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Research Article

Changes in antioxidant properties of pepper leaves (*Capsicum annuum* L.) upon UV radiation

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Abstract: Bell pepper (*Capsicum annuum*) is one of the most popular vegetables consumed worldwide. The leaves of pepper are rich in phenolics, including phenolic acids and flavonoids. These compounds are well known for their ultraviolet (UV) absorbing and antioxidant properties. While the change of the phenolic pattern is an intensive research subject, it is not yet well-known in pepper leaves, particularly in outdoor conditions. In this experiment, we examined the effect of UV radiation on the leaves of outdoor grown peppers, focusing on the UV-absorbing properties and antioxidant capacities. Three different total antioxidant capacity (TAC) measurements have been compared: (I) Folin-Ciocalteu Reactivity (FC), (II) Ferric Reducing Antioxidant Power (FRAP) and (III) Trolox Equivalent Antioxidant Capacity (TEAC). Moreover, non-enzymatic hydrogen peroxide scavenging antioxidant capacity was measured. Significant increase was detected only in FRAP, suggesting an elevation exclusively in the level of phenolic acids in case of UV exposed outdoor grown pepper leaves.

1. INTRODUCTION

Phenolic compounds like phenolic acids and flavonoids are synthetized via the shikimic acid pathway (Casañal *et al*., 2013), and are also referred to as phenylpropanoids (Deng & Lu, 2017). Phenolics usually occur mainly as flavonoid glycosides in plant tissues (Yang *et al.,* 2018). These metabolites protect the plants against ultraviolet (UV) radiation and act as UV-screeners because of their partly different UV absorption spectra (Csepregi & Hideg, 2018). UV radiation, especially UV-B radiation is a potential stressor (Ballaré, 2003). Depending on irradiance and dose, UV-B can induce reactive oxygen species (ROS) and the cellular hydrogen peroxide level (Czégény *et al*., 2014). ROS compounds can cause cell damage through the oxidation of DNA, lipids, and proteins (Hernández *et al*., 2009). However, UV-B can also elicit increase the production of phenolic compounds an effect which has been demonstrated across many species including, *Arabidopsis thaliana* (Hectors *et al*., 2014), *Betula pendula* (Morales *et al*., 2010), *Hordeum* spp. (Klem *et al*., 2015) and *Capsicum* spp. (Rodríguez-Calzada *et al*., 2019). The induction of phenolic compound biosynthesis is correlated with the stimulation of the UVR8 photoreceptor by UV-B (Jenkins, 2014; Morales *et al*., 2013; Rizzini *et al*., 2011) and the cryptochrome photoreceptors by UV-A (Siipola *et al*., 2015). Typically, UV enhances the total

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concentration of phenolics as well as the individual concentration of phenolic acids and flavonoids, a response which has been linked to protection against oxidative stress. In fact, the primary function of phenolics (especially the dihydroxy-B-ring flavonoids) is to counter oxidative stress (Agati *et al*., 2020) via the scavenging of reactive oxygen species (ROS). Phenolics have a high total antioxidant capacity *in vitro* (Csepregi *et al*., 2016; Hernández *et al*., 2009) and are capable of neutralizing high efficiency ROS molecules (Csepregi & Hideg, 2018). Moreover, a positive correlation has been detected between phenolic content and antioxidant capacity in grapevine leaves (*Vitis vinifera*) (Csepregi *et al*., 2019).

Capsicum annuum, a member of the Solanaceae family is one of the most economically important crop plants in the world (Kim *et al*., 2014). The fruit is full of a source of nutrients in human diet containing carotenoids, ascorbic acids, flavonoids, and other phenolic compounds (Assefa *et al.,* 2021) with the leaves also being consumed (Kim *et al.,* 2014). Moreover, leaves also utilized for their antimutagenic and antimicrobial activity in the cosmeceutical (Kim *et al.,* 2014), pharmaceutical and medicinal industries (Rodríguez-Calzada *et al.,* 2019). Intriguingly, little is known about the UV absorption and antioxidant capacity of metabolites found in pepper leaves, and the protective role they play against individual ROS. In this experiment, we focused on the antioxidant and UV-absorbing properties of pepper leaf extracts (*Capsicum annuum*) grown under outdoor conditions. To have a more comprehensive knowledge, we applied three various total antioxidant capacity (TAC) methods, to account for discrepancies in results caused by differences in methods (Csepregi *et al*., 2016; Csepregi *et al*., 2019).

