption was confirmed by HPLC analysis, as the number of certain flavonoids increase (probably in the epidermal layer), while other flavonoids decreased (probably in the mesophyll layer). Localization could provide an even more accurate overview of the real location and role of the phenolics, so it would be an exciting research area in the nearly future.

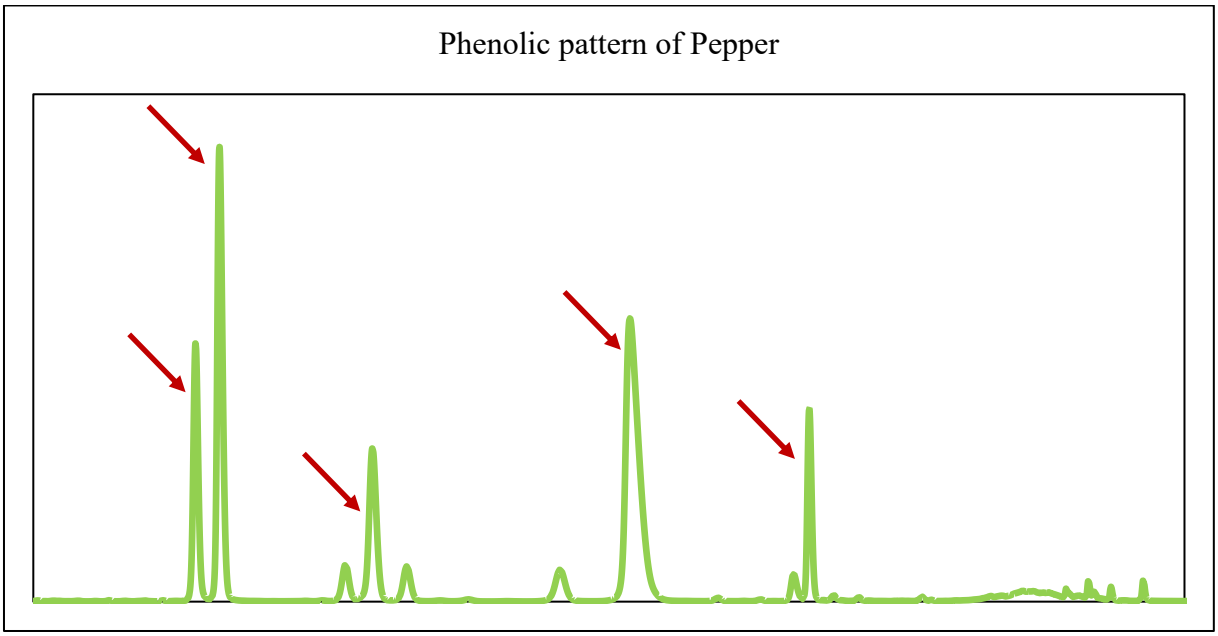

Figure 4: Chromatogram of the known phenolics (green line) in Capsicum annuum cv Amy pepper leaf

The data were collected from this study and the results of which we plan to publish in an international journal (paper writing is in progress).

During the growing season (spring and summer) of the past year, the experiment with glass filters and plastic filters could not be performed due to the extreme weather. As a result of the extreme heat and extremely high PAR, our plants did not develop properly, they stopped growing, kind of almost dying and our photosynthesis measurement results also showed that the plants were damaged. We planning to carry out the experiment again this growing year (seeds were sowed) and publish the results at an international conference and in an international journal.

Phenolic profiling of tobacco samples from pilot experiments in the first year confirmed that while Nicotiana tabacum cultivars were equally good for model experiments indoors, cv. Xanthi and especially cv. Wisconsin-38 was better suited for outdoor work than cv. Petit

Havana SR1. Absolute and relative quantities of the phenolic acid and flavonoid change in response to UV radiation, therefore UV is applied to modify the phenolic pattern of different species of plants grown indoors as well. It remains to be seen how the extent and quality of changes induced such indoor studies compares to those brought about by solar UV. In this experiment phenolic composition of tobacco (cv. Xanthi) were compared indoors and outdoors, in the absence and in the presence of UV. Three different filters foils were used: UV-blocking, UV-A-transparent, and total UV-transparent plastic filters. In the outdoor experiment tobacco plants were grown from seedling to harvest from mid-June until the end of July. Meanwhile, the same filters were used indoors condition, but plants were grown in a growth chamber under 110 μmol^{-2} s^{-1} PAR and added UV radiation for 5 days. The specific metabolites pattern was analyzed with HPLC system which are the following: chlorogenic acid, neochlorogenic acid, chryptochlorogenic acid, p-coumaric acid, quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside. The main goal of this experiment was to observe potential UV related alterations indoors and outdoors. Among the above mentioned phenolics, chlorogenic acid is the most important phenolic acid, while the two rutinosides are the most important flavonoids in tobacco leaves.

