Acta Sci. Pol. Hortorum Cultus, 17(5) 2018, 23–36

www.acta.media.pl 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2018.5.3

ORIGINAL PAPER

Accepted: 24.01.2018

NITROGEN METABOLISM IN CUCUMBER COTYLEONS AND LEAVES EXPOSED TO THE DROUGHT STRESS AND EXCESSIVE UV-B RADIATION

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ABSTRACT

Plants absorb light energy for photosynthesis, and some amount of potentially damaging range of solar radiation, ultraviolet B. It accounts for less than 0.5% of the total solar radiation and the ambient current level of UV-B during the growth season provide 10 kJ·m^{-2} energy per day on the Earth's surface. In the field conditions, increased UV-B radiation is often accompanied by drought, but negative effect of combined stresses is not so deleterious as the effect of one of them alone. We studied some changes in nitrogen me deficit and UV-B radiation. The stresses generally decreased the biomass and total dry matter production. Combination of water deficit and UV-B activated a stress tolerance mechanism in cucumber seedlings. The NR activity and synthesis of UV-B absorbing compounds were induced. Protection against UV-B radiation can partially involve increased production of UV-B protective pigments – slightly increased the SPAD value in cucumber cotyledons and leaves was observed. The deleterious effect of combined stresses was weaker than their additive or individual effects.

Key words: biomass production, cucumber, nitrate reductase, nitrate, SPAD

INTRODUCTION

Plants are rather sessile autotrophic organisms; they absorb light energy for photosynthesis, including considerable amounts of damaging range of solar radiation, in particular ultraviolet B (280–315 nm). In nature crops growing in open ground, they are from early stages exposed to environmental stress factors. Thus, they need to achieve balance between optimal light capture and UV-B protection [Ulm and Jenkins 2015]. The cucumber is a vegetable that prefers higher temperatures for germination and growth. For this reason, it is sown relatively late, at higher temperature and under intensive solar radiation. The plant develops a shallow root system and in a shallow soil layer it suffers the stress of water deficit as a result of insufficient and irregular rainfall.

Solar radiation in the UV-B range accounts for less than 0.5% of the total solar energy reaching the Earth's surface [Favory et al. 2009]. Current level of UV-B radiation during the growth season ranges from 2 to 12 kJ·m^{-2} (ambient 10 kJ·m^{-2}) per day on the Earth's surface [UNEP 2002, Singh et al. 2017]. In recent decades, UV-B dose-rates in the biosphere have increased due to the thinning of the protective ozone layer caused by human activities. CO_2 and other heat-trapping gases emitted into the atmosphere warm the troposphere and cool the stratosphere, indi-

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rectly depleting the ozone layer and giving access to the direct effect of potentially ozone-destroying halogenated chemicals, including chlorofluorocarbons (CFCs). In the stratosphere, UV-mediated activation of CFCs generates halogen radicals, which initiates a chain reaction of ozone depletion [Andrady et al. 2010]. Increase in the UV-B radiation has been estimated since the 1980s, and projections like the Kyoto protocol estimate that even after the implementation of these protocols, returning to the pre-1980 levels will be possible by 2050–2075 [UNEP 2002, Singh et al 2017]. Since the impact of UV radiation on genes and genomes is one of the driving forces of evolution [Tuteja et al. 2001], plants have evolved various strategies to moderate this stress [Gupta et al. 2011].

The lack of water is still a major limitation to crop productivity. Basic understanding of the physiological, biochemical and gene regulatory network is essential for growing crops with enhanced tolerance to the water deficit stress [Valliyodan and Nguyen 2006]. In the last 20 years, there has been a significant increase in the rate of transgenic or mutant plants tested for drought resistance [Blum 2014]. In many agricultural areas, drought is often accompanied by increased UV-B radiation [Ballaré et al. 2011].

Recent studies have revealed that the response of plants to a combination of two different abiotic stresses is unique and different from the response when each stress is applied individually. One stress may increase or alleviate the negative effect of the other [Mittler 2006]. Sequential or simultaneous action of stress factors may result in cross-resistance. The negative effect of combined stresses is not so deleterious as the additive effect or even as the effect of one of them alone [Alexieva et al. 2003]. There can be an antagonistic effect as well – decreased resistance to another factor acting at the same time [Mittler 2006]. Plants' responses to combined drought and UV-B radiation were reviewed in detail by Bandurska et al. [2013]. The impact of ozone depletion on other climate factors may result in an increase in the interactive effects of UV-B radiation with drought and temperature [Bornman et al. 2015].

Apart from water, nitrogen is an important element that limits the crop productivity. Nitrate reductase (NR) is a key enzyme in nitrogen metabolism, which reduces nitrate (NO_3^-) into nitrite (NO_2^-) and forms an amino acid. Reducing agents (NADH or NADPH) make leaf NO_3^- assimilation dependent on the photosynthetic process. The availability of NO_3^- from soil is believed to be the most important factor controlling the NR activity [Campbell 1999, Canovas et al. 2007]. NR in foliage may also depend on the water availability, temperature, light intensity and UV-B radiation [Norby 1989, Krywult et al. 1996, 2002, 2008].

To our knowledge, there is very little information on two or more abiotic stress factors interacting together in nature. Therefore, we studied some physiological and biochemical changes in nitrogen metabolism of cucumber at two developmental stages – cotyledons and leaves. The plants were exposed to two abiotic stress factors, i.e. water deficit and UV-B radiation, applied individually and in combination. The nitrate metabolism was investigated on the basis of NR activity and the nitrate content. The water conditions, i.e. relative water content, and chlorophyll content, i.e. SPAD, were also measured.

