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Effect of seed irradiation on the content of antioxidants in leaves of Kidney bean, Cabbage and Beet cultivars

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Abstract

Seeds of two varieties of kidney bean (*Phaseolus vulgaris* L., var. oratus and var. ellipticus), cabbage (*Brassica oleracea* L., var. capitata alba and var. capitata rubra) and beet (*B. vulgaris* L., saccharifera Alef. and ssp. esculenta (Salisb) Gurke, var. rubra) were exposed to ultraviolet (UV) irradiation (460-760 μW/cm^2 for 30, 60 and 90 min). The purpose of the study was to evaluate the importance of pre-sowing treatment with UV for activation the antioxidative system and building up adaptive mechanisms against unfavorable environmental conditions in crops. In particular, we studied the effects of exposing seeds to UV irradiation prior to sowing on antioxidant contents in leaves of experimental plants developed from the irradiated seeds and on their physiological state and growth indices (height, biomass, photosynthetic activity of leaves, amount of plastid pigments and total proteins). Treatment of seeds with low doses of UV caused increase in ascorbic acid content in leaves of kidney bean varieties (32-35%). Applied doses of UV significantly enhanced the amount of tocopherol in leaves of both varieties of kidney bean (2-4 times and more), and white beet (5-9 times). Seed UV irradiation with high doses (90min) had clear effect on the content of plastid pigments in kidney bean varieties (about 35%), while in cabbage and beet stimulative were both doses (30min and 90min) (45% and more). UV treatment of seeds also stimulated synthesis of anthocyanins in leaves of kidney bean varieties (3-6%) and white beet (14-21%). In the case of cabbage and red beet low doses of irradiation were effective (9-20%). We conclude that UV irradiation of seeds stimulated stress adaptive mechanisms in tested plants. These effects, however, depend on the intensity of irradiation, also on plant species and varieties. Treatment of seeds with optimal doses of UV irradiation may used for stimulation of antioxidant synthesis in plants and enhance their nutritional value and tolerance to environmental stress factors.

Key words: *anthocyanins; ascorbic acid; tocopherol; seeds; vegetables; ultraviolet irradiation*

Abbreviations: UV- ultraviolet

Introduction

Environmental pollution increases the risk of influence of different stressors on a living organism (Pickering and Owen, 1997; Hoffman and Pertons, 1997). Compared to animals and microorganisms, plants are remarkably resistant to a number of stresses due to their ability to synthesize specific protective substances, most of which are antioxidative (Takahama and Oniki, 1997; Zagoskina et al., 2003). Different antioxidants like glutathione, ascorbic acid, tocopherols, anthocyanins, carotenoids combine into a united antioxidative system and determine the adaptability of plants to unfavorable environmental conditions (Takahama and Oniki, 1997; Kagan et al., 2000; Zagoskina et al., 2003). Some stress factors may stimulate biosynthesis of antioxidants, and by this improve the value of agricultural products (Barka et al., 2000; Kruhova et al., 2007).

Pre-sowing treatment of seeds with high energy rays (γ, lazer, UV) is effectively used to increase crop productivity (Jdanova, 1962; Dubrov, 1977; Ghallab and Omar, 1998; Delibaltova and Ivanova, 2006). Although the influence of UV radiation on plants has been intensively investigated, there was surprisingly little focus on influence of seeds irradiation on antioxidants (Rogozin et al., 2000). Free radicals produced by UV irradiation of seeds change cell membrane permeability and electric

potential, presumably initiating diverse metabolic responses including biosynthesis of antioxidants.
Here our goal was to evaluate the importance of pre-sowing treatment with UV for activation the antioxidative system and building up adaptive mechanisms against unfavorable environmental conditions in crops such are kidney bean, cabbage and beet. In particular, we studied the effects of exposing seeds to UV irradiation prior to sowing on antioxidant contents in leaves of experimental plants developed from the irradiated seeds and on their physiological state and growth indices (height, biomass, photosynthetic activity of leaves, amount of plastid pigments and total proteins). Plants may differ in their sensitivity to UV irradiation not only at species but also sub-species levels (Lercari et al., 1989; Janukashvili et al., 2001). Sub-species differ in their antioxidant activity (Prakash et al., 2007). We deliberately compared two cultivars of the same species but with evidently different contents of anthocyanins, which are one of the principal antioxidants in plant (Rice-Evans, 1997). Such a comparison presumably could reveal new links between stress factors and antioxidants.

