PHYSIOLOGICAL RESPONSE OF COMMON BEAN (*Phaseolus vulgaris* L.) TO DROUGHT STRESS

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Abstract

Exposure to drought stress induces a cluster of physiological changes and has detrimental effects on several cell functions. Common bean is sensitive to drought stress, which can cause yield losses of more than 50 %. Different approaches to study physiological changes in leaves of two common bean varieties, Tiber and Starozagorski, subjected to drought stress were implemented. These included Total Radical-Trapping Potential (TRAP) test, Pulse-Amplitude-Modulation (PAM) fluorometry and reflectometry. The results of TRAP measurements and reflectometry indicate higher drought tolerance of the variety Tiber. Drought stress was not so intense as to cause irreversible damage of the photosynthetic apparatus in any of the varieties.

Keywords: common bean, *Phaseolus vulgaris* L., drought, physiological response

FIZIOLOŠKI ODZIV NAVADNEGA FIŽOLA (*Phaseolus vulgaris* L.) NA SUŠNI STRES

Izvleček

Izpostavljenost sušnemu stresu povzroči niz fizioloških sprememb v rastlinah in ima lahko uničujoč vpliv na številne celične funkcije. Navadni fižol je občutljiv na sušni stres, pridelek se lahko zmanjša do 50%. Za študij fizioloških sprememb v listih dveh sort navadnega fižola, Tiber in Starozagorski, so bili uporabljeni različni pristopi, ki so vključevali TRAP (Total Radical-Trapping Potential) test, PAM (Pulse-Amplitude-Modulation) fluorometrija in reflektometrija. Rezultati meritev TRAP in reflektometrije so pokazali, da je sorta Tiber bolj odporna na sušni stres. Vendar sušni stres pri nobeni sorti ni bil tako močan, da bi povzročil nepovratne spremembe fotosintetskega aparata.

Ključne besede: fižol, *Phaseolus vulgaris* L., suša, fiziološki odziv

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1 INTRODUCTION

Drought is a major factor affecting the growth and development of plants and may cause severe reductions in crop yields in many countries in the world. Its importance is likely to increase in response to the effect of global change and increased competition for water. The first signs of drought are visible in leaves, which appear prematurely senescent, although earlier changes, both morphological and metabolic, occur in roots, the first tissues to experience the reduction in water supply. These changes reflect, not merely a progressive reduction of water content in the plant, but qualitative and quantitative changes in its metabolism, suggesting a number of mechanisms by which plants can, within different limits, tolerate drought and recover from its effects. During evolution, plants have developed both physiological and biochemical responses to promote their survival under stress.

Drought stress can lead to increased reactive oxygen species (ROS) production. Drought induced stomatal closure inhibits CO_2 uptake, which under high light conditions results in over-reduced electron transport chains leading to photo-oxidative stress [27]. The plant cells can contain drought induced imbalance between pro- and antioxidants only to a certain degree, after which irreversible oxidative damage occurs [8]. Some plants, however, are more adapted to drought. Pastore et al. [18] have demonstrated that durum wheat mitochondria have mechanisms for dissipation of membrane potential, which inhibits ROS production. Other mechanisms of drought avoidance and oxidative damage control include solute accumulation, cell wall modification and the synthesis of protective proteins [31]. ROS are present in all aerobic cells, in equilibrium with antioxidants. When this balance is disturbed oxidative stress occurs. Aerobic organisms respond to oxidative stress by either non-enzymatic or enzymatic defence responses. Non-enzymatic defence reactions involves glutathione, ascorbic acid, α-tocopherol, β-carotene and other compounds capable of quenching ROS. Enzymes involved in defence include superoxide dismutases, catalases, peroxidases, glutathione reductase and $NADP^+$ reducing enzymes [14, 21, 24]. One of the methods for detecting ROS, particularly the formation of H_2O_2, is luminol assisted chemiluminescence [3]. With a slight modification of this method it is possible to determine ROS quenching activity in a biological sample, if an exogenous source of ROS is added. The resulting parameter, Total Radical Antioxidant Potential (TRAP), represents the cumulative action of intracellular and intercellular enzymatic and non-enzymatic antioxidants active under assay conditions [30]. The assay of luminol assisted chemiluminescence has been used for determining the antioxidant activity of pure chemicals [9] as well as biological samples [17, 20].