MATERIAL AND METHODS

Plant material

Cucumber seeds (*Cucumis sativus* cv. Dar) were sown in perlite, 5 pieces per one-liter capacity pot. They were allowed to germinate and develop in a growth chamber under the following conditions: fluorescent light intensity of 120 μmol m^{-2} s^{-1}, photon flux density of 400–700 nm supplied by Osram LUMILUX L18/840 lamps, at a photoperiod of 14/10 h, temperature of 25/20°C day/night and 60–70% relative humidity. The PAR intensity was measured with a phytophotometer FF-01 (Sonopan). The water content was maintained at 60% of total capacity. Seedlings were divided into four groups: a control sample and three groups subjected to the stress of UV-B radiation, water deficit, and a combination of water deficit and UV-B radiation. The control plants were well watered and kept under PAR only.

Cotyledons: 7-day-old seedlings were exposed to the stresses for 3–10 days. Leaves: 21-day-old seedlings were exposed to the stresses for 9 days, respectively.

Stresses: UV-B radiation supplied by Philips TL 20 W/01 RS lamps, with max. 315 nm at an intensity of 16 kJ m^{-2} day^{-1} for 8 h daily (3.25 μmol m^{-2} s^{-1} – photon flux density), during a 14 h light period. UV-B irradiation intensity was controlled using VLX 3W radiometer. Water deficit was applied by withholding the water supply, reducing the water content to about 40% of the total capacity of perlite. Both stresses were applied individually or in combination for 9 days. The cotyledons or third fully expanded leaf of each seedling were used for analysis. Each time, a dose sample contained cotyledons from 25 plants (5 pots, 5 plants per pot) and leaves of 15 plants (3 pots, 5 plants per pot).

Relative water content (RWC), indicating the content of water in leaves during dehydration, was estimated according to Weatherley's method [1950], and calculated according to the formula ((fresh mass – dry mass) / (weight at full turgor – dry mass)) · 100%.

Nitrate content: A 250 mg plant sample (fresh basis) was cut into small pieces. 10 cm^3 of distilled water was added and it was boiled for 20 min. At the second step of the extraction procedure, cooled extract was spin-dried at 4,000 g for 15 min (18–20°C). Concentration of nitrates in the supernatant was calculated with the method developed by Cataldo et al. [1975]. Then, 0.4 cm^3 of 5% salicylic acid in concentrated H_2SO_4 was added to 0.1 cm^3 of the supernatant and left for 20 min. Next, 9.5 cm^3 of 2 mol dm^{-3} NaOH was added and samples were cooled at room temperature. The absorbance was measured at 410 nm. The results were expressed as mg of NO_3 per 1 g^{-1} of tissue.

Nitrate reductase (NR; EC 1.6.6.1) activity. The method described by Jaworski [1971] was used. Plant samples (200 mg fresh mass) were placed in flasks. Then 5 cm^3 of the incubation mixture containing nitrates was added, and it was left for 1 h at 30°C. At the second step of the procedure, 1 cm^3 of SSA (1% sulphanilamide in 1 mol dm^{-3} HCl) and 1 cm^3 of NED (0.01% N-(l-naphthyl) ethylenediamine dihydrochloride) were added to 1 cm^3 of the incubation solution, and then, the mixture was left for 15 min. The absorbance of the mixture was measured at 540 nm. The activity of nitrate reductase was expressed as nmol of NO_2 per 1 g of fresh leaf mass per 1 h.

SPAD. Chlorophyll content index expressed in Soil Plant Analysis Development (SPAD) units was measured in fully developed cotyledons and leaves using a portable chlorophyll meter SPAD-502 (Konica Minolta Ltd., Osaka, Japan) [Scharf et al. 2006].

Statistical analyses were based on 3–5 replications. The data were presented as a mean ± standard deviation. The experimental data were subjected to one-way ANOVA and significant differences between the means were determined applying Tukey's multiple range test, using the Statistica program. Means followed by different letters are significantly different at $P = 0.05$. Data that differed significantly from the respective control sample were marked with asterisks * $P < 0.05$, ** $P < 0.01$.

RESULTS AND DISCUSSION

Many studies have shown the detrimental effects of UV-B and drought on plants' physiological processes. Plants stimulate protection and activate repair mechanisms to cope with different stresses [Frohnmayer and Steiger 2003]. In nature, stresses hardly ever occur separately. Water deficit, excessive radiation and heat often appear simultaneously. Our earlier studies showed that supplemental UV-B radiation reduced the deleterious effects of drought in cucumber plants. Antioxidant defense was activated at the stage of cotyledons and leaves when plants were exposed to the stress of water deficit and excessive UV-B radiation [Kubiś and Rybus-Zając 2008, Rybus-Zając and Kubiś 2010]. Our findings also suggest that the water deficit and UV-B radiation applied together generally alleviated the effect of individual stresses within the cellular membrane [Rybus-Zając et al. 2017]. In this study we analyzed changes in the nitrogen metabolism of cucumber cotyledons and leaves exposed to water deficit and UV-B radiation applied individually and in combination.

RWC

Applied stresses of water deficit and excessive UV-B radiation decreased the RWC in cucumber seedlings (Fig. 1). At the end of the progressive 9-day-long stress period, the water deficit in cotyledons (Fig. 1a) was considerably lower than in leaves (Fig. 1b). The water content in cotyledons (Fig. 1a) was reduced to 88% by UV-B radiation, to 75% by drought, and to 79% by both stresses. The water content measured in leaves (Fig. 1b) at the end of the stress period, i.e. on the ninth day, lowered to 90% under UV-B, 64% under drought, and 79% under both stresses. During the experiment, the water content in the well-watered control organs of seedlings exceeded 90%.

