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Impact of drought stress on physiological characteristics and isolation of chloroplasts in common bean (*Phaseolus vulgaris* L.)

Tanja Zadražnik[1], Jelka Šuštar-Vozlič[2]

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ABSTRACT

Chloroplasts are involved in many cellular processes and play an important role in plant stress response. In this study, the effect of drought on different physiological characteristics and the isolation of chloroplasts from leaves of drought-stressed and control plants of common bean (*Phaseolus vulgaris* L.) are reported. Two cultivars differing in the response to drought were analysed - Tiber and more sensitive Starozagorski čern. The results of relative water content showed that plants were under mild stress conditions after six days without watering. Negative effects of drought stress on the photosynthetic rate and chlorophyll fluorescence parameters of both cultivars were observed. The yield of isolated chloroplasts in control samples of both cultivars was at least three times higher compared to drought-stressed samples. The intactness of the isolated chloroplasts was checked and used for protein isolation. The results of the study suggested that even mild drought stress strongly affected the isolation of intact chloroplasts. Still, intact chloroplasts were enriched and were suitable for downstream proteomic analysis.

Key words: Chloroplast, Drought, *Phaseolus vulgaris*, Photosynthesis, Proteins.

INTRODUCTION

Plants are often exposed to unfavourable environmental conditions that affect their growth, development and productivity. Drought is one of the major stress factors that limit agricultural productivity of many crops, including legumes and among them common bean (Montero-Tavera *et al*., 2017). Development of cultivars with improved tolerance to drought stress is a goal of many common bean breeding programs throughout the world, due to its importance for human consumption, high nutritional value and positive effect on human health (Boros and Wawer, 2018; Boschin and Arnoldi, 2011). Detailed molecular mechanisms of common bean response to drought and drought tolerance are not yet well understood.

Plants respond to drought stress by various cellular and tissue specific physiological and molecular mechanisms, including changes in gene expression and abundance of specific proteins. Chloroplasts have a central role in plant stress response, because drought stress causes the alterations in photosynthesis through the damage of the photosynthetic systems (Farooq *et al*., 2009). To obtain comprehensive and deeper insight into the molecular mechanisms involved in the response to drought stress, the role of cellular organelles, such as chloroplasts should be considered in stress-related molecular studies.

The aim of this study was to determine plant physiological status and to compare the efficiency of the isolation of chloroplasts from drought-stressed and control plants of common bean. Two cultivars were included in the study- drought susceptible cultivar Starozagorski čern (Starozagorski) and cultivar Tiber, shown in the previous study to be the most tolerant to drought among all the genotypes tested (Hieng *et al*., 2004). Plant water status and plant physiological traits were recorded. For the chloroplasts isolation minimal amount (the third trifoliate leaves) of plant material was used. The intactness of the isolated chloroplasts was checked and used for protein isolation. The described procedures are suitable for further proteomic analysis.

[1]Agronomy Department, University of Ljubljana, Biotechnical Faculty, SI-1000 Ljubljana, Slovenia.

[2]Crop Science Department, Agricultural Institute of Slovenia, SI-1000 Ljubljana, Slovenia.

Corresponding Author: Tanja Zadražnik, Agronomy Department, University of Ljubljana, Biotechnical Faculty, SI-1000 Ljubljana, Slovenia. Email: tanja.zadraznik@kis.si

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MATERIALS AND METHODS

Experimental design and growth conditions

Two cultivars of *P. vulgaris* (Starozagorski čern and Tiber) were studied. Plants were cultivated in the greenhouse from May till June 2017. Growth conditions were as follows: 16 h illumination under natural lighting at 25°C during the day and 20°C at night and relative humidity from 40 to 70 %, on average. Three seeds were sown in each of 14 cm pots containing a mixture of fertilized substrate (Klasmann, Germany) and vermiculite (1:1, v/v). Ten days after germination, seedlings were thinned to one plant per pot.

Plants were regularly irrigated with tap water and were subjected to water deficit 4 weeks after sowing. Half of the plants were not watered and the other half was regularly irrigated. The analyses of soil and plant water status and physiological parameters of plants were determined on day 6 after the beginning of water withdrawal. Both stressed and control plants were collected for chloroplast isolation. The third trifoliate leaves were harvested, frozen immediately in liquid nitrogen and stored at -80°C until further analysis. There were 5 replicates of drought-stressed plants and control plants.

