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RESEARCH ARTICLE

Biochemical and Physiological Analysis of Flag Leaf Senescence in Field-Grown Barley (*Hordeum vulgare*)

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ABSTRACT: Barley is an important feed grain in many areas such as Canada, Europe and in the U.S. It has also so important for animal feed and has several other usages such as alcohol industry, malt productive and biomass-fuels. The characteristics of physiological and biochemical events during leaf senescence either flag leaf or other leaves have been investigated in field-grown barley (*Hordeum vulgare* cv. *Hordea*). The traits included grain yield, chlorophyll and protein levels, CO_2 assimilation rate, fluorescence, α-tocopherol and carotenoid levels, cellular oxidative level and chloroplast-encoded gene expression. The result showed significant role of flag leaf in grain yield. The chlorophyll and protein levels decline dramatically during senescence particularly in other levels. This result was quite similar for net CO_2 assimilation rate and maximal efficiency of PSII photochemistry (Fv/Fm). This suggests that PSII apparatus remains functional in senescent flag leaf. Two main lipid-soluble antioxidant agents (α-tocopherol and carotenoid) showed significant increase particularly in flag leaf at late senescence stage around 25 days after anthesis, the level of lipid and other macromolecular-derived peroxidation has been measured by TBARM assay. The TBARM levels increased gradually during flag leaf and particularly other leaves senescence. The transcript levels of the chloroplast-encoded psbA decrease during senescence especially in other leaves. However, the transcript level was significantly higher in flag leaf leading to high level of photosynthesis capacity at the molecular level.

KEYWORDS: photosynthesis, senescence, flag leaf, fluorescence, TBARM

INTRODUCTION

Barley was the first domesticated grain in the near east [3]. It is a widely adaptable crop and more tolerant of soil salinity than wheat. It has a short growing season and is also relatively drought tolerant [6, 26]. Barley has so important for animal feed and has several other usages such as alcohol industry, malt productive and biomass-fuels. Barley grain contains about 10% protein with eight essential amino acids [30]. Over a recent span of 25 years, barley cultivated area increased by over 80%, illustrating barley's increasing economic importance [26]. In cereal crops, the most-leaf i.e., flag leaf is the primary source of carbohydrates production [15]. The physiological traits of flag such as chlorophyll content and photosynthesis parameters have been considered to be the important determinants of grain yield in cereals [1].

Senescence in plants is a complex, highly genetic regulated process that involves a decline in photosynthesis, degradation of macromolecules and mobilization of nutrients to developing parts of the plant e.g. seeds [16, 21]. In last few years, the study of leaf senescence resulted in the identification of a number of senescence-enhanced genes [20, 22]. Characterization of

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these has helped to elucidate some of the processes that occurring. The most remarkable event in leaf senescence is the disassembly of the photosynthetic apparatus within chloroplasts and thus the concomitant decrease in photosynthetic activity [2]. Many studies have shown that decrease in photosynthetic activity is associated with the decline in dark reaction of the Calvin cycle [27], which is mainly due to the degradation of Rubisco [25]. On the other hand, it has been shown that the decrease in photosynthetic activity in senescent leaves may be associated with the decrease in photochemical activity of PSI and PSII as well [25]. It has been reported that PSII is more susceptible to senescence than PSI and a greater decrease in PSII activity is observed in senescent leaves [17]. The decreased PSII electron transport may be due to inactivation of the oxygen evolution system [18] and PSII reaction center complex [12] as well as the inhibition of energy transfer from carotenoids to chlorophyll [8].

Chlorophyll a (Chla) fluorescence quenching analysis has been shown to be a non-invasive, powerful and reliable method to assess the changes in the function of PSII under different environmental condition [8, 28]. It is of interest to use this method to assess PSII photochemistry during leaf senescence since detailed information on the fundamental processes of energy absorption, is not quite clear. It is also important to find out the expression pattern for some photosynthetic genes during the leaf flag senescence.