One of the most spectacular results of the experiment is that there are significant differences in the total phenolic content between inside and outside conditions (Figure 5). The amounts of these metabolites were higher at outside. However, the total phenolic acid content did not show any significant change during outdoor conditions, but under UV exposure, their amount approximately doubled during indoor conditions (the increasing only reached about 2/3 of the outside level).

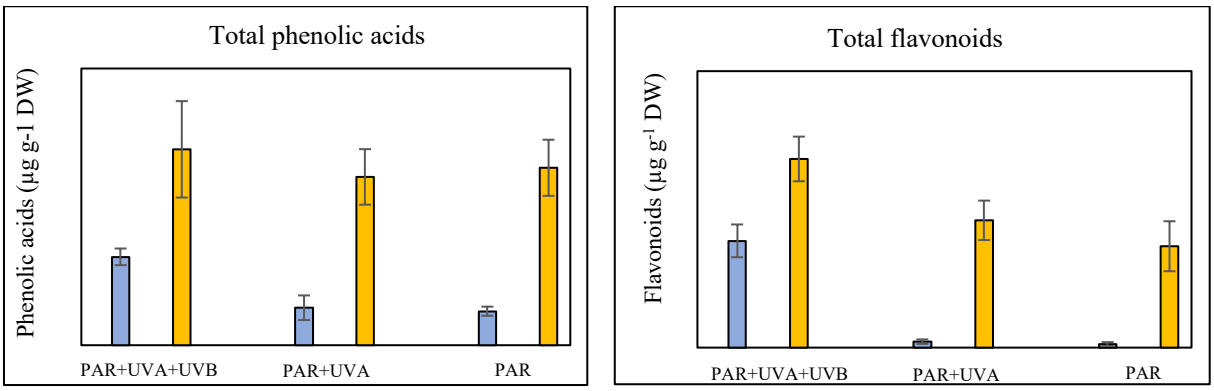

Figure 5: Indoor conditions (blue columns) and outdoor conditions (orange column) phenolic pattern of tobacco leaves. The plants were grown under UV blocking (PAR), UV-B blocking (PAR+UV-A) and UV transparent (PAR+UV-A+UV-B) filters. Bar plots represents average of 6 replicates and error bars the standard deviation.

The change in case of total flavonoid content showed a continuous increase in outdoor conditions, even UV-A radiation also increased their level to a small extent while UV-B radiation increased it further. Nevertheless, during indoor conditions, UV-B radiation was a factor which could be able to induce kind of from the zero level to accordingly 2/3 of the outdoor level (Figure 5). Based on our results, the UV radiation quantitatively induces the phenolic compounds in tobacco leaves, but this induction is selective. The different circumstances (indoor or outdoor conditions) cause similar responses. During the evaluation process an exciting result was found, which can be observed in the case of intermediate compounds (Figure 6) both phenolic acids and flavonoids.