There were similar relations observed by Feng et al. [2007] in three water-stressed and UV-B irradiated wheat cultivars, where the RWC was significantly higher than under the exposure to drought only. UV-induced changes in the permeability of membrane ion channels and loss of K^+ from guard cells caused the loss of stomatal conductance [Eisinger et al. 2000]. This might explain lower evaporation from cotyledons/leaves exposed to supplementary UV-B radiation during drought.

Nitrate

The stresses caused changes in the nitrogen metabolism of cucumber seedlings. The content of nitrate (NO_3^-) in the leaves of control cucumber seedlings was higher than in cotyledons, i.e. 0.350–0.680 vs. 0.100-0.190 mg $NO_3^- \cdot 1g^{-1}$ fresh mass (Fig. 2). It exhibited an upward trend during the 9 days of the experiment. The nitrate content in cotyledons (Fig. 2a) exposed to stress was lower than in the respective control sample (100%). The nitrate content measured at the end of the stress period, i.e. on the ninth day, dropped to 68% under UV-B, to 37% under drought, whereas both stresses reduced it to 39%. UV-B increased the nitrate content in leaves (Fig. 2b), but the drought and drought/UV-B decreased it, as compared with the respective control-time sample (100%). At the end of the stress period, i.e. on the ninth day, the nitrate content increased to 137% under UV-B, but it dropped to 79% under drought and to 65% under both stresses.

In the foliage of silver birch seedlings grown under control, ambient and UV exclusion, there were no significant differences in the total nitrogen content [Krywult et al. 2002]. The amount of nitrate in maize leaves treated with UV-B was lower than in the control plants [Quaggiotti et al. 2004]. Rajendiran and Ramanujam [2006] observed similar relation in green gram (*Vigna radiata* L) plants, where the content of nitrate was slightly suppressed by UV-B stress.

NR

The nitrate reductase activity (Fig. 3) in cotyledons and leaves of the control cucumber seedlings was similar at the starting time. During the following 9 days, this parameter exhibited a downward trend. UV-B did not cause any changes in the cucumber cotyledons (Fig. 3a), but the drought and drought/UV-B generally decreased the nitrate reductase activity. In comparison with the respective control-time sample – 100%, excessive UV-B reduced the nitrate reductase activity to 98%, drought – to 35%, whereas the combination of drought and UV-B radiation reduced the nitrate reductase activity to 36%, measured at the end of the stress period, i.e. on the ninth day. The effect of the stresses on leaves (Fig. 3b) was opposite to the effect observed in the cotyledons. At the end of the stress period, i.e. on the ninth day, the NR activity increased to 306% under UV-B, to 115% under the drought and to 200% when both stresses were applied simultaneously.

In green gram (*Vigna radiata* L) plants, Rajendiran and Ramanujam [2006] observed reduced NR activity under UV-B radiation, whereas Ghisi et al. [2002] reported the same relation in barley, Sudaroli Sudha and Rajendiran [2013] in *Sesbania grandiflora* (L), and Vijayalakshmi and Rajendiran [2014] in cluster bean. The activity was altered in *Vigna unguiculata* L [Balakumar et al. 1999]. UV-B radiation significantly reduced the NR activity in maize. The analysis of transcript accumulation of genes encoding maize NR did not show significant differences between the control tissue and UV-B treated seedlings. The results of the research conducted by Quaggiotti et al. [2004] indicate that the effect of UV-B radiation on NR activity does not involve a transcriptional mechanism.