MATERIALS AND METHODS

Plant material

Winter barley (*Hordeum vulgare* L. cv. *Hordea*) was grown in field condition. Each plot contains five rows with 4m length. Three middle rows have used for trait measurement and sampling to avoid border effect. Detached flag leaf plants were used as negative control for grain yield analysis. Three biological replications were carried out for statistical analysis. The statistical analysis has performed by using Statistical Analysis System (SAS), in order to increase accuracy, standard error has been calculated (n=3) for traits mean. Water and nutrient fertilizer were routinely supplied as well as pest and weed control throughout the growing season. Flag leaves (each sample include 5 random flag leaf plants) were used for all analysis, as well as three samples of other green leaves at anthesis stage (five random green leaves for each sample). Flag leaves samples were collected every five days starting at anthesis stage and ending at final maturity stage. All samples were collected in the early morning to avoid photo inhibition effects.

Chlorophyll Determination

Chlorophyll determinations were made on leaf samples based on the method of Porra et al., (1989) [24]. Leaf samples (0.3 g) were extracted in 10 mL of 80% (v/v) acetone. After centrifugation, absorbance (A) was read at 646.6, 663.6 and 750 nm on a Uvikon spectrophotometer (Kontron Instruments).

Protein Determination

Protein was extracted from barley leaves (0.5 g) in 1.5 mL of extraction buffer (50 mm Tris [tris(hydroxymethyl) aminomethane]-HCL, pH 7.5, 2 mm EDTA, pH 8, 0.04% [v/v] mercaptoethanol). Samples were centrifuged at 12,000g for 20 min at 4°C, and proteins determined using a Coomassie protein assay reagent (Pierce Chemical, Rockford, IL) based on a modified Bradford method [4] with bovine serum albumin as a standard.

CO_2 assimilation rate

An infrared gas analyzer (Ciras System, UK) was used to estimate net CO2 assimilation rates. Measurements were made on attached flag leaves in the field. Flow rate was 200mL min-1 and external humidity 65-75% was used. The temperature inside leaf chamber was maintained at 24±1oC. To obtain standard light-saturation curves a halogen lamp attached on the leaf chamber window. A range of light intensities between 0 – 2000 µmol m-2 s-1 were given to achieve steady-state photosynthesis at each light intensity.

Fluorescence determination

Fluorescence parameters were measured at room temperature (about 26 ºC) with a portable flourometer (PAM – 2000, Walz, Germany) after the leaves were dark-adopted for 30 min. The data has transferred to a computer with acquisition software (OA – 2000 Walz) and following parameters were estimated. The Minimal fluorescence level (Fo) with all PSII reaction centers open was determined by measuring modulated light, which was (< 0.1 µmol m-2 s-1) not to induce any significant variable fluorescence. The maximal fluorescence level (Fm) with all PSII reaction center closed was determined by a 0.8 s saturating pulse at 8000 µmol m-2 s-1 on dark-adopted leaves. Using these data, the maximal efficiency of PSII photochemistry in dark-adopted state (Fv/Fm) was calculated [13].

RNA isolation

Plant material (5 g) was ground to a fine powder in liquid N2 using a prechilled mortar and pestle. The frozen powder was transferred to a 50 ml polypropylene tube containing 10 ml phenol and 15 ml extraction buffer (100 mM Tris-HCl pH 9, 200 mM NaCl, 5 mM dithiothreitol, 1% (w/v) sarcosyl, 20 mM EDTA) and mixed gently. The mixture was centrifuged at 3,000 rpm for 10 min. The upper aqueous layer was extracted two or three times with an equal volume of chloroform, until it became clear. The solution was transferred into a 30 ml Corex tube and adjusted to 2 M LiCl by addition of 1/3 volume of 8 M LiCl and left overnight at 4ºC. Precipitated RNA was pelleted by centrifugation at 10,000 rpm, 4ºC, for 10 min. The pellet was washed twice with 1.5 ml of 2 M LiCl and recentrifuged each time. The pellet was dissolved in 1.5 ml water. A 10 µl aliquot was diluted with 990 µl water, and used to evaluate RNA concentration and purity by spectrophotometry. The remainder of the solution was mixed with 1/10 volume 3 M sodium acetate pH 5.2 and 2.5 volumes 100% ethanol in a Corex tube, and left at -70ºC for 30 min. Precipitated RNA was pelleted by centrifugation and washed in 70% ethanol, and after air drying, dissolved in sterile distilled water at a concentration of 2 µg/µl.