Chlorophyll fluorescence has become a common tool in plant physiology and ecophysiology to access photosynthetic performance of plants non-invasively. Monitoring of primary photochemistry is extremely useful in the cases of plant stress caused by high temperature, chilling, high light and drought [12]. The photosynthetic parameters Fv/Fm, qP and qN are well described [22].
The decrease in relative water content of leaves cause stomatal closing inhibiting the supply of CO_2 to the mesophyll cells. Such conditions are not expected to influence fluorescence induction parameters such as Fv/Fm [2]. After severe water stress the inhibition of the photosynthesis occurs resulting in the reduction of photochemical quenching qP [16] and increase in nonphotochemical quenching [11]. Fv/Fm is the indicator of the extreme stress causing the photoinhibition of PSII [12].

Reflectometry in the visible and near-infrared spectrum is a popular tool for studying plant stress, yield and biomass in the field conditions. Reflectometry is especially suited for the remote sensing applications from airplanes and satellites. One of the major goals for the remote sensing is detection of the water stress caused by drought. There are several vegetation indexes used to indentify leaf water content including normalized difference vegetation index (NDVI), developed by Rouse et al. [23]:

$$NDVI = \frac{NIR - R}{NIR + R},$$

where R and NIR are reflectances in the red (0.6-0.7 μm) and Near-IR wavelengths (0.7-1.1 μm) respectively. NDVI was used for several field studies including screening of drought tolerance in *Solanum* [25], peanut genotypes [26] and mixed vegetation [7].

Common bean is widely exposed to drought and many modifications associated with stress responses generally affect plant growth and yield. Abiotic stress resistance is by its nature more complex, is typically subject to large environmental effects and has been less well studied than biotic stress resistance in common bean [19]. Therefore, compared to pest and disease resistance, much less is known about the genetics of resistance to abiotic constraints or physiological stress [13] Abiotic stress resistance is typically governed by polygenic inheritance and may be conditioned by multiple, interacting mechanisms. These and other factors make abiotic stress resistance especially difficult to study, both physiologically and genetically. Several genes whose expression responds to drought have already been identified in common bean [28, 29]. In a study that was performed at the Agricultural Institute of Slovenia changes in gene expression in the leaves at different levels of dehydration were identified by differential display reverse transcriptase PCR and confirmed by quantitative real-time PCR [10].

A better understanding of the physiological basis of differences in drought stress resistance is very important in selection of new varieties of crops to obtain a better productivity under stress conditions. Studies of the effect of drought on the yield, photosynthesis, growth, osmotic adjustment and also about adaptation of common bean were described by Montalvo-Hernández et al. [15] and Aydi et al. [1]. In many studies the identification of tolerant and susceptible cultivars is based on a few physiological measures related to drought response. The physiologically relevant integrator of drought effects are the water content and the water potential of plant tissues which are widely published [6]. Experience in other crops indicates that abiotic stress tolerance may be the key to improving yields of common bean in both stressed and unstressed environments. Although disease resistance remains an important objective for most bean breeding programs, selection for greater tolerance to abiotic stress such as drought is expected to gain importance in response to climate change [4]. Breeding for drought resistance has a long history in many national bean programmes but only limited progress has been made in the improvement of cultivars [4]. The reasons for slow improvement are genetic complexity and the complex mechanism of water stress tolerance. However, there have been a few recent reports on development of cultivars and breeding lines with enhanced tolerance to drought [5]. Local adaptation is an important component of drought resistance in common bean. Development of cultivars with improved resistance to abiotic stress is one of the goals of the common bean breeding program at the Agricultural Institute of Slovenia.