Materials and methods

Plant material

For experiments were selected common food crops including kidney bean (*Phaseolus vulgaris* L.), cabbage (*Brassica oleracea* L., var. capitata) and beet (*Beta vulgaris* L.). In particular, two varieties of each crop species were tested; the two varieties of beans were tendrillar (*Ph. vulgaris* L., var. oratus) and field (*Ph. vulgaris* L., car. ellipticus), correspondingly with white and red seeds; the two forms of cabbage were white (*B. oleracea* L., var capitata, alba) and red (*B. oleracea* L., var. capitata, rubra); the two forms of beet were sugar (*B. vulgaris* L., saccharifera Alef.) and red (*B. vulgaris* L., ssp. esculenta (Salisb) Gurke, var. rubra).

Preparation of samples and irradiation

Seeds of experimental plants were first soaked in water for 24h and then irradiated with UV rays from an artificial source (lamp ДРТ-400, Russia) which was situated at 50cm from the seeds. The intensity of irradiation was measured with a UVP radiometer (UVP Inc. USA) and exposure was 460μW/cm^2, 494μW/cm^2 and 760μW/cm^2 for A, B and C sections of UV radiation, respectively. Irradiation durations for kidney bean were 60 and 90 min, and for cabbage and beet – 30 and 90 min.
After UV treatment seeds were sown in outdoor experimental plots. We collected leaves for the analysis two months later, when beans were in flowering phase and biannual cabbage and beet had fully expanded leaves.

Ascorbic acid and glutathione determination

A titration method was used (Ermakov et al., 1987) to measure the content of ascorbate and glutathione. First, 2g of leaves were ground in 15ml of 2% hydrochloric acid and 19ml of 2% metaposphoric acid, and filtered. To determine ascorbic acid contents, 1 ml of the filtrate was added 24 ml of distilled water and titrated with 0.001N solution of dichlorphenolindophenol. To determine glutathione contents, 5 ml of the same filtrate was mixed with 5 ml of 15% KI and 5 drops of 1% starch was added and was titrated with 0.001N KIO_3 till bright grey color.

Tocopherol determination

Two g of ground leaves were extracted with 20-25ml of pure ethanol three times at room temperature, till leaves were fully discolored. The combined extract was mixed with 20 ml of 60% potassium hydroxide, and saponificated on water bath for 2h. Tocopherol was extracted from the obtained hydrolyzate using diethyl-ether. Extraction was performed three times, first adding 50ml ether for the first time, and then 25 ml for the next two repetitions. The combined extract was washed with distilled water until a complete removal of alkaline residuals detected by indicator paper. Water was removed with H_2SO_4, the obtained solution was evaporated on the water bath, cooled, mixed with alcohol-nitric acid (1 ml of concentrated HNO_3: 5ml of 96^o alcohol), and boiled during 3 min till the color became dark red. Extinction of the extract was measured at 470nm by the spectrophotometer (SPEKOL 11, KARL ZEISS, Germany) (Filippovich et al., 1982).

Photosynthesis

Photosynthetic activity of leaves was measured using a portable infrared gas analyzer (Ciras-1, PP systems, Hitchin, Herts, UK). The rate of photosynthetic uptake was calculated (Caemmerer and Farquhar, 1981).

Plastid pigments and anthocyanins

Plastid pigments (chlorophylls and carotenoids) were determined spectrophotometrically (Ermakov et al., 1986). Fresh leaves (100-200mg) were ground together with quartz sand and $CaCO_3$ with acetone in a mortar. The mixture obtained was filtered through a glass filter N3. The total volume of the filtrate was 50ml. The optical density of the

Table 1. The effect of seed UV treatment (no treatment *versus* 30, 60 and 90 min UV radiation) on contents of ascorbic acid, glutathione, and tocopherol in studied plants.