α-Tocopherol content

(α-TOC) was extracted as described by Munné-Bosch et al. (1999) [19]. Leaf segments (0.5g) were ground in liquid N2 using a pestle and mortar with ice-cold 5 ml of methanol containing 1% ASC. The macerate was homogenized and α-TOC was extracted by vigorous mixing for 1 min with 4 ml of hexane. After the samples were centrifuged at 1,500×g for 10 min, the upper hexane layer was carefully removed and evaporated to dryness under N2. Samples were dissolved in 2 ml of methanol and injected into a HPLC apparatus in 100 µl portions. α-TOC was separated at room temperature using an Ultra sphere ODS 5 µm column (250×4.6 mm) with 100% methanol as an eluant a flow rate of 1.5 ml min-1. UV detection was carried out at 295 nm and fluorescence detection was carried out at an excitation wavelength of 298 nm and an emission at 328 nm. Pure (±)-α-TOC was used as a standard.

Carotenoid content

Leaf samples were taken and immediately frozen in liquid nitrogen. Samples were extracted in ice-cold 100% acetone and pigment extracts were filtered through a 0.45 µM membrane filter. Carotenoid were separated and quantified by HPLC as described by Thayer and Bjorkman (1990) [32].

Northern hybridization analysis

RNA was transferred onto a Hybond N+ (Amersham) nylon membrane with 0.05 M NaOH as the transfer solution. Transfer occurred overnight and then the filter was removed and washed in 2×SSC. The probe psbA was denatured in a boiling water bath for 4 min, it was then added to hybridization buffer and mixed thoroughly. The filter was replaced in the box and incubated on a shaker at 65ºC overnight. The hybridization solution was removed and the filter rinsed in 2×SSC/ 1% SDS, and in some cases in 0.2×SSC/ 1% SDS, and washed twice for 20 min at 65ºC. The filter was dried briefly on filter paper, wrapped in Saran wrap and relative gene transcript quantified by phosphor-image analyzer (Norton KX11 UK).

Measurement of thiobarbituric acid reactive material (TBARM)

TBARM (of which malondialdehydes (MDAs) are considered to be a significant component) was measured using an assay modified from Hagege et al., (1990) [10]. Plant material (0.5 g) was homogenized with 1 ml trichloroacetic acid (10% w/v). The homogenate was washed with 10 ml acetone, vortexed then centrifuged at 4,750 rpm for 15 min. The pellet was washed in 5 ml acetone, vortexed and then centrifuged at 4,750 rpm for 10 min (4 times). The pellet was dried under nitrogen and incubated at 100oC for 30 min with 3 ml H3PO4 (1%) and 1 ml thiobarbituric acid (0.6%). The reaction was terminated by rapidly cooling the tubes on ice. Butan-1-ol (3 ml) was added and the mixture vortexed then centrifuged at 5,500 rpm for 20 min to achieve separation of the phases. Absorbance of the aqueous phase was measured at 532 nm and 590 nm using a Uvikon 930 Spectrophotometer (Kontron Instruments, Watford, UK).

RESULTS

Grain Yield

The amount of grain yield showed considerable decrease by detaching flag leaves (Fig.1). It was somehow expected and has been reported in other studied [11, 14]. So, it was out of interest to find out more molecular and physiological details in flag leaves, as follow.

Chlorophyll and Protein

Chlorophyll and protein content for flag leaves were greater than their amount in other leaves, at all periodic samples. Chlorophyll content of flag leaves did not change considerably until 23 days after anthesis whereas for other leaves this was declined about 16 days after anthesis. There was not relatively much difference between Chl content in flag leaves and other leaves at anthesis day, but this difference became greater during leaf senescence (Fig. 2a). In contrast, for protein this difference was almost same at all periodic sample times (Fig. 2b).