The aim of the research was to implement different approaches to study physiological changes in common bean plants subjected to drought stress, which included TRAP test, PAM fluorometry and reflectometry.

2 METHODS

2.1 Plant material

Two common bean varieties, Starozagorski (susceptible to drought) and Tiber (tolerant to drought) were included in the analysis. The samples for TRAP measurement were excised from the 5th pair of leaves (bottom up), whereas PAM and NDVI measurements were performed on the 3rd or 4th pair of leaves (bottom up), each time on the same leaf.

2.2 The experiment in growth chamber

The experiment was conducted under controlled conditions in the growth chamber (RK - 13300CH, Kambič Laboratory equipment, Slovenia) in the year 2009. Plants were grown in pots (substrate Gramoflor, Germany; volume 4 l); in each pot one plant was grown. At the beginning of the experiment the same volume density of substrate was provided. Plants were subjected to three different levels of dehydration followed by a regeneration phase. Different regimes were applied for each treatment, control (well-watered plants) and drought (plants under different levels of dehydration) (Table 1). Twenty plants were used per each treatment. For the control treatment the regime was as follows: normal lightness (15000 lux); relative air humidity 70 %; the day length 15 h; day temperature: 26 °C, night temperature: 20 °C. The regime for drought treatment was: normal lightness (15000 lux); relative air humidity 70 % in the beginning of the experiment and 55 % under drought conditions.

The plants were optimally irrigated based on tensiometer measurements and with the pot weighting every three days. Control plants followed these regimes until the end of the experiment, while watering of drought-stressed plants was stopped when plants were 24, 31 and 38 days old. After 21 of water deprivation all plants (control and drought treatment) were optimally watered again every third day (310 ml/pot) till the end of the experiment. At each sampling the soil humidity was determined gravimetrically for each treatment.

Table 1: Plant age at collection for control (well-watered), drought-stressed plants and at the regeneration phase with duration of water deprivation.

Preglednica 1: Starost rastlin ob vzorčenju kontrolnih rastlin (dobro zalite), rastlin pod sušnim stresom in rastlin v fazi regeneracije s trajanjem odvzema vode

Treatment	Plant age at collection	Water deprivation days
Control	24, 31, 38, 57	
Drought - week 1	24	7
Drought - week 2	31	14
Drought - week 3	38	21
Regeneration phase	57	21

2.3 TRAP

The TRAP assay is based on a chemiluminescence signal, which is produced when horseradish peroxidase is oxidized by H_2O_2 and loses two electrons. When the horseradish peroxidase oxidizes luminol to recover the missing electrons, the latter becomes unstable and emits chemiluminescence [3]. The samples for TRAP measurement were prepared in the following manner: Approximately 100 mg of fresh leaf tissue per sample was frozen in liquid nitrogen, homogenized in 1 ml of potassium phosphate buffer (50 mM, pH 7.0) and centrifuged (10 min, 10000 RPM, 4 °C). The supernatant was frozen and kept at -80°C until the measurement of luminescence. The luminescence reaction mixture contained horseradish peroxidase (0.13 units ml^{-1}) and 350 µM luminol in 0.1 M potassium phosphate buffer, pH 8.5. To the reaction mixture, the sample and then hydrogen peroxide (final concentration of 870 mM) were added to start the reaction. The mixture was shaken for 15 s followed by 5 s of incubation at 20°C. Chemiluminescence was then recorded as relative light units every 20 s for 25 min with a Victor X5 Multilabel reader 2030 (Perkin Elmer). The TRAP value of plant tissue homogenates was determined as the quotient of blanks to treatments according to

$$TRAP = \frac{\sum I_0}{\sum I},$$

where I_0 represents the measured chemiluminescence of the blank sample (luminol, buffer, horseradish peroxidase and H_2O_2) and I represents the chemiluminescence of the tested sample (supernatant of homogenized plant tissue, luminol, buffer, horseradish peroxidase and H_2O_2).