2. MATERIAL and METHODS

2.1. Chemicals

Test compounds for the calibrations were purchased from Merck (Merck Industrial and Laboratory chemicals, Darmstadt, Germany). All other chemicals were purchased from VWR (VWR International Kft., Debrecen, Hungary).

2.2. Experimental Design

Capsicum annuum cv. amy seeds were sown on garden soil in 12x12 cm pots and germinated outside. Research was started in the month of May at the Research Institute Viticulture and Oenology, University of Pécs (latitude: 46°04’ N, longitude: 18°11’ E). The experimental work included seven plants which were not exposed to UV radiation (covered by Rosco 3114 UV filter, Roscolab Ltd, London, UK) and seven plants that were exposed UV radiation (covered by cellulose-diacetate plastic foil, Courtauds Chemicals, Derby, UK) since seeding. The average global irradiances in May, June and July were 68.27 Wm^{-2}, 63.72 Wm^{-2} and 60.68 Wm^{-2} while relative humidity were 16.79 %, 21.18 % and 24.12 % respectively. Air temperatures were 18.03 °C, 23.12 °C and 26.65 °C. All environmental data was provided by the meteorological station on the premises of the Faculty of Natural Sciences of the University of Pécs (joido.ttk.pte.hu).

At the end of July, the leaves were collected. Two fully developed pepper leaves were collected from the 3rd node of each plant and pooled together. Plant leaves were frozen in liquid nitrogen and stored at -60 °C for further antioxidant capacity measurements. The frozen leaves were lyophilized (SCANVAC CoolSafe 110-4, LaboGene, Denmark) to make a more homogeneous extracts from the dried material.

The leaf samples extracted in methanol aqueous (70:30 v/v) and sonicated in a sonic bath for 15 minutes (RoHS JP-020, Shenzhen, China) and finally centrifuged (Thermo Fisher Scientifc Inc., Waltham, MA, USA) for 10 minutes at 15000xg. The supernatant was collected used to pipettes, and the process was repeated two more times. All supernatants were subsequently combined.

2.3. Folin-Ciocalteu Reactivity (FC)

The FC measurement was carried out with some modifications (Csepregi *et al*., 2016). 90 µL aqueous Folin-Ciocalteu (1:10 with distilled water) reagent is added to the 20 µL diluted sample. After 5 minutes at room temperature, another 90 µL of Na_2CO_3 solution was added. After 90 minutes of incubation at room temperature, the absorbance of the solution was measured (Multiskan FC plate reader, Thermo Fischer Scientific, Shanghai, China) at 651 nm. The calibration curve was prepared with gallic acid solution using the above method, then the results are given in µM gallic acid equivalent per mg leaf dry weight.

2.4. Trolox Equivalent Antioxidant Capacity (Teac)

The TEAC measurement was carried out according to Rice -Evans *et al.,* (1997). $ABTS^{\bullet+}$ (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic cation radical) solution was prepared by mixing 100 µL ABTS (0.1 mM), 100 µL H_2O_2 (0.1 mM) and 100 µL horse radish peroxidase (0.0125 µM) in a 9.7 mL 6.0 pH sodium phosphate buffer (50 mM). During the 15 minutes incubation at room temperature, the formation of $ABTS^{\bullet+}$ was indicated by its blue-green color. 190 µL of this solution was mixed with 10 µL diluted plant extract and the absorption at 651 nm was recorded. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used to prepare a calibration and the results are given as µM Trolox equivalent per mg leaf dry weight.

2.5. Ferric Reducing Antioxidant Power (Frap)

FRAP method was performed following (Csepregi *et al*., 2016). The reaction mixture contained 1.25 mL TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (10 mM with 40 mM HCl) and 1.25 mL mM $FeCl_3$ (20 mM with distilled water) in 12.5 mL 3.6 pH acetate buffer (300 mM). 10 µL diluted leaf extract was added to 190 µL of reagent, and absorptions were measured at 620 nm, followed by an incubation at room temperature for 30 minutes. FRAP values are given as µM ascorbic acid equivalent per mg leaf dry weight.