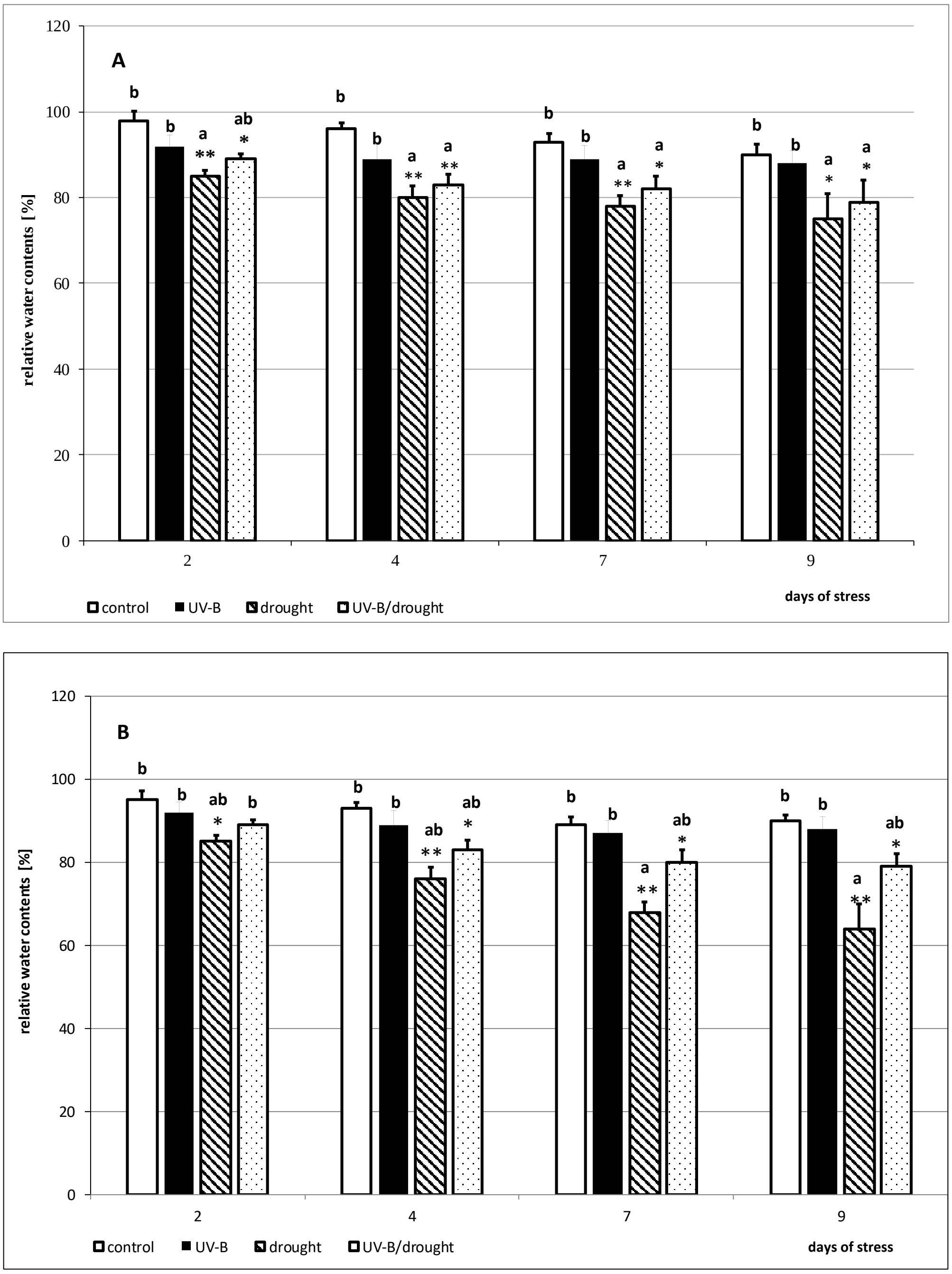


Fig. 1. Influence of progressive UV-B, water deficit and co-stresses on relative water content (RWC) in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 3. Data (stressed plants) significantly different from respective control: * $P < 0,05$, ** $P < 0,01$. Means followed by different letters are significantly different at $P = 0.05$, n = 5

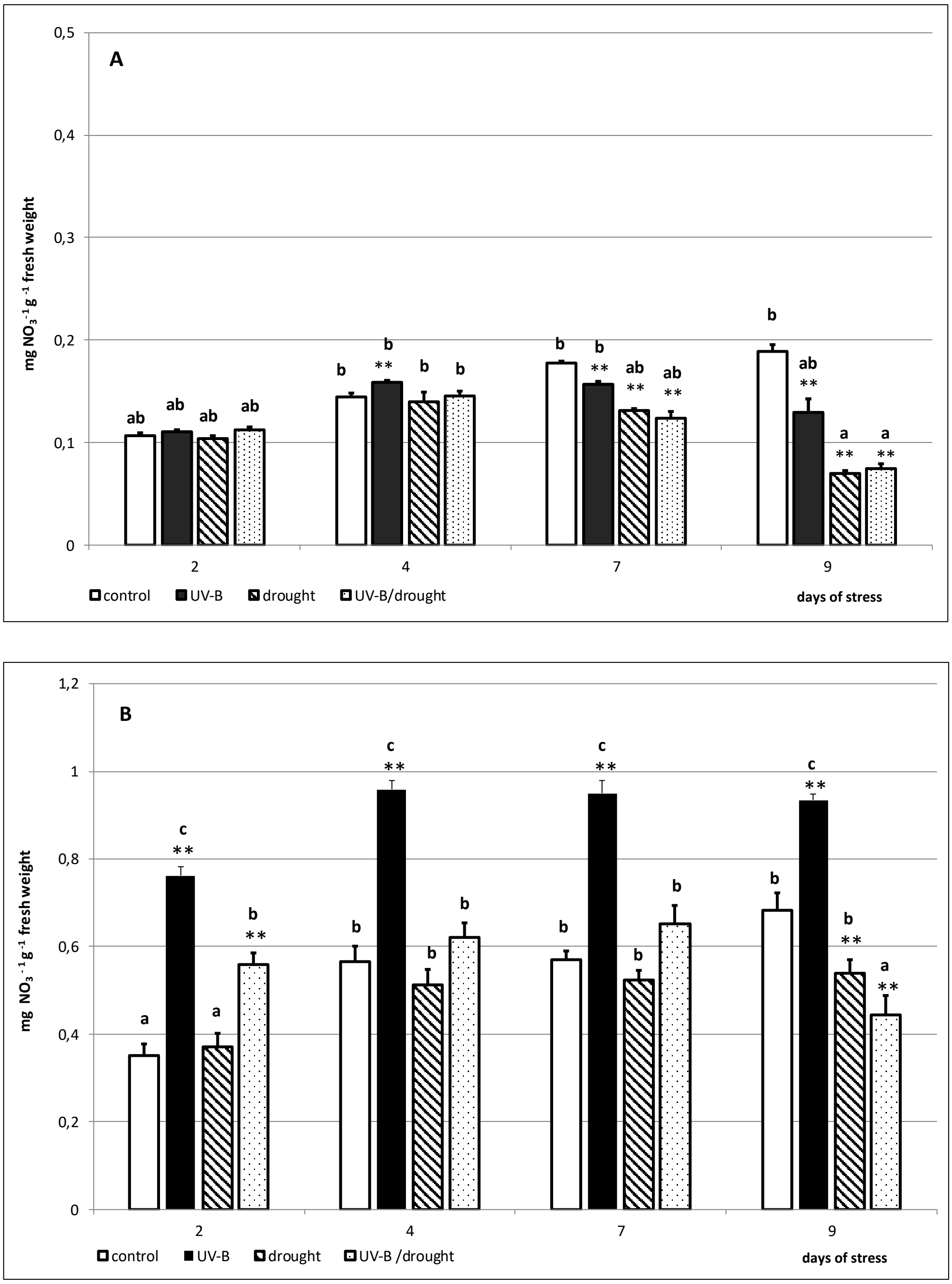


Fig. 2. Influence of progressive UV-B, water deficit and co-stresses on nitrate content in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 5. Data (stressed plants) significantly different from respective control: * P < 0,05, ** P < 0,01. Means followed by different letters are significantly different at P = 0.05, n = 5