The samples of variety Starozagorski from the first week of the experiment unfortunately thawed during the transportation to the laboratory, therefore we omitted those results.
Note: The results of TRAP test of Starozagorski variety in the first week of drought were excluded from the analysis due to power failure of the ultra-freezer in which they were stored.

2.4 Chlorophyll fluorescence

Chlorophyll fluorescence was measured using a pulse amplitude modulated fluorometer from Opti Sciences (USA) OS-5 using a kinetic test protocol. Before measurement a dark clip was utilized for 10 minutes, and then the F_0 of dark adapted leaves was obtained. The saturation pulse of the intensity 8000 µmol $(m^2s)^{-1}$ was switched on for 0.8 s and Fm was obtained. The steady state parameters Fs and Fm were measured after 3 minutes of actinic illumination of 160 µmol $(m^2s)^{-1}$.

From the measured fluorescence several physiological parameters were obtained: maximum quantum efficiency of PSII photochemistry $F_v/F_m=(F_m-F_0)/F_m$, photochemical quenching $qP=(F_m'-F')/(F_m'-F_0)$ and nonphotochemical fluorescence quenching $qN=(F_m-F_m')/(F_m-F_0)$ [22].

2.5 Reflectometry

Reflectance spectra was measured using a Ocean Optics HL-2000 Tungsten Halogen Light Source, the reflectance probe Ocean optics QR400-7-UV/BX and Ocean optics USB2000

spectrometer with the wavelength range from 400 – 1200 nm. Reflection probe was mounted 20 mm from the leaf at the 45 degrees incidence angle.

Reflection spectra were obtained using SpectraSuit application software. First the dark spectrum was subtracted. Light spectra were measured when 1 mm thick teflon sheet was inserted instead of the leaf. Reflection spectra were calculated as follows:

$R=(I_{leaf}-I_{noise})/(I_{teflon}-I_{noise})$

Every leaf was illuminated for 30 s before measurement to allow for the stabilization of reflectance in the red part of the spectrum.

NDVI was calculated as

$$NDVI = \frac{NIR - R}{NIR + R},$$

where NIR was taken at 800 ± 5 and R at 580 ± 5 nm.The NDVI values were averaged between 5 plants with the same treatment.

2.6 Statistical analysis

The TRAP, Fv/Fm, qP, qN and NDVI values were averaged from five plants within the same treatment. The statistical significance of plants exposed to drought compared to the control plants was evaluated using a student t-test. The results were considered statistically significant when $p<0.05$.

3 RESULTS

3.1 TRAP

A transient significant difference between control and drought stressed plants of variety Tiber was measured in week 3 of drought stress (Figure 1). The TRAP value of the drought stressed plants was 154% of the control value. The TRAP value in the leaves of the variety Tiber increased in the regeneration phase, but the difference between the control and drought-stressed plants diminished.

The TRAP value of the drought-stressed plants of variety Starozagorski insignificantly decreased from week 2 to week 3 (Figure 1). The TRAP value significantly differed between the control and the drought-stressed plants at the regeneration phase; it was 2.57-fold higher in drought stressed plants compared to control plants.