2.6. Hydrogen Peroxide Scavenging Capacity

This assay is based on the oxidation of potassium iodide (KI) but instead of the iodine absorption maximum the reaction is evaluated at 405 nm as described earlier (Csepregi & Hideg, 2016). The KI solution (0.85 M) is prepared in 7.0 pH potassium-phosphate buffer (100 mM), to 140 µL of which added 10 µL diluted plant extract, finally added 50 µL of H_2O_2 during an orange color reaction can be observed, which was characterized by measuring the absorption two times (zero and three minutes) at 405 nm (Csepregi & Hideg, 2016). Thus, the extent of antioxidant activity can be deduced from the absorption decreasing. In this way, the 50% inhibitory concentration (IC_{50}) can be specified, which is given as Trolox equivalents.

2.7. UV Absorption Measurements

Leaf extracts were diluted with a mixture of acidified ethanol and their absorption spectra were measured between 280-400 nm (Shimadzu Corporation UV-1800, Kyoto, Japan). The area under the curve was identified with a spectrophotometer and separately determined in the UV-A (315-400 nm) and UV-B (280-315 nm) ranges (Csepregi & Hideg, 2018). The results are given in µM quercetin equivalents, based on the literature (Csepregi & Hideg, 2018).

2.8. Statistical Analyses

Statistical analyses of data were carried out using Excel (Version 2007, Microsoft Corporation, Redmond, WA, USA) while graphs were prepared using OriginPro (OriginLab Corporation, Northampton, Massachusetts, USA). The leaf samples were characterized by means and standard deviations of seven biological samples for the UV deprived and seven for the full sunlight exposed plants. The significance of differences was assessed using Student's t-tests where the level of significance was $p < 0.05$.

3. RESULTS

Pepper leaf extracts were measured using various total antioxidant (TAC) and UV absorbing capacity assays (Figures 1 and 2). In addition, specific ROS scavenging capacity were also measured (Figure 3). The three different TAC methods and UV absorbing capacity have been calibrated with the appropriate standards. Full sunlight exposed plants showed similar TAC values in case of TEAC (Figure 1A) and FC (Figure 1B), while only FRAP (Figure 1C) measurement displayed significantly higher (p<0.05) antioxidant capacity values (Figure 1). Phenolics, like flavonoids and phenolic acids are strong antioxidants, although there can be differences in their *in vitro* TAC reactivity because of intramolecular differences (Csepregi *et al*., 2016). The B ring hydroxylation pattern of flavonoids significantly affect TEAC, FC and FRAP results. Nevertheless, the presence of -OH group in the C ring (C3 position) is likely affect TEAC and FRAP, but not FC values in case of flavonoids. The hydroxylation pattern of phenolic acids significantly alters FRAP, but not TEAC and FC (Csepregi et al., 2016). Based on these, we can postulate that the higher FRAP value of full sunlight exposed pepper leaves is more likely due to the accumulation of phenolic acids and not flavonoids.

In another experiment, phenolic content of UV-B exposed pepper leaves was examined with HPLC. In this work, the flavonoid level did not increase significantly, moreover, the level of apigenin-8-C-hexoside even decreased. In contrast, the level of chlorogenic acid (a phenolic acid) increased, therefore the compound which is responsible for the higher FRAP value can be this compound (Rodrígez-Calzada *et al.,* 2019). In our experiment, a different pepper variety were chosen, but it is hypothesized that they may have the same or similar special metabolites in the leaves, only to a different extent.

This study shows that under outdoor conditions, the UV-A and UV-B absorbing properties of leaf extracts were unchanged (Figure 2). From previous works were well known that phenolic acids absorbing the UV radiation mostly more efficiently than flavonoid glycosides (Csepregi & Hideg, 2018). However, chlorogenic acid occurs in relatively low concentration in *Capsicum annuum* cv. Coronel pepper leaf, compared to the total phenolic content (Rodríguez-Calzada *et al.,* 2019).