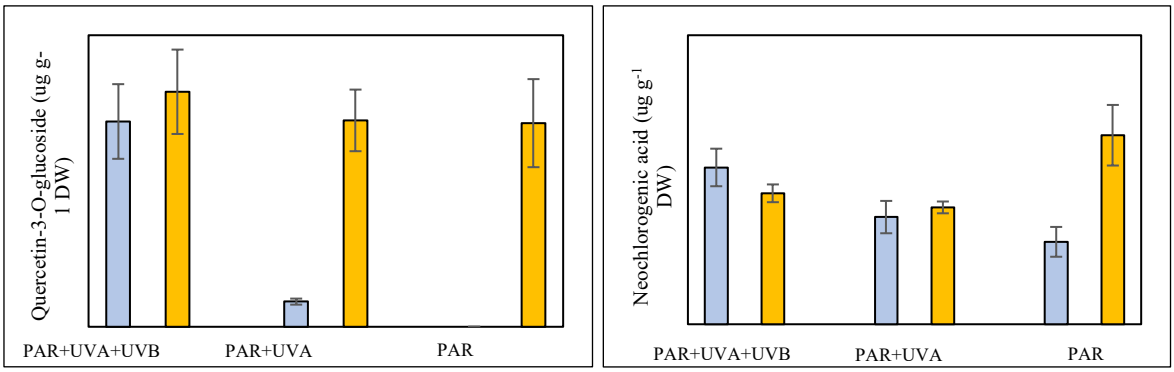

Figure 6: Indoor conditions (blue columns) and outdoor conditions (orange column) intermediate compounds of tobacco leaves. Quercetin-3-O-glucoside (left) and neochlorogenic acid (right). The plants were grown under UV blocking (PAR), UV-B blocking (PAR+UV-A) and UV transparent (PAR+UV-A+UV-B) filters. Bar plots represents average of 6 replicates and error bars the standard deviation.

The level of neochlorogenic acid continuously decreased under the influence of UV radiation, but in indoor conditions it significantly increased and the level of which exceeded the outdoor level (Figure 6) meanwhile, the amount of quercetin-3-*O*-glucoside reached the outdoor level, and an evolved form of this flavonoid is quercetin-3-*O*-rutinoside that is one of the main flavonoids in tobacco leaves. As a result of indoor conditions, total phenolic acids are less, while total flavonoids are more inducible by UV radiation. In the outside conditions, the high PAR may have been one of the driving forces, while the effect of UV radiation had less influence on the amount of specific metabolites. The main flavonoids in tobacco leaves are the rutinosides, which simple increased because of UV radiation, while the intermediate quercetin-3-*O*-glucoside flavonoid reached the outside level. The situation is similar for neochlorogenic acid and p-coumaric acid cases. An interesting question is, why did UV radiation increase the quantity of the small amount of intermediate compounds so drastically? Probably, these compounds act as buffers, from which compounds with extremely good antioxidant properties

can easily be formed. In this study, during indoor conditions, several phenolics were significantly increased and from these some of also reached the outdoor levels.

The data were collected from this study and the results of which we plan to publish in an international journal (paper writing is in progress).

Due to the pandemic situation, we were only able to carry out experiments with indoor glass filters to a limited extent, therefore plastic foils were used to as these results were presented above. Nonetheless, outdoor experiments with glass filters were crerated with Arabidopsis, Capsicum and Nicotiana model plants. The pepper experiment was destroyed due to last year extreme weather, and this must be planned again this growing season. The Arabidopsis leaves samples are in the deep freezer (-80 °C) while with tobacco plants results of HPLC analysis is recorded from this pilot experiment (Figure 7). Most of the measurements and evaluation are still in progress. In this study seven different glass filters were used which allowed only part of the UV radiation to pass through: 280 nm (full UV transparent), 295 nm, 305 nm, 316 nm, 320 nm, 324 nm, 395 nm (kind of UV excluding). In the outdoor experiment tobacco plants were grown from seedling to harvest under outdoor conditions. In the case of neochlorogenic acid, the results of our previous experiment were confirmed in which used to plastic foils and experienced that the UV-B radiation decreased them level during outside conditions. A similar result was experienced when used to the above mentioned glass filters (Figure 7).