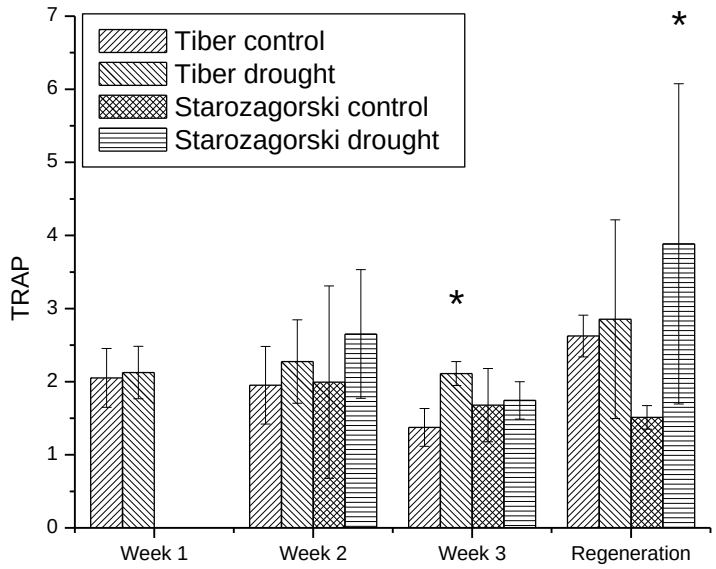

Figure 1: Total radical antioxidative potential of common bean plants, varieties Tiber and Starozagorski in relation to the duration of drought stress. The watering was discontinued seven days prior to 'Week1' time point. After three weeks of drought, the plants were watered to test their regeneration capability. Data shown are the mean values ± standard error of a single experiment performed in 5 replicates (n=5). Asterisk (*) represents statistically significant difference between control and drought exposed plants of the same variety at the same time point.

Slika 1: Rezultati TRAP testa fižolovih rastlin, sort Tiber in Starozagorski, v odvisnosti od trajanja sušnega stresa. Zalivati smo prenehali sedem dni pred "Week1" časovno točko. Po treh tednih suše smo rastline zalili, da smo preizkusili njihovo sposobnost regeneracije. Prikazani podatki so povprečne vrednosti ± standardna napaka enega poskusa, izvedenega v petih ponovitvah (n = 5). Zvezdica (*) predstavlja statistično značilne razlike med rastlinami, izpostavljenim suši in kontrolnimi rastlinami, iste sorte ob istem času.

3.2 Chlorophyll fluorescence

We observed a decrease of qP in week 3 for the stressed samples in both varieties. The difference was statistically significant in the variety Starozagorski. The effect was reversed but non significant in the regeneration phase (Figure 2a).

There was no statistically significant difference of nonphotochemical quenching qN between the control and drought exposed plants during the drought induction (Figure 2b). During the regeneration phase qN of the drought exposed variety Starozagorski dropped significantly compared to the control.