Overall, leaf extracts displayed higher UV-B absorbing capacity when compared with UV-A absorbing capacity (Figure 2). This could be due to the phenomena that both phenolic acids and monohydroxy B-ring flavonoid glycosides have higher UV-B absorbing capacity (Csepregi & Hideg, 2018).

The specific ROS scavenging capacities were not changed significantly (Figure 3). Phenolic acids like chlorogenic acid have relatively low H_2O_2 neutralizing capacity compared to dihydroxy B-ring flavonoids and monohydroxy B-ring flavonoids with higher neutralizing activity (Csepregi & Hideg, 2018) Potentially the increase in phenolic acid level is not necessarily elevating the non-enzymatic H_2O_2 neutralizing capacity of outdoor grown pepper leaves.

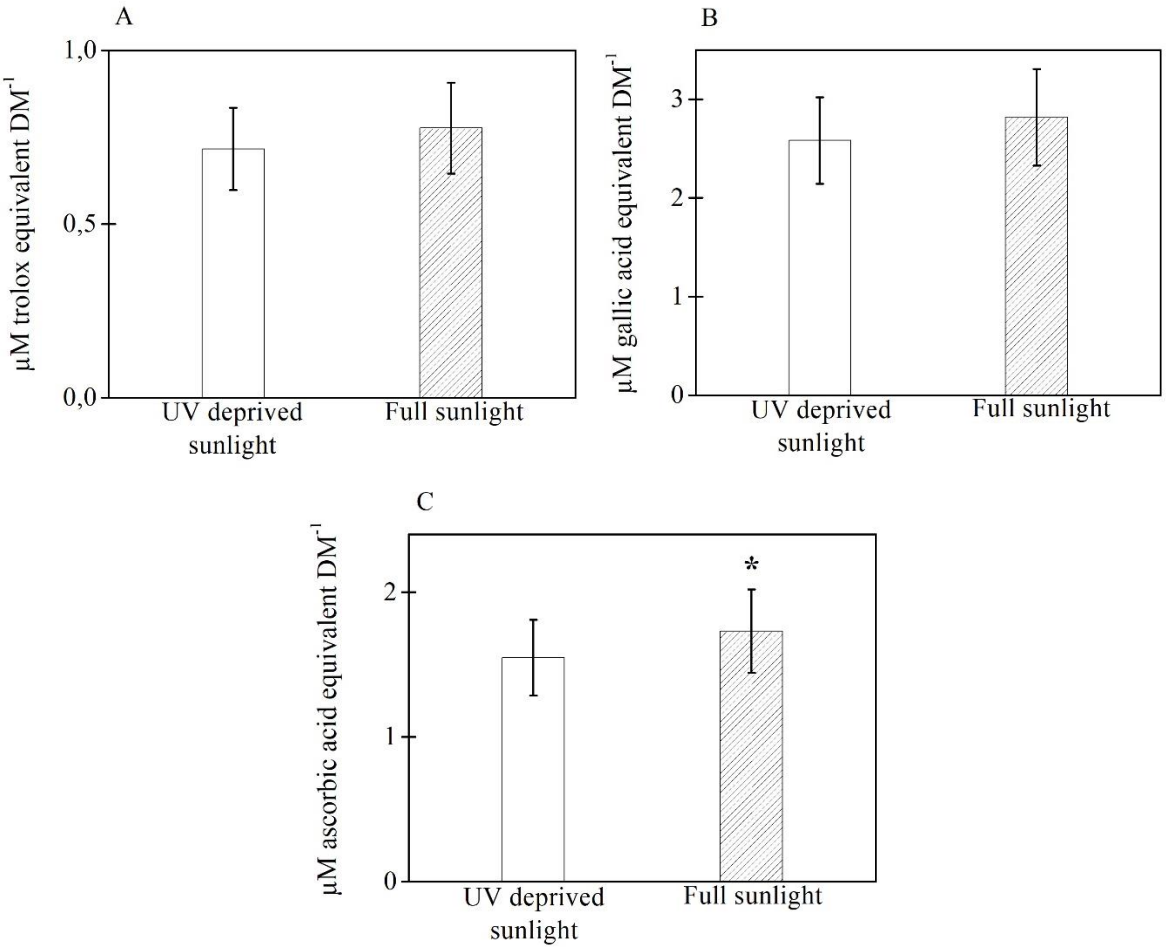

Figure 1. A) TEAC (Trolox Equivalent Antioxidant Capacity); B) Folin-Ciocalteu reactivity (FC); C) FRAP (Ferric Reducing Antioxidant Power). White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