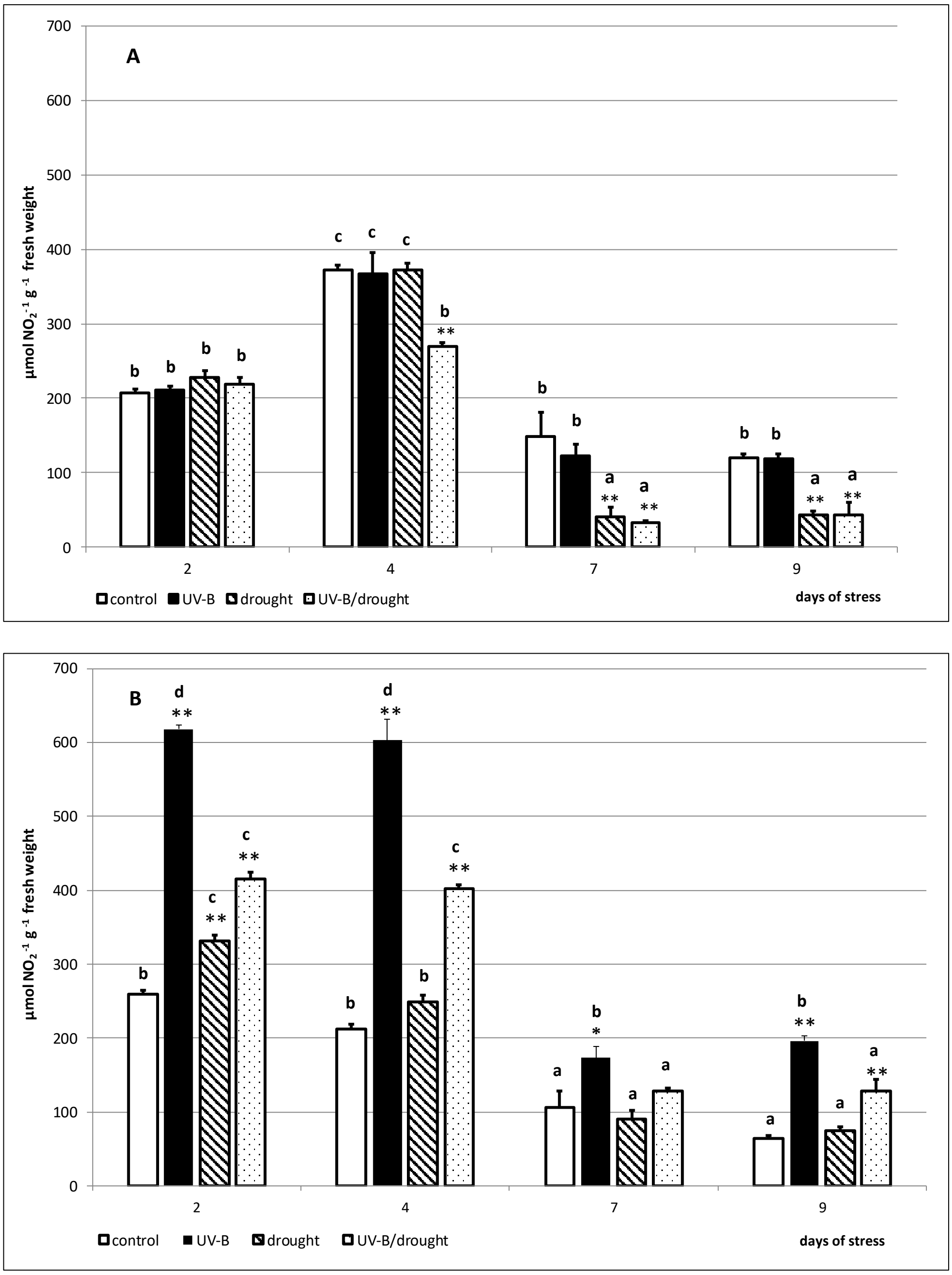


Fig. 3. Influence of progressive UV-B, water deficit and two stresses on nitrate reductase activity in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 5. Data (stressed plants) significantly different from respective control: * $P < 0,05$, ** $P < 0,01$. Means followed by different letters are significantly different at $P = 0.05$, $n = 5$