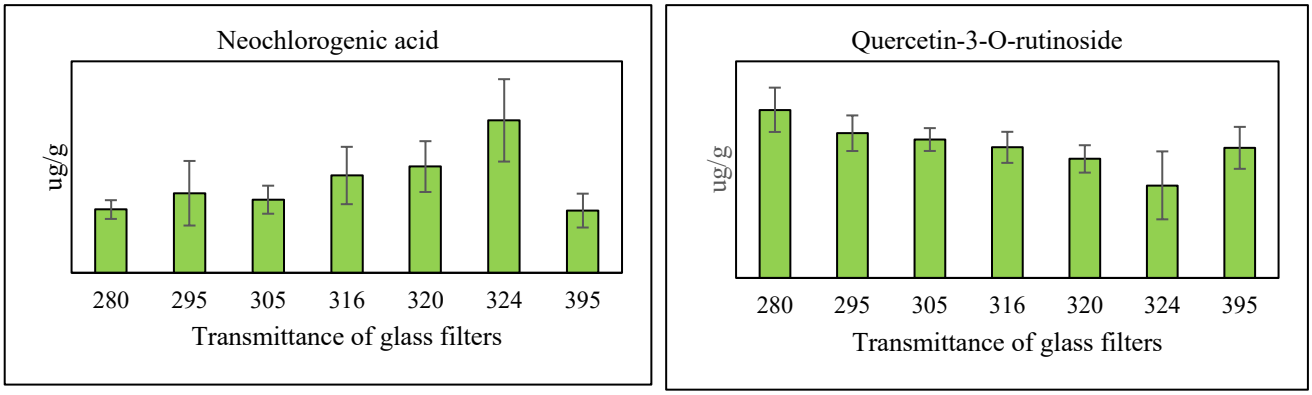

Figure 7: Neochlorogenic acid (left) and quercetin-3-O-rutinoside (right) levels under different glass filters during outdoor conditions. Bar plots represents average of 5 replicates and error bars the standard deviation.

In parallel the case of quercetin-3-*O*-rutinoside (main flavonoid in tobacco leaf) was also similar, which we experienced in a previous plastic filter study. The UV-A radiation is also significantly changed the flavonoid level (Figure 7). Later on, the experiment will be carried out with a large number of sample, paying attention to the intermediate compounds (chlorogenic

acid and quercetin derivatives) as well, which probably a very important buffer products for plants life.

A comparison of broad band UV-B and narrow band 311 nm UV-B treated tobacco leaves showed that although the two treatments resulted in diverse antioxidant responses, both stimulated the bioproduction of the same flavonoids (primarily quercetin-3-*O*-rutinoside) and phenolic acids (chlorogenic acid). Because the applied 311 nm treatment is outside the known absorption range of the plant UV-B photoreceptor UVR8, this observation confirmed the heterogeneous nature of UV inducible changes in phenolic biosynthesis.

Results of this experiment with UV treated tobacco plants were published an international journal with the following title "Narrow-band 311 nm Ultraviolet-B radiation evokes different antioxidant responses from broad-band ultraviolet" (accepted).

The following experiment was carried out in the UCC, where the effect of UV radiation and the effect on drought stress were investigated used to mint as a model plant. Mint seeds were grown for 30 days in plastic boxes in a chamber where the conditions were the following 180 μmol m^{-2} s^{-1} PAR with a 14/10 hours light/dark regime at 20 °C and around at 98% relative humidity inside the tissue culture boxes. After development, seedlings were exposed to a UV treatment for 8 days and wrapped them UV-B blocking and UV-B transmitting filters. Half of the plants were sampled immediately after 8 days of UV exposure while the other half were transplanted on soil and monitored for further 7 days. After the recovery time plants were harvested for analysis. I took part in the antioxidant, UV absorbing measurements and the evaluation process, the results of which are as follows. Total antioxidant capacity is increased in non UV exposed mints which measured with Folin-Ciocalteu assay. However, UV had a positive effect for the phenolic resulting in significantly higher concentration in UV treated plants compared with the non-exposed plants in fact, this effect was just temporary. After the above mentioned recovery stage, the phenol concentration was significantly decreased in case of UV treated plants but increased in case of non UV treated mints. A similar trend was observed in the case of TEAC. Nevertheless, UV exposed leaves showed higher antioxidant activity with FRAP method compared with the non UV treated plants but after the transplantation FRAP results almost tripled. During the recovery stage, plants decreased the antioxidant compounds, probably the plants concentrated on stress from transplanting and not from UV stress. In parallel with the antioxidant measurements, UV absorbing pigment compounds had a significant increasing in case of the UV treated plants after the UV treatment. On the contrary, overall similar contents of UV, UV-A and UV-B absorbing capacity were displayed after the recovery. Results of this experiment which in examined the effect of UV

radiation and drought stress are publishing to an international journal with the following title “From stressor to protector, UV-induced drought resistance” (accepted at the week before the deadline for the final report, there is no page number yet).

Overall, considering the success of the project, it was successful videlicet, several posters and presentations were also produced in addition accepted, under review and under writing publications. During the grant period, existing collaborations were strengthened, while acquired new ones in our and foreign country. At the beginning of the report, I mentioned that due to the pandemic (SARS-CoV-2) we had to deviate from the work plan, despite this carried out successful experiments both in the outdoor and indoor conditions used to several model plants. Publications expected in the near future, which I would like to attach to the application because, the experiments were carried out, and the results were collected and evaluated.

Pécs, 2023.04.26

Dr Csepregi Kristóf
egyetemi adjunktus