variant	State of vegetation	Total ascorbic acid, mg%,dry weight	Glutathion, mg% dry weight	Tocopherol, mg/g, fresh weight	Total ascorbic acid, mg%,dry weight	Glutathion, mg% dry weight	Tocopherol, mg/g, fresh weight
Contr.	flowering	kidney bean var. oratus (red bean)			kidney bean var. ellipticus (white bean)		
60'		411.3±32.9	719.4±35.9	7.2±0.7	575.6±23.1	633.1±39.8	4.0±0.8
90'		543.4±27.2	815.4±40.8	13.8±1.1	776.4±38.8	416.5±24.9	13.0±1.3
		736.8±36.8	479.0±23.9	11.2±0.9	592.2±29.6	732.0±36.6	26.0±2.1
Contr.			red beet			white beet	
30'	First year of vegetation	557.3±27.9	104.2±10.4	16.7±1.3	408.3±20.4	390.5±31.2	1.5±0.1
90'		427.7±21.4	109.9±9.8	12.6±1.6	371.1±18.5	125.8±10.1	8.4±0.8
		526.4±26.3	135.2±10.8	4.2±0.4	713.0±35.6	162.6±13.1	13.9±1.6

extract was measured with spectrophotometer (SPEKOL 11, KARL ZEISS, Germany). The concentrations of chlorophyll a and b, and carotenoids ($mg \cdot g^{-1}$ fresh weigh) were calculated using the equation of von Wettstein.
For determination of anthocyanins, 1g of leaf material was grinded with 20 ml of ethanol and 2% HCl solution, and filtered. The extinction of the extract was measured at 529nm (Caldwell, 1968).

Total proteins

Content of total proteins was studied after Lowry (Lowry et al., 1951). Bovine serum albumin served as a standard.

Statistical analysis

The results are mean values of 5 biological replicates with indication of standard error and p. We used three-way ANOVA to analyze the general effects of UV irradiation: the doses of UV irradiation, crops species and crop species variety being, respectively, the first, second and third factors. Separately, for each variable (the contents of antioxidants, pigments and proteins, rate of photosynthesis, height, wet and dry weight), one-way ANOVA was used to test the effect of UV radiation doses. Tukey's multiple comparison tests were used to test differences between the means. All calculations were performed using statistical software Satistix8 (Analytical Software, Tallahassee, FL).

Results

Ascorbate, glutathione and tocopherol

Experimental results have shown that differences between cultivars of one and the same species were significant for the content of ascorbic acid

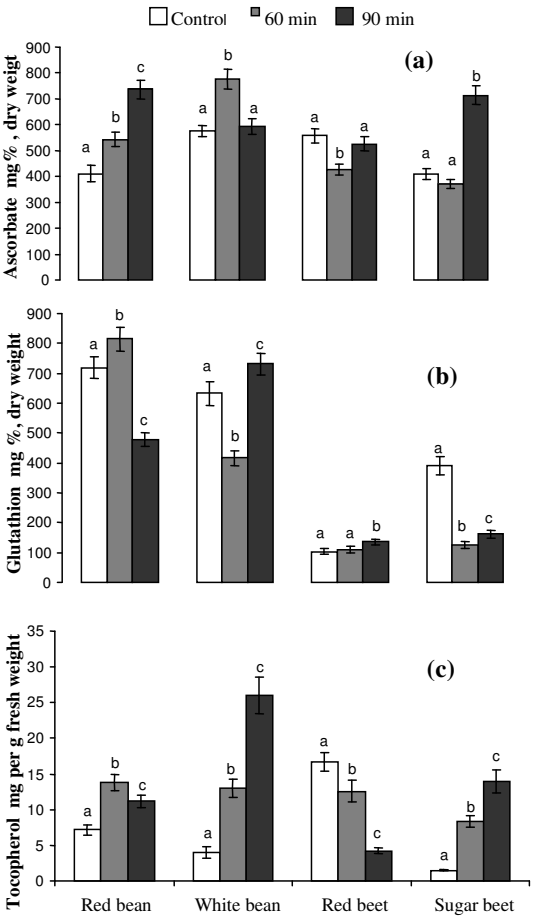

Fig1. The effect of seed UV treatment (no treatment *versus* 30 and 90 min UV radiation) on contents of (a) ascorbic acid (b) glutathione and (c) tocopherol in studied plants. Error bars show standard deviation. Different letters indicate significant differences ($P<0.05$) according to Tukey's multiple comparison test.