Fresh mass/dry mass

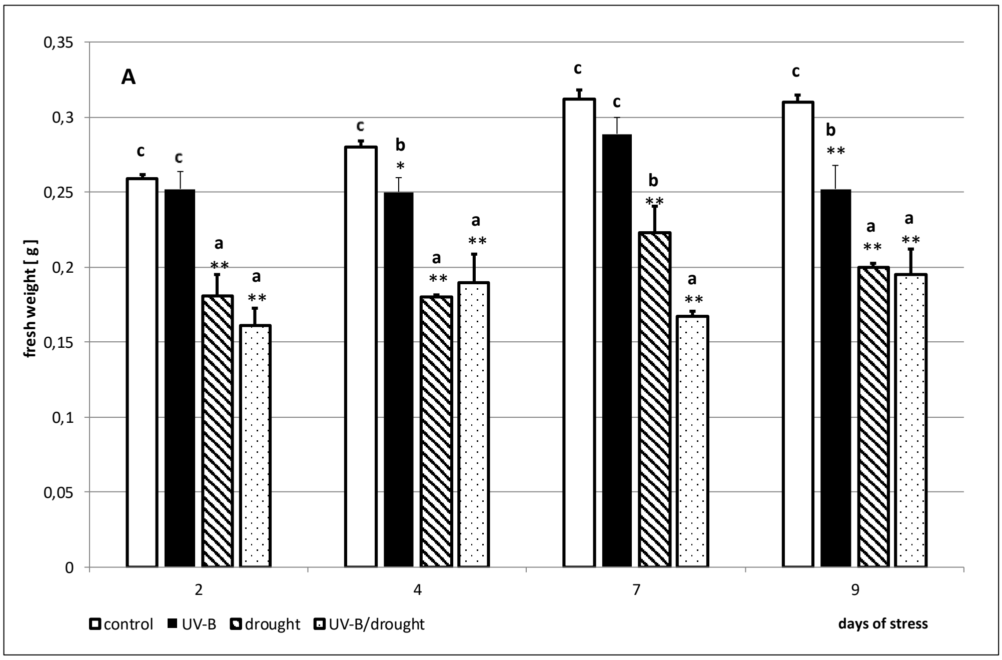
The fresh mass of the cucumber cotyledons (Fig. 4a) increased by 20% during the 9 days of the experiment, as compared with the starting point. The stresses decreased the growth intensity of cotyledons. Excessive UV-B reduced it by 18.7%, drought – by 35.5%, whereas the combination of drought and UV-B radiation – by 37.1%, as compared with the respective control-time sample – 100%, measured at the end of the stress period, i.e. on the ninth day. There was similar relation observed in the dry mass data. The dry mass of cotyledons (Fig. 4b) increased by 13% during the experiment, as compared with the beginning of the experiment. The stresses decreased these parameters as follows: excessive UV-B radiation – by 16.6%, drought – by 19.4 %, whereas the combination of drought and UV-B radiation – by 22.2%, as compared with the respective control-time sample – 100%, as measured at the end of the stress period, i.e. on the ninth day.

Leaves of the cucumber seedlings (Fig. 5a) grew by 117.2% during the 9 days of the experiment, as compared with the fresh weight at the starting point. The stresses decreased the leaf growth intensity as follows: excessive UV-B – by 21.6%, drought – by 26.6%, whereas the combination of drought and UV-B radiation – by 29.9%, as compared with the respective control-time sample – 100%, measured at the end of the stress period, i.e. on the ninth day. There was similar relation observed in the dry weight. During the experiment, the dry weight of leaves (Fig. 5b) increased by 147.2%, as compared with the dry weight at the beginning of the experiment. The stresses decreased this parameter as follows: excessive UV-B radiation – by 13.6%, drought – by 36.6%, whereas the combination of drought and UV-B radiation – by 31.8%, as compared with the respective control-time sample – 100%, as measured at the end of the stress period, i.e. on the ninth day.

The stresses decreased the biomass and total dry matter production. The growth in the cucumber cotyledons was more limited than in the leaves. The increase in total dry matter was considerably limited in the leaves. This effect was reviewed in detail by Zlatev et al. [2012]. Some opposite effects were also observed, especially under the short-term exposure [Gwynn-Jones 2001]. Several studies have shown evidence of plant resistance to UV-B radiation. Sullivan and Teramura [1990] reported that supplemental UV-B and drought did not result in additive effects on total soybean plant growth or seed yield compared to reductions under either stress independently. However, the stomatal limitations on photosynthesis were only significantly affected by the combination of UV-B and drought, but ribulose biphosphate regeneration limitations were observed under either stress. Under UV-B radiation, cucumber leaves become slightly less sensitive to water stress, but the mechanism of sensitivity or tolerance to combined stress has not been explained yet [Zlatev et al. 2012].

Spad

In general, there were higher chlorophyll meter readings – the SPAD (Fig. 6) value in cotyledons than in leaves of the control cucumber seedlings. Protection against UV-B radiation can partially involve increased production of UV-B protective pigments [Van de Staaij et al. 1995]. During the 9 days of the experiment, the SPAD index exhibited an upward trend in the control cotyledons, but it remained at the same level in leaves. In maize plants, the chlorophyll content decreased slightly under UV-B, but it dropped sharply in plants exposed to drought [Javadmanesh et al. 2012]. In the cucumber cotyledons (Fig. 6a), only the UV-B treatment increased the SPAD, as compared with the respective control-time sample (100%). Under the drought and UV-B/drought, the chlorophyll content remained almost the same as the control level, i.e. 101% and 93%, respectively. In leaves (Fig. 6b), the SPAD index measured at the end of the stress period, i.e. on the ninth day, increased to 123% under UV-B. It decreased slightly under drought, whereas the combination of UV-B and drought did not decrease the index. The respective values of 90% and 101% were measured, as compared with the corresponding control-time sample (100%).