Plant physiological status

Physiological measurements were performed on third trifoliate leaves between 10 and 12 am. Net photosynthesis rate (A_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration (E) were measured using a portable photosynthesis measuring system, Li-COR 6400 (Li-COR, Lincoln, USA). The system also enables photochemical efficiency (Fv'/Fm') and electron transport rate (ETR) measurements. The parameters for photosynthesis measuring were as follows: leaf temperature 21°C, flow rate 300 mmol s^{-1}, photosynthetically active radiation 600 µmol m^{-2} s^{-1} and reference CO_2 concentration 380 µmol mol^{-1}.

The middle of the third trifoliate leave was cut for the determination of the leaf area and relative water content (RWC). The leaf area of each plant was measured with an area meter (LI-3100, Li-Cor, inc, Lincoln, Nebraska, USA). The hydration state of the leaves was defined by RWC as described by Zadražnik *et al*. (2013). The soil water content (SWC) was determined from the middle of the pots (Bittelli, 2011).

Isolation of chloroplasts, estimation of intactness and chlorophyll concentration

Chloroplasts were isolated using Percoll gradient according to Kley *et al*. (2010) with minor modifications. Third trifoliate leaves were homogenized using mortar and pestle in cold homogenisation buffer (0,45 M sorbitol, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 50 mM HEPES-KOH pH 8.0, 10 mM $NaHCO_3$, 1,9 mM ascorbic acid and 0,5 mM DTT). The homogenate was rapidly filtered through two layers of miracloth and loaded on top of preformed 40 and 80% Percoll gradient. Chloroplast separation was achieved by 30 min centrifugation at 3220 g at 4°C in a swing-out rotor (accelleration set 2, brakes set off). Upper layers containing broken chloroplasts were removed and the intact chloroplasts were collected from the interphase between 40 and 80% Percoll, washed with three volumes of homogenisation buffer and centrifuged for 5 min at 3220 g. The supernatant was removed and the chloroplast pelet was resuspended in homogenisation buffer using paint brush.

The intactness of chloroplasts was confirmed by ferricyanide dependent photoreduction, in presence and absence of osmotic shock as previously described by Lande *et al*. (2017). Chloroplasts were checked by epifluorescent microscope Nicon Eclipse 80i (Nikon Corporation, Japan). Chlorophyll content of the intact chloroplast fractions was estimated according to Lande *et al*. (2017).

Protein extraction and 2D-PAGE

Extraction of the chloroplast proteins was carried out according to the modified method by He *et al*. (2013). Chloroplasts stored in homogenisation buffer were centrifuged for 10 min at 3220 g. The supernatant was removed and the chloroplast pelet was suspended in extraction buffer (8 M urea, 7 M thiourea, 4% CHAPS, 30 mM Tris, 1% Triton X-100 and 1% DTT). After vigorous shaking for 20 min and 3 cycles of 5 min sonication, samples were centrifuged at 5000 g for 15 min. The supernatant was precipitated by acetone with trichloroacetic acid and proteins were extracted as described by Zadražnik *et al*. (2013). Protein concentration was quantified by 2D Quant kit (GE Healthcare) according to manufacturer's protocol. Preliminary 2D-PAGE of isolated protein was performed as described by Zadražnik *et al*. (2013). After electrophoretic separation, gels were stained with Coomassie R-250.

RESULTS AND DISCUSSION

Plant physiological responses to drought

Drought stress can cause harmful changes to physiological, biochemical, morphological and structural processes in plants (Kusvuran and Dasgan, 2017; Marcinska *et al*., 2013). In our study, drought stress significantly decreased the RWC of the leaves and the SWC in both analysed cultivars of common bean. RWC decreased from 91.3% in well-watered plants to 68,3% in water-stressed plants of Starozagorski. In Tiber, the decrease of RWC was similar; it decreased from 92,9% in control plants to 75,3% in drought stressed plants (Table 1). In addition, the SWC was measured and was 69,9% (Starozagorski) and 75,9% (Tiber) for well-watered plants, reaching 34,1% for Starozagorski and 31,9% for Tiber under drought stress. There were no significant differences in RWC and SWC in drought-stressed plants between the two cultivars after 6 days of drought. According to previous studies, where RWC was also measured on the third leaf of common bean plants under drought (Budič *et al*., 2013), plants included in our study were in mild drought stress.