Table 2. The effect of seed UV treatment (no treatment *versus* 30, 60 and 90 min UV radiation) on the content of plant pigments (mg/g fresh weight): chlorophyll, carotenoids, and anthocyanins.

Variant	State of vegetation	Chlorophyll	Carotenoids	Anthocyanins	Chlorophyll	Carotenoids	Anthocyanins
		kidney bean var. oratus (red bean)			kidney bean var. ellipticus (white bean)		
Cintr.	Flowering	2.96±±0.2	1.02±0.08	0.213±0.02	3.27±0.4	1.15±0.09	0.109±0.02
60'		3.16±±0.25	1.09±0.09	0.220±0.02	3.68±0.3	1.12±0.09	0.119±0.02
90'		3.33±0.27	1.38±0.11	0.227±0.02	4.50±0.4	2.14±0.2	0.124±0.01
		red cabbage			white cabbage		
Contr.	First year of vegetation	0.38±0.04	0.16±0.02	0.244±0.03	1.66±0.2	0.69±0.03	0.130±0.02
30'		0.61±0.06	0.26±0.03	0.278±0.03	2.96±0.35	1.08±0.13	0.157±0.03
90'		0.56±0.08	0.21±0.02	0.240±0.02	2.41±0.29	0.88±0.11	0.129±0.02
		red beet			white beet		
Contr		0.36±0.04	0.15±0.01	0.191±0.02	0.33±0.04	0.11±0.01	0.114±0.01
30'		0.90±0.07	0.65±0.05	0.209±0.02	0.63±0.08	0.31±0.04	0.138±0.01
90'		0.60±0.05	0.48±0.04	0.194±0.02	0.51±0.06	0.24±0.03	0.130±0.01

(p=0.003, Fig.1, a). Exposing seeds to UV irradiation before sowing however significantly changed the content of ascorbic acid in leaves of experimental plants (p=0.0001), but differently among species and varieties. For both varieties of kidney bean the total amount of the vitamin increased (35% and 32% for 60' variant, and 3% and 79% for 90' var.), but the beet varieties responded differently to UV irradiation: the content of ascorbic acid was reduced in red beet leaves whilst in sugar beet 90 min irradiation increased the vitamin content (74%) (Fig.1, a).

Irradiation dose had a significant effect on glutathione contents. Both species (p>0.0001) and their varieties (p=0.002) responded differently. For field bean plants longer irradiation stimulated glutathione synthesis (16%), while in var. oratus the maximum amount of glutathione happened after 60 min irradiation (13%)(Fig.1, b). In sugar beet both doses of irradiation appeared to inhibit glutathione synthesis (2.5-3 times), whilst a contrary was observed in red beet (1.3 times, 90'var.) (Fig.1, b). Tocopherol contents also differed between the crop species (p= 0.026) and their varieties (p= 0.045): tocopherol increased in both kidney bean varieties (3 times in 60'var. and 6 times in 90'var) and sugar beet plants (5.6 times in 30'var. and 9 times in 90'var. in ellipticus and 2 times – 60' var., 1.5 times – 90' var in oratus)), but decreased in red beet (1.3 times in 30' var. and 4 times in 90'var) (Fig 1, c).