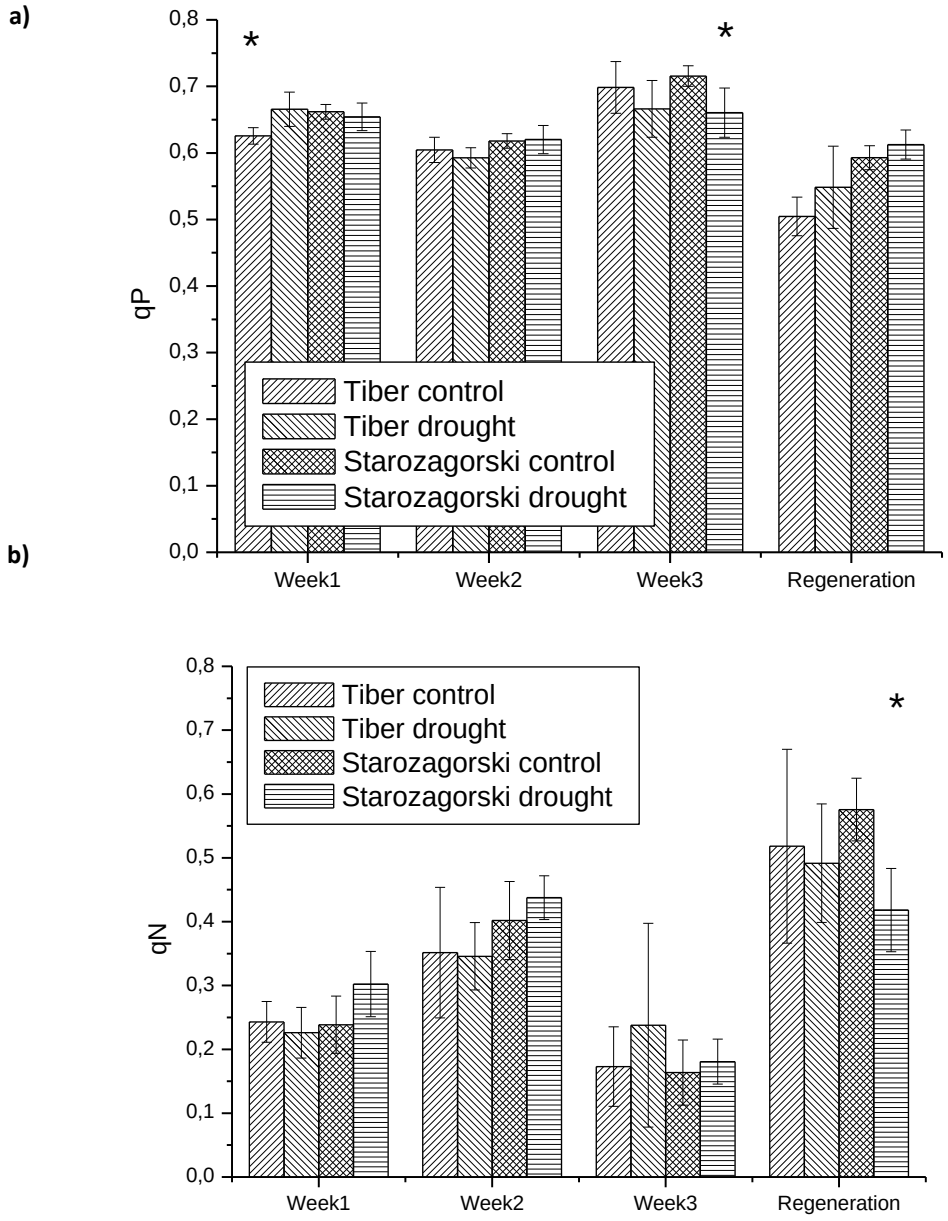

Figure 2: Photochemical parameters qP (a) and qN (b) of two common bean varieties, Tiber and Starozagorski after different duration of drought. Data shown are mean values ± standard deviation of a single experiment performed in 5 replicates (n=5). Asterisk (*) represents statistically significant difference between control and drought exposed plants of the same variety at the same time point.

Slika 2: Fotokemična parametra qP (a) in qN (b) dveh sort navadnega fižola, Tiber in Starozagorski, po različnem času trajanja suše. Prikazani podatki so povprečne vrednosti ± standardni odklon enega poskusa, izvedenega v petih ponovitvah (n = 5). Zvezdica (*) predstavlja statistično značilne razlike med rastlinami, izpostavljenim suši in kontrolnimi rastlinami, iste sorte ob istem času.

Parameter Fv/Fm was stable at the theoretical optimum value 0.83 except for the drought exposed plants of the variety Starozagorski in week 3, where the measured Fv/Fm value was 0.8 (Figure 3). The effect diminished during the regeneration.