Drought stress can inhibit and even damage plant photosynthetic physiology, such as photosynthetic rate and chlorophyll fluorescence parameters (Mathobo *et al*., 2017). In the present study, negative effects of drought stress on the photosynthetic physiology of both cultivars were noticed and there was no statistical significant difference between them, showing equal response on stress of Starozagorski and Tiber (Table 2). For instance, the net CO_2 assimilation rate or net photosynthetic rate (A_N) dropped by 93 % in both cultivars. The reduction of photosynthesis due to drought stress has been reported in grain legumes (Farooq *et al*., 2017), dry bean (Lanna *et al*., 2016) and faba bean (Girma and Haile, 2014).

Table 1: Effect of drought stress on RWC, SWC and leaf area in cultivars Starozagorski čern and Tiber.

Sample	Treatment	SWC (%)	RWC (%)	Leaf area (cm^2)
Starozagorski čern	control	69,9±2,0 a	91,3±1,0 a	104,7±6,4 a
	drought	34,1±4,5 b	68,3 ± 11,7 b	103,9±15,1 a
Tiber	control	75,4±4,7 a	92,9±1,7 a	136,1±12,1 b
	drought	31,9±2,0 b	75,3±7,7 b	112,3±13,6 a

Mean of five replicates ± standard deviation. The values within one parameter not sharing the same letters are significantly different ($P \leq 0.05$).

Table 2: Effect of drought stress on photosynthetic parameters of common bean cultivars subjected to drought stress.

	Sample	Control	Drought
A_N (µmol CO_2 $m^{-2}s^{-1}$)	Starozagorski čern	10,49±1,40 a	0,71±0,10 b
	Tiber	9,20±0,90 a	0,64±0,14 b
g_s (mmol H_2O $m^{-2}s^{-1}$)	Starozagorski čern	0,27±0,06 a	0,00±0,00 b
	Tiber	0,23±0,07 a	0,01±0,01 b
C_i (µmol CO_2 mol^{-1})	Starozagorski čern	301,41±7,90 a	305,39±85,51 a
	Tiber	300,93±14,38 a	420,71±46,41 b
Fv'/Fm'	Starozagorski čern	0,56±0,02 a	0,46±0,03 b
	Tiber	0,55±0,02 a	0,47±0,01 b
qP	Starozagorski čern	0,45±0,04 a	0,16±0,06 b
	Tiber	0,41±0,05 a	0,12±0,02 b
qN	Starozagorski čern	2,29±0,13 a	1,87±0,09 b
	Tiber	2,22±0,10 a	1,90±0,05 b
ETR	Starozagorski čern	66,41±7,92 a	20,19±8,51 b
	Tiber	59,21±6,87 a	14,40±2,70 b
E (mmol H_2O $m^{-2}s^{-1}$)	Starozagorski čern	2,32±0,44 a	0,02±0,01 b
	Tiber	1,93±0,43 a	0,06±0,01 b

Presented parameters: net photosynthetic rate (A_N), stomatal conductivity (g_s), intercellular CO_2 concentration (C_i), maximal PSII light energy transformation efficiency (Fv'/Fm'), photochemical quenching coefficient (qP), non-photochemical quenching coefficient (qN), apparent photosynthetic electron transport rate (ETR) transpiration rate (E). Values are the mean of five replicates ± standard deviation. The values within one parameter not sharing the same letters are significantly different ($P \leq 0.05$).

In addition, there was a decrease in stomatal conductivity (g_s) and transpiration rate (E) in Starozagorski and Tiber after drought stress. A decrease in g_s is in agreement with previous results where a reduction of g_s after drought stress was observed in dry bean (Mathobo *et al.*, 2017). However, the intercellular CO_2 concentration (C_i) changed only slightly in both cultivars.