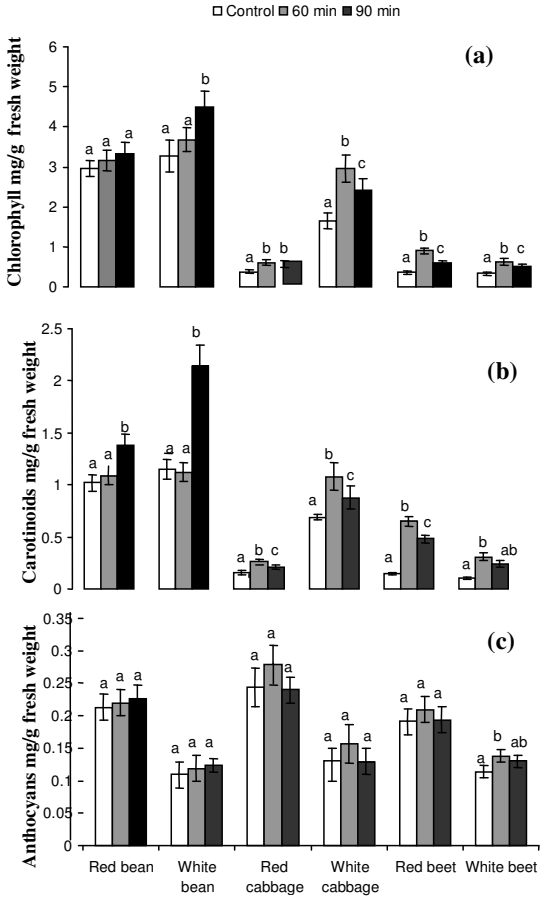

Fig 2. The effect of seed UV treatment (no treatment *versus* 30 and 90 min UV radiation) on the content of plant pigments (a) chlorophyll (b) carotenoids (c) anthocyanins. Error bars show standard deviation. Different letters indicate significant differences (P<0.05) according to Tukey's multiple comparison test.

Pigments

The effects of UV irradiation on plastid pigments content were different both in studied species (p<0.0001) and in their varieties (p= 0.0125). UV treatment of seeds both with low and high doses

Table 3. The effect of seed UV treatment (no treatment *versus* 30, 60 and 90 min UV radiation) on net photosynthesis ($\mu molm^{-2}s^{-1}$) and content of total protein (mg/g dry weight).

Variant	State of vegetation	Photosynthesis	Total protein	Photosynthesis	Total protein
		kidney bean var. oratus (red bean)		kidney bean var. ellipticus (white bean)	
Contr.	Flowering	8.1	18.4±0.92	7.1	29.6±1.5
60'		9.04	30.1±1.5	5.4	32.1±1.6
90'		8.02	54.8±2.7	5.4	37.8±1.9
		red cabbage		white cabbage	
Contr.	First year of vegetation	3.3	9.6±0.5	6.4	3.2±0.2
30'		6.5	8.0±0.3	6.8	2.4±0.12
90'		3.3	8.8±0.4	6.4	3.1±0.15
		red beet		white beet	
Contr.		11.2	2.4±0.12	8.4	2.4±0.12
30'		8.3	3.3±0.16	10.5	2.2±0.11
90'		7.8	2.0±0.1	7.9	1.6±0.08

increased pigment contents (p=0.0035) in all tested plants (Fig. 2, a). In kidney bean varieties more effective was 90' irradiation (37% and 12%), while in cabbage and beet 30' variant was more stimulative.

Both high and low doses of UV irradiation stimulated carotenoid synthesis in cabbage and beet (more effective was 30' irradiation – 62% red cabbage, 20% white cabbage, 4 times in red beet and 2.8 times in white beet); but for kidney bean only the 90 min irradiation was effective (Fig. 2, b). Although species (p=0.035) and their varieties (p= 0.014) differed strongly for anthocyanin content in leaves, UV irradiation changed this index in cultivars of one and the same species in a similar way: in bean and beet cultivars both doses of irradiation were stimulative (9-21%), while in cabbage – only 60min exposition did (13% - red, 20% - white) (Fig. 2, c).

Photosynthesis and Total proteins

Pre-UV treatment photosynthetic activity in leaves was evidently different among species (p< 0.0001, Fig 3, a). Brief UV irradiation of seeds reduced the rate of photosynthesis in kidney bean var. oratus and red beet (1.3 times), but increased it in other plants (6% and more). After 90 min irradiation, however, the rate of photosynthesis was either similar (field kidney bean, red and white cabbage, white beet) or less (white kidney been) than the control level.

Kidney bean, as a legume plant, was clearly distinguished from other species by high content of proteins (p< 0.0001). In both forms of kidney bean UV treatment of seeds stimulated protein synthesis in leaves, with a stronger effect on the red form (1.6-3 times)(Fig.3, b). In other plants UV irradiation generally reduced protein contents, although there was small and temporary increase found in the case of red beet (1.4 times)(Fig. 3, b).