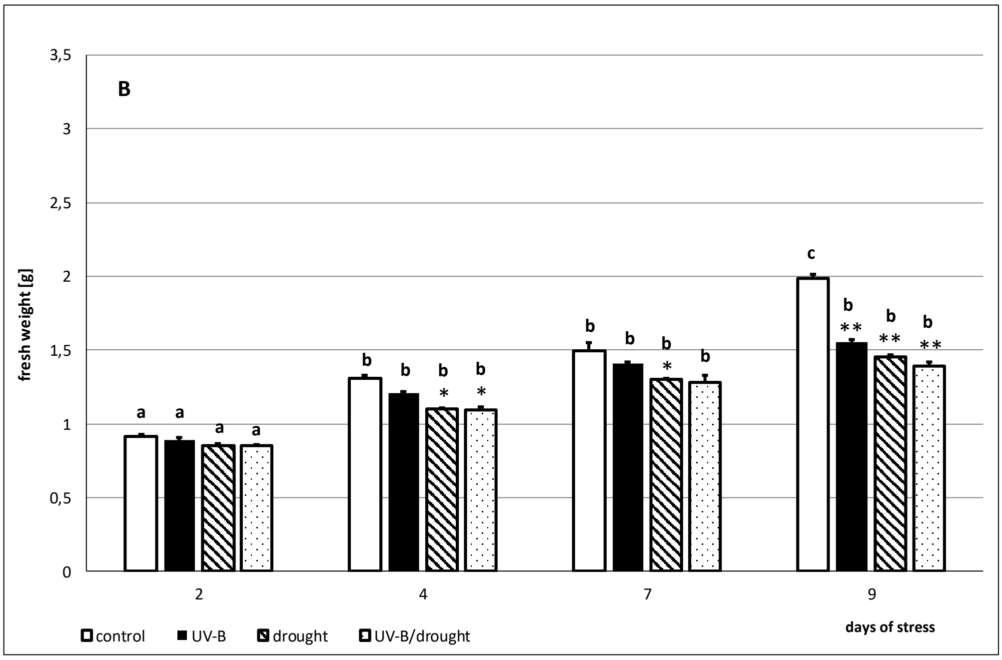


Fig. 4. Influence of progressive UV-B, water deficit and both stresses applied together on the fresh mass in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 3. Data (stressed plants) significantly different from respective control: * P < 0,05, ** P < 0,01. Means followed by different letters are significantly different at P = 0.05, n = 3

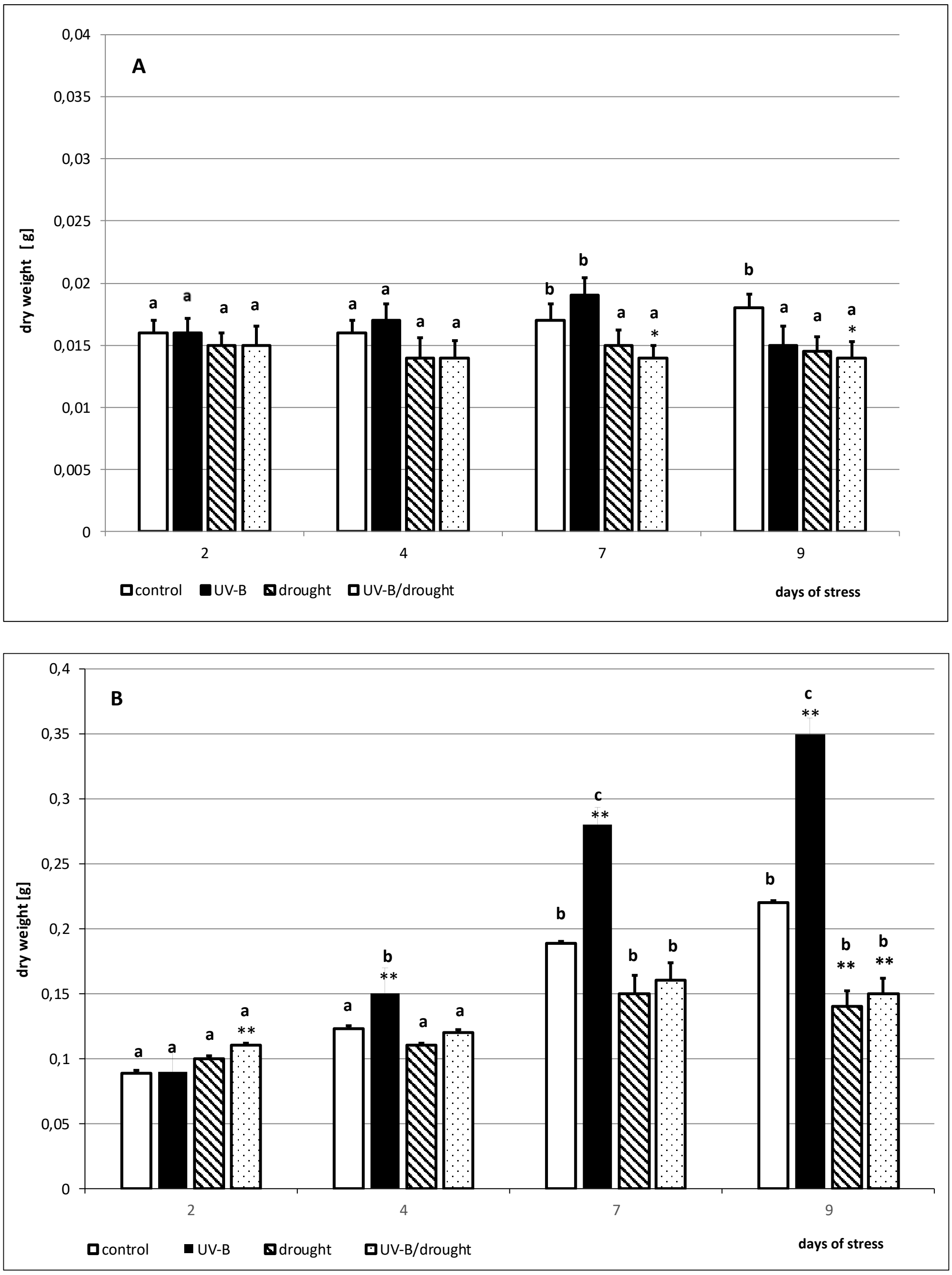


Fig. 5. Influence of progressive UV-B, water deficit and both stresses applied together on the dry mass in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 3. Data (stressed plants) significantly different from respective control: * $P < 0,05$, ** $P < 0,01$. Means followed by different letters are significantly different at $P = 0.05$, $n = 3$