Some chlorophyll fluorescence parameters, such as Fv'/Fm', qP, qN and ETR were also negatively affected by drought stress, but there was no difference in reduction of those parameters between cultivars after drought stress. Fv'/Fm' is widely considered to be a sensitive indication of plant photosynthetic performance and drought stress resulted in a decrease in this parameter in our study by 18% and 15% in Starozagorski and Tiber, respectively. In our study, qP in Starozagorski significantly decreased after drought stress by 65% and in Tiber by 70%. Values of qN significantly decreased by 18% and 14% in Starozagorski and Tiber, respectively. The decrease in qN indicated that the thermal dissipation capacity of PSII was damaged. The electron transport rate (ETR) which mainly reflects the circumstances of actual electron transport in the PS II reaction center under light adaptation conditions (Zhang *et al.*, 2017), was decreased by 70% and 77% in Starozagorski and Tiber, respectively.

The results in this study were in agreement with the conclusions from previous studies (Mathobo *et al.*, 2017; Li *et al.*, 2015; Zhang *et al.*, 2012). To sum up, results of present study suggest that the photosynthetic function of both cultivars was similarly damaged, since no statistical significant difference of the measured photosynthetic parameters between the cultivars was observed.

Isolation of intact chloroplasts and its proteins

Chloroplasts were isolated from leaves of control and drought-stressed plants applying a common method involving a centrifugation on Percoll gradient (Salvi *et al.*, 2008). Here, the third trifoliate leaves were selected and minimal amounts of leaf tissue were used for the isolation; the amount of leaf material was between 2 and 4 g. Isolated chloroplasts formed a typical, narrow band within the Percoll gradient. Based on visual estimation, the yield of chloroplasts isolated from control plants was as least five times higher compared to drought-stressed samples (Fig 1).

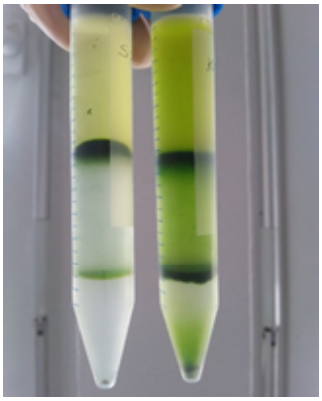

Fig 1: Percoll gradient purification of plant chloroplasts.

Intactness of the chloroplasts isolated by Percoll gradient was visualised by microscopy (Fig 2). After centrifugation in Percoll gradient, the 40/80 % interface (Fig 2A, 2B) contained mainly intact chloroplasts and only a few broken chloroplasts. Intact chloroplasts were considered those with pale yellow-green color and refractive, with a bright halo appearance around each plastid, whereas broken chloroplasts (Fig 2C, 2D) in the upper phase of Percoll gradient were those with a less pronounced halo and non-refractive appearance (Vieira *et al*., 2014).

The yield of isolated chloroplasts was expressed as mg of chlorophyll. Average chlorophyll content of intact chloroplasts for control samples was 2,63±0,84 mg/ml and 2,61±0,85 mg/ml for Starozagorski and Tiber, respectively.

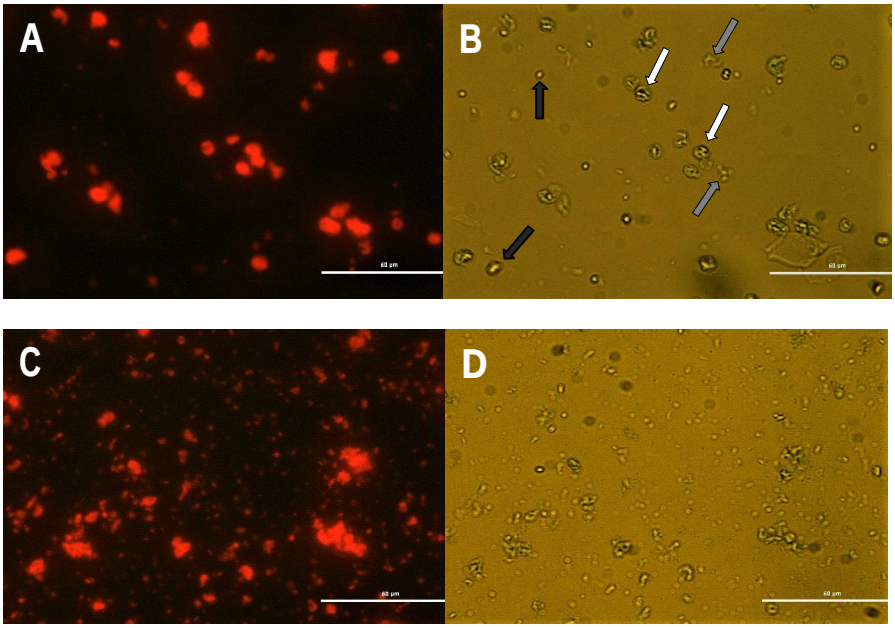

Fig 2: Phase contrast microscopy of chloroplasts of common bean leaves.
A, B: Intact chloroplasts in the 40/80 % interface after Percoll gradient centrifugation;
C, D: Broken chloroplasts in upper 40 % phase after Percoll gradient centrifugation. White arrows point to intact chloroplasts, grey arrows point to broken chloroplasts and black ones to non-chloroplast bodies. Scale bars: 60 µm.