Growth

Without UV treatments species differed in height (p< 0.0001), but mainly there was not strong difference between varieties. The metabolic alterations caused by UV irradiation of seeds correlated with altered growth and development (Fig. 4, a). Generally, UV irradiation increased plant height in kidney beans (11-39%) and for other plants short UV irradiation periods increased height (11-16%) and longer irradiation periods reduced plant height (insignificantly in the case of white beet) (Fig. 4, a).

The effect of UV irradiation on plant fresh weight overall was significant (p=0.0046) and very similar to the responses of plant height (Fig. 4, b): increase in kidney bean varieties (16-53%), and an increase followed by decrease in other plants; the differences were significant between species (p=0.0001). Dry mass accumulation in response to seed UV pre-treatment was significant (p<0.0001, Fig. 4, c): short UV irradiation caused increase in dry mass practically in all plants (6% and more), but longer UV treatment was indistinguishable from the control level. However, the rate of dry mass accumulation was significantly different between species (p=0.0002) as well as between varieties (p=0.0001).

Discussion

Different forms of stress cause the production of reactive oxygen species such as superoxide and peroxide radicals in the cells. To detoxify these oxy-products, plants have elaborated antioxidative

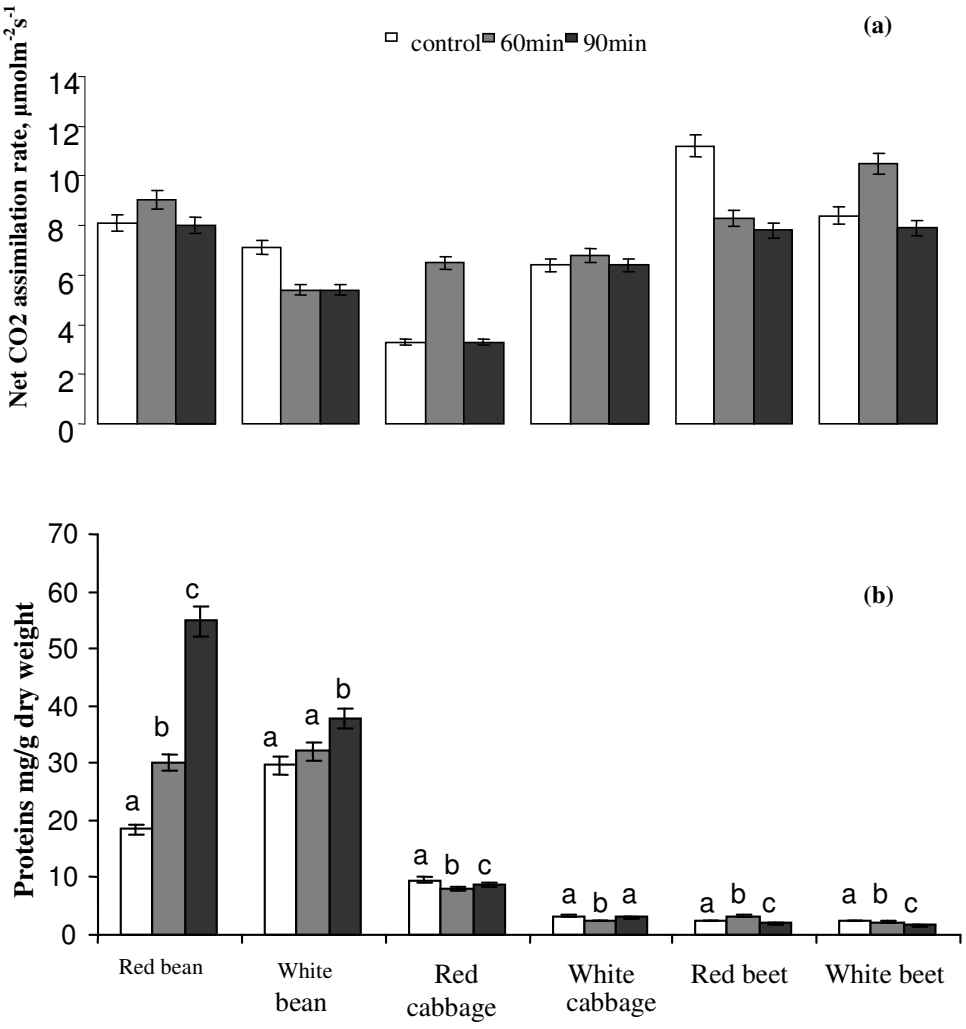

Fig 3. The effect of seed UV treatment (no treatment *versus* 30 and 90 min UV radiation) on (a) net photosynthesis and (b) content of total protein. Error bars show standard deviation. Different letters indicate significant differences ($P<0.05$) according to Tukey's multiple comparison test.

systems, which are composed of both lipophilic (tocopherol, carotenoids) and hydrophilic (glutathione, ascorbic acid) substances (Polle and Rennenberg, 1994; Noctor and Foyer, 1998; Tausz et al., 2004). For example, ascorbic acid is one of the strongest antioxidants, a source of electrons for many enzymatic and non-enzymatic reactions (Blokhina et al., 2002). Tightly connected to this antioxidative function of ascorbate is three-peptide glutathione, which serves for the regeneration of reduced ascorbate in the ascorbate-glutathione cycle (Noctor and Foyer, 1998). According to generally accepted views, the initial response to the stress is then followed by an acclimation phase in which the new stable state of the stressed system is