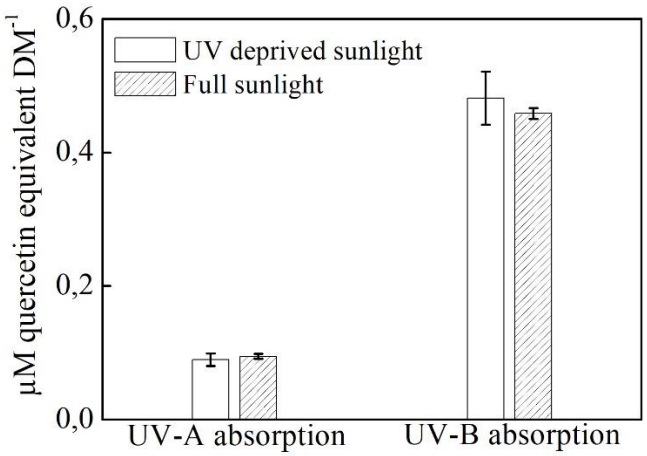

Figure 2. UV absorption measurements: UV-A absorption (315–400 nm) and UV-B absorption (280–315 nm) of the samples. White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

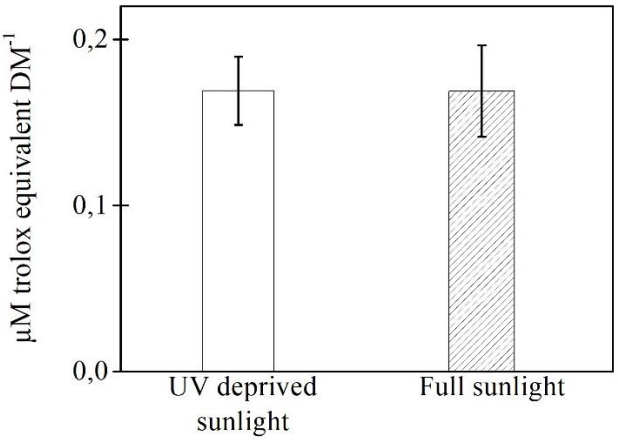

Figure 3. Non-enzymatic hydrogen peroxide scavenging capacity. White columns: plants were deprived from UV radiation, dashed columns: plants were received the full sunlight.

4. DISCUSSION and CONCLUSION

In outdoor experiments, there are several highly fluctuating environmental factors which may be stress inducing. Factors including temperature (Csepregi *et al*., 2019), drought (Rodríguez-Calzada *et al.,* 2019) and UV radiation can alter the phenolic profile of leaves. Indeed, both UV deprived, and full sunlight exposed plants were continuously exposed to potential stressors like high temperature or low air humidity. Therefore, we attribute the unchanged TEAC, FC, UV absorbing capacity and H_2O_2 scavenging capacity to the experimental conditions. These results suggests that UV might not the most important environmental stress factor considering the overall level of plant secondary metabolites. But nor negligible either, because the elevated level of FRAP suggesting a slightly modification in the phenolic content presumably the level of chlorogenic acid. This is corresponding to the most recent findings, that plants defend themselves firstly against other environmental stress factors rather than UV radiation (Agati *et al*., 2020). In accordance with the literature, our findings support the hypothesis that UV induces changes in the phenolic content of plant, elevating level of chlorogenic acid which is thought to be a key component in the specific defense against UV stress in case of *Capsicum annuum* L. cv. Amy pepper leaves.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: Department of Biology, Faculty of Sciences, University of Pécs, H-7624 Pécs, Ifjúság útja 6. Hungary

Authorship Contribution Statement

Valér Góra: Investigation, Visualization, Formal Analysis, and Writing. **Kristóf Csepregi**: Methodology, Supervision and Writing. Authors may edit this part based on their case.

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