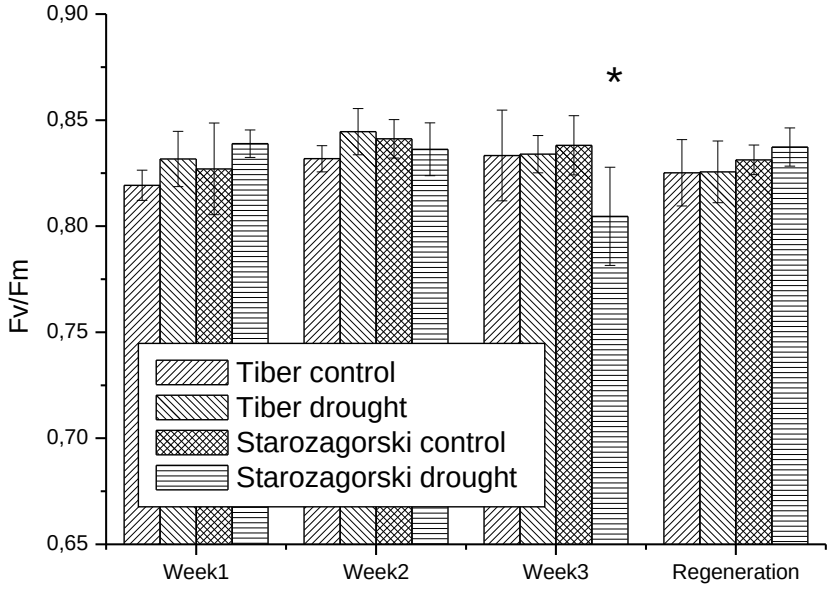

Figure 3: Photochemical parameter Fv/Fm of two common bean varieties, Tiber and Starozagorski after different duration of drought. Data shown are mean values ± standard deviation of a single experiment performed in 5 replicates (n=5). Asterisk (*) represents statistically significant difference between control and drought exposed plants of the same variety at the same time point.

Slika 3: Fotokemični parameter Fv/Fm dveh sort navadnega fižola, Tiber in Starozagorski, po različnem času trajanja suše. Prikazani podatki so povprečne vrednosti ± standardni odklon enega poskusa, izvedenega v petih ponovitvah (n = 5). Zvezdica (*) predstavlja statistično značilne razlike med rastlinami, izpostavljenim suši in kontrolnimi rastlinami, iste sorte ob istem času.

3.3 Reflectometry

In the first two weeks (week 1, week 2) there was no difference between the groups with the NDVI being approximately 0.91. In week 3 there was a slight drop of NDVI in the drought exposed variety Starozagorski. The difference was reduced in the regeneration phase.

4 DISCUSSION

The results of TRAP measurements of common bean plants under drought stress indicate higher drought tolerance of the variety Tiber. The TRAP value in the leaves of drought stressed Tiber plants was significantly higher than in the control plants in the third week of drought stress. This indicates active struggle against drought-induced oxidative stress [20]. During the regeneration phase the difference of TRAP value between drought-stressed and control plants diminished. The opposite is true for the variety Starozagorski, where the TRAP

value was significantly induced in the drought stressed plants during the regeneration phase. This indicates that the antioxidative network was still induced after three weeks of regeneration and that the plants were still struggling against drought-induced oxidative stress [20].