established. If this second phase fails, the system starts to degrade. The stress effect implies progressive degradation of the metabolites and decrease in their pool through oxidation (Tausz et al., 2004). Accordingly, the increase of ascorbic acid and glutathione found in our experimental plants may be understood as indication of their ability to adapt to environmental stress, especially in kidney beans. Evidently, sensitivity of our crop species and their varieties to UV doses were different, pointing to the importance of finding optimal doses for seed pre-treatment for each species and its variety. The function of ascorbic acid as of antioxidant is also linked with lipophilic antioxidant – α-tocopherol (vitamin E). The later is

Table 4. The effect of seed UV treatment (no treatment *versus* 30, 60 and 90 min UV radiation) on plant height, fresh and dry masses

Variants	State of vegetation	Plant height, cm	Green mass. g	Dry mass, g (per g of fresh weight)	Plant height, cm	Green mass. g	Dry mass, g (per g of fresh weight)
		kidney bean var. oratus (red bean)			kidney bean var. ellipticus (white bean)		
Contr.	Flowering	67.3±11.6	15.3±2.6	0.16±0.03	43.0±7.1	12.9±1.9	0.16±0.07
60’		80.4±3.4	18.6±2.8	0.21±0.01	47.8±6.1	14.1±2.4	0.17±0.01
90’		93.6±5.7	23.8±3.6	0.17±0.01	52.6±13.1	22.3±3.3	0.21±0.01
		red cabbage			white cabbage		
Contr.	First year of vegetation	15.2±1.4	60.0±10.2	0.18±0.01	18.7±1.3	62.0±9.3	0.18±0.01
30’		17.6±1.5	65.0±9.7	0.20±0.01	21.9±2.6	71.0±12.8	0.33±0.03
90’		13.5±1.7	44.0±7.9	0.17±0.01	12.2±0.9	50.0±7.5	0.20±0.02
		red beet			white beet		
Contr.		23.8±2.7	52.8±9.5	0.14±0.01	24.0±2.1	65.0±11.0	0.17±0.01
30’		27.6±1.5	58.7±10.6	0.17±0.01	28.0±3.9	74.3±12.6	0.23±0.01
90’		20.0±1.4	50.9±9.1	0.16±0.02	23.0±2.8	61.0±9.1	0.18±0.01

an essential component of bio-membranes, giving to lipid radical an oxygen ion and turning itself into a tocoperoxy-radical, which further is restored by ascorbic acid or glutathione (Freyer, 1992). Accordingly, kidney bean responded to UV irradiation with increased levels of tocopherol, although the responses of beet cultivars were not similar. Apparently, the lipophilic antioxidative system (in the form of tocopherol) is more sensitive to UV irradiation in red beet, while the hydrophilic antioxidative system based on ascorbate-glutathione cycle is more sensitive in sugar beet. In sum, we conclude that there is a positive effect of pre-sowing UV-irradiation on the content of tocopherol for kidney bean and sugar beet.