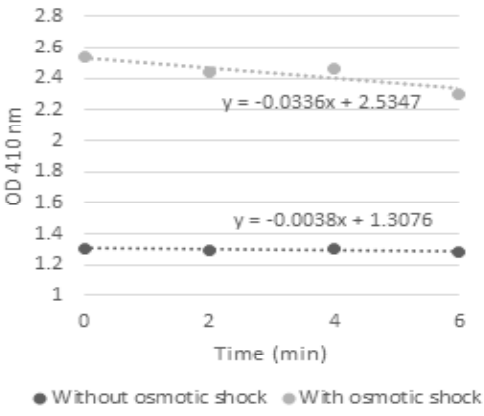

Fig 3: The degree of integrity of prepared chloroplast.

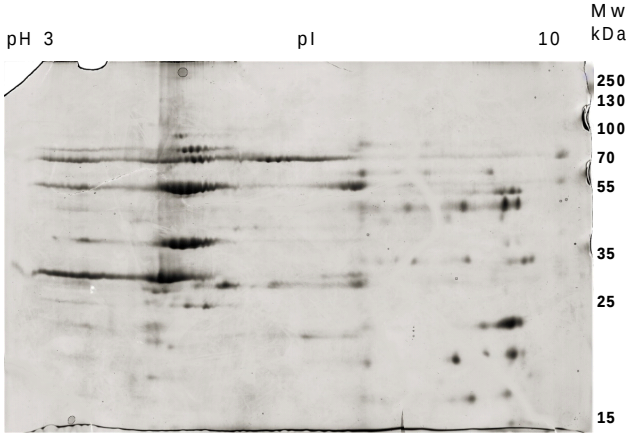

Fig 4: 2D electrophoresis gel of chloroplast proteins from common bean leaves.

In drought-stressed samples of Starozagorski the average chlorophyll content was 0,32±0,08 mg/ml, while in Tiber it reached 0,46±0,18 mg/ml. The yield of isolated chloroplasts was in control samples at least three times higher compared to drought-stressed samples in both cultivars and was similar between the cultivars. There was no significant difference in the average chlorophyll content in drought-stressed plants between the two cultivars after 6 days of drought.

In the present study, the results of intactness of the isolated chloroplasts confirmed by ferricyanide photoreduction assay were around 88% (Fig. 3), which is in agreement with the study of Aronsson and Jarvis (2002) where the chloroplasts from the lower band after Percoll gradient centrifugation were estimated to be above 85 % intact by phase-contrast microscopy.

The isolated chloroplasts were used for protein isolation and proteins were preliminary separated by 2D-PAGE (Fig 4). These results altogether suggest that the isolated fractions of chloroplasts, despite minimal amounts of starting material used, were enriched in chloroplast proteins and highly suitable for further proteomic analysis.

CONCLUSION

In drought stressed conditions, chloroplasts in the plant cells became damaged, which strongly affected the isolation of intact chloroplasts even in mild drought stressed plants as shown in our study. Mild stressed conditions were indicated by results of RWC and also by the negative effects of the photosynthetic rate and chlorophyll fluorescence parameters. In the present study, Percoll gradient centrifugation enabled to obtain pure intact chloroplasts and the intactness was checked by ferricyanide photoreduction assay and microscopy. The yield of isolated intact chloroplasts was determined by chlorophyll quantification and was in control samples at least three times higher compared to drought-stressed samples in both cultivars due to negative effects of drought on chloroplasts and its isolation. The results of the average chlorophyll content in drought-stressed plants between the two cultivars after drought stress showed no significant difference. Nevertheless, these results suggest that the isolated intact chloroplast proteins are suitable for downstream proteomic analysis.

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