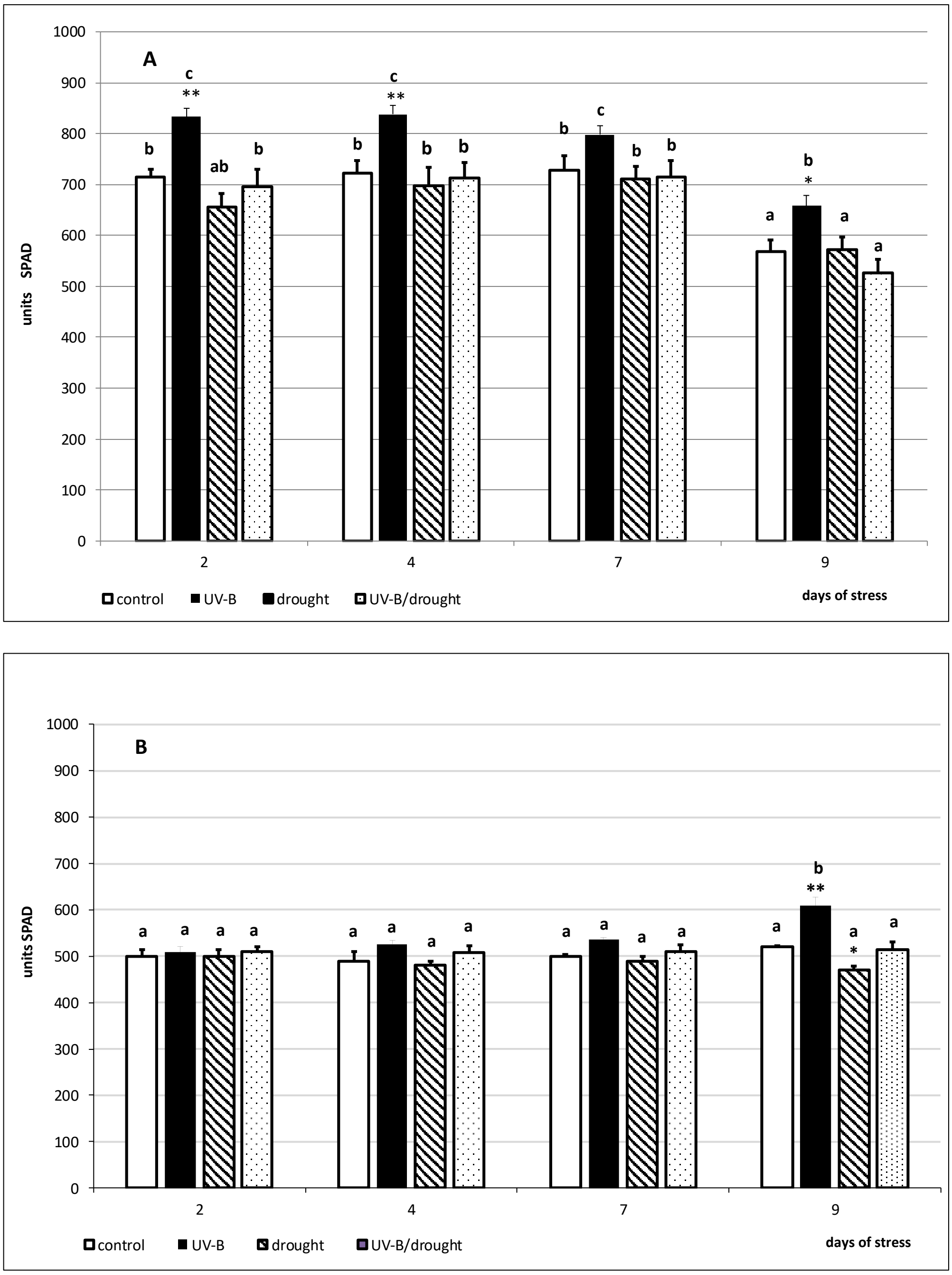


Fig. 6. Influence of progressive UV-B, water deficit and both stresses applied together on the SPAD measurements in: A – cucumber cotyledons and B – cucumber leaves. Measurements were made 2 to 9 stress's day. Values indicate means ±SE with n = 5. Data (stressed plants) significantly different from respective control: * P < 0,05, ** P < 0,01. Means followed by different letters are significantly different at P = 0.05, n = 5

Summarizing results obtained in our study, the combination of water deficit and UV-B activated a stress tolerance mechanism in cucumber seedlings. The NR activity and synthesis of UV-B absorbing compounds were induced in leaves. We observed that the deleterious effect of the combined stresses was weaker than their summative or individual effects. Zang et al. [2011] results demonstrate that UV-B induced up-regulation of *NIA 1* may lead to the enhancement of NR activity. NR is involved in UV-B triggered NO generation and NR-mediated NO generation is essential for UV-B induced nitrate reductase-mediated nitric oxide signaling and may resulted in UV-B tolerance [Gupta et al. 2011]. An alternative oxidative pathways are based on nitric oxide synthase (NOS)-like enzyme and polyamine mediated NO generation [Arasimowicz-Jelonek et al. 2009, Kubiś et al. 2014]. Bornman et al. [2015] suggesting that UV-B radiation has specific regulatory roles in plants growth and development that in turn can have beneficial consequences for plant productivity *via* effects on plant hardiness, and improved quality of agricultural product with subsequent implication for food security.

CONCLUSIONS

The stresses decreased the biomass and total dry matter production, generally the growth in the cucumber cotyledons was more limited than in leaves.

In the leaves, drought and drought/UV-B decreased the nitrate content, but these stresses tended to increase the nitrate reductase activity.

Under UV-B radiation, cucumber leaves become slightly less sensitive to water stress, protection against UV-B radiation can partially involve increased production of UV-B protective pigments – slightly increased the SPAD value in cucumber cotyledons and leaves was observed.

The combination of water deficit and UV-B activated a stress tolerance mechanism in cucumber seedlings and the deleterious effect of the combined stresses was weaker than their additive or individual effects.

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