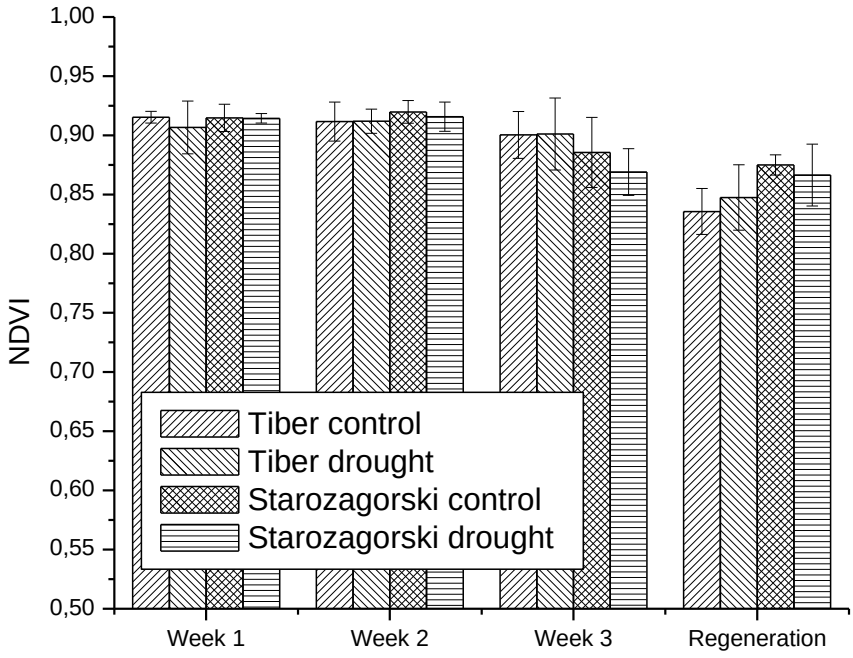

Figure 4**:** Measured values of the reflectometric index NDVI of leaves of two common bean varieties Tiber and Starozagorski s after different times of drought induction. Data shown are the mean values ± the standard deviation of a single experiment performed in five replicates (n=5). Asterisk (*) represents statistically significant difference between control and drought exposed plants of the same variety at the same time point.

Slika 4: Izmerjene vrednosti reflektometričnega indeksa NDVI v listih dveh sort fižola, Tiber in Starozagorski, po različnem času trajanja suše. Prikazani podatki so povprečne vrednosti ± standardni odklon enega poskusa, izvedenega v petih ponovitvah (n = 5). Zvezdica (*) predstavlja statistično značilne razlike med rastlinami, izpostavljenim suši in kontrolnimi rastlinami, iste sorte ob istem času.

First effects of drought on photosynthesis were observed after three weeks of drought. The stress manifested itself via the decrease of the photochemical quenching, which can be a direct consequence of stomatal closing and lower mesophyll CO_2 concentration [2]. There was no observable increase in nonphotochemical quenching showing the ability of the stressed plants to utilize the actinic illumination for the photosynthesis. The damage caused by drought was indicated in Starozagorski variety where parameter Fv/Fm was decreased. Such effect is not expected in the case of mild water stress according to Lima et al. [11]. The drought stress was not so intense as to cause irreversible damage to the plants. During the regeneration phase the qP value in the drought-stressed plants was higher than in the control plants. The opposite was

true at week 3. Additionally, the Fv/Fm value of the drought-stressed plants of the variety Starozagorski surpassed the control values during the regeneration indicating that the plants actively repaired the photosynthetic apparatus.

NDVI was very stable in the first two weeks of the experiment, showing the decrease in the variety Starozagorski in third week. The lower value in the drought exposed plants of Starozagorski indicates a drop in the leaf water content. The drop of NDVI values in the regeneration phase indicates damage to the structure of the leaves in the variety Starozagorski since the effect was not fully reduced during the regeneration phase. For the variety Tiber there was no observable difference between the plants before and during the regeneration phase. NDVI shows higher drought resistance in the variety Tiber and indicates that reflectometry can be used to distinguish drought tolerant and intolerant plants as in the study of Sullivan and Holbrook [26] conducted on peanuts plants.

The decrease in NDVI value in the variety Starozagorski in the third week showed the onset of drought stress. This is in accordance with a significant decrease of the Fv/Fm parameter. During the regeneration phase the photosynthetic performance of the drought-stressed plants of variety Starozagorski improved as represented by the increase of qP and Fv/Fm and reduction of qN. However, the significantly increased TRAP value shows that the antioxidant apparatus of plants of the variety Starozagorski was still active in protecting the plants from drought-induced oxidative damage. In contrast, in the variety Tiber we did not measure any significant decrease of the Fv/Fm value and the average NDVI index was very similar in the control and drought stressed plants in the third week of drought. Additionally, the TRAP value was not induced during the regeneration phase. This indicates that the strategy of the variety Tiber for coping with drought stress is stress avoidance.

The results provide new insights into physiological pathways of drought tolerance in common bean and revealed that the methods applied to measure physiological changes in leaves of plants exposed to drought could be applicable in the selection for drought tolerance in the breeding program of common bean.

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