Increasing of the total chlorophyll’s content in leaves of experimental plants corresponds with the results of other authors (Correia et al., 1999). Insensitivity of chlorophyll to UV irradiation may be linked with the activation of carotenogenesis, or the stimulation of anthocyanidins synthesis. The latter substances are almost ideal protection against free radicals and have been shown to be more effective antioxidants *in vitro* than tocopherol and ascorbate (Rice-Evans et al., 1997).

Carotenoids are the principal metabolites of xanthophyll cycle, which neutralize active oxygen and, by this, protect photosynthetic apparatus from excessive energy (Muller et al., 2001). Evidently, optimally selected doses of pre-sowing UV treatment may be very beneficial as it increases the contents of carotenoids – important antioxidants.

The increased levels of protective pigments after treatment probably were responsible for low sensitivity of photosynthesis to UV irradiation. Remarkably, protein synthesis appeared comparatively more sensitive to UV treatment, as shown by significantly reduced general protein contents in leaves.

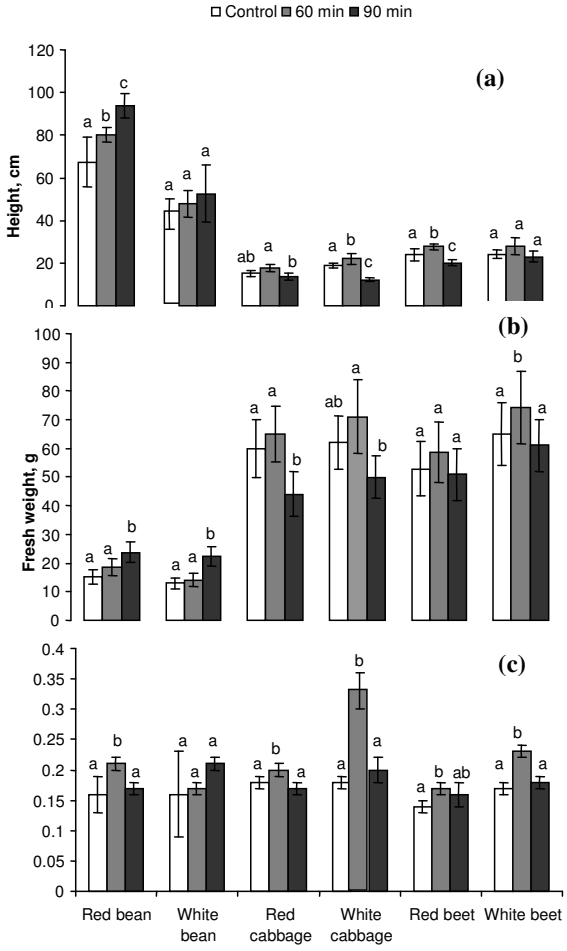

Fig 4. The effect of seed UV treatment (no treatment *versus* 30 and 90 min UV radiation) on (a) plant height (b) fresh and (c) dry masses. Error bars show standard deviation. Different letters indicate significant differences (P<0.05) according to Tukey’s multiple comparison test.

Optimal UV doses improved growth and biomass accumulation in plants: kidney bean required longer irradiation, while biennial crops responded positively to shorter, but negatively to longer UV treatments. The negative effects of high UV doses on plant growth can be explained by inhibition of photosynthetic activity and protein biosynthesis.

Overall, our results show that pre-sowing UV-treatment of seeds affects plant vital processes and activates stress adaptive mechanisms, via stimulating synthesis of antioxidants in leaves. The effect depends on the intensity of irradiation, plant species and the type of the antioxidative system. It should be possible to determine the optimal UV treatment for any crop and stimulate its defensive antioxidative systems aiming at improving stress resistance and hence crop productivity. Besides, antioxidants are increasingly used by humans as effective protectors against different stresses. Making synthesized antioxidants now is not a problem, but their natural analogues are still much more useful and friendly for living organisms. Vegetable food is the main source of antioxidants; therefore, vegetable food with higher antioxidant contents will also have more nutritional value.

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