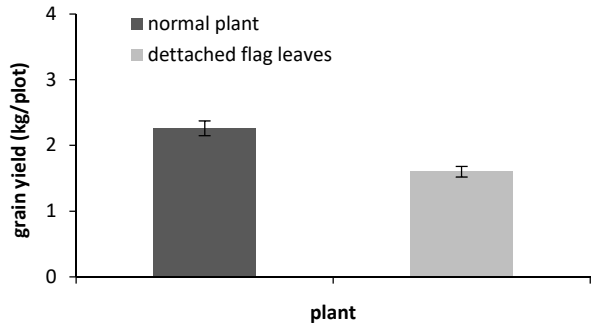

Figure 1. The amount of grain yield on basis of plot means ±SE (n = 3) in field-grown barley plants in the condition of attached and detached flag leaves.

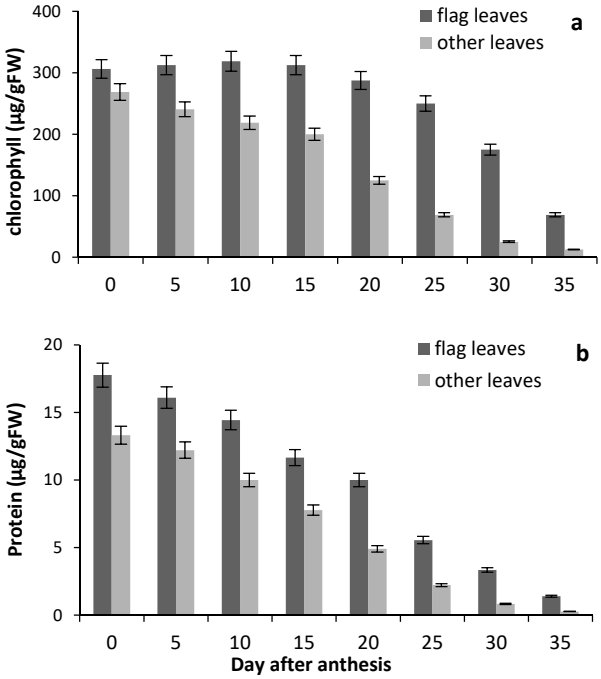

Figure 2. Changes in Chlorophyll (a) and protein (b) during leaf senescence of field-grown barley plants. Data are means ±SE of 3 independent measurements.

Net CO_2 assimilation and PSII efficiency

The amount of net CO2 assimilation rate and particularly Fv/Fm ratio for flag leaves were quite similar to their amount for other leaves at anthesis date. The difference for those amount became greater during leaf senescence (Fig.3a, b). The net assimilation rate remained almost at high until around the 20 days after anthesis in flag leaves. Whereas, this was around the 15 days after anthesis in other leaves (Fig.3a). The maximal efficiency of PSII photochemistry (Fv/Fm) in flag leaves remained constantly at high level up to 25 days after anthesis. While, this was around the 18 days after anthesis in other leaves (Fig.3b).

α-Tocopherol and Carotenoid content

During leaf senescence the amount of both α-Tocopherol and Carotenoid smoothly increased in both flag leaves and other leaves up to around 30 days after anthesis (Fig. 4a, b).

There was quite similar illustration pattern for both α-Tocopherol and Carotenoid in flag leaves and other leaves

Measurement oxidative levels (TBARM) and photosynthesis gene (psbA) activity

Measurement of thiobarbituric acid reactive material (TBARM) showed dramatic increased during leaf senescence (Fig.5a). The levels of TBARM were nearly equal for both flag leaves and other leaves until 10 days after anthesis. After 10 days the TBARM levels of other leaves were significantly greater than their amount of flag leaves (Fig.5a). Indicating during senescence stage the potential oxidative damage in flag leaves is lower than other leaves.

The transcript levels of psbA gene (DI polypeptide of photosystem II) declined dramatically during leaf senescence. At all time period after anthesis, the relative activity of the gene in flag leaves was greater than its activity in other leaves (Fig. 5b).

DISCUSSION

The results of the present study showed significant decline in the grain yield of detached flag leaf barley plants (Fig.1), indicating the important role of flag leaf in assimilation and gain in grain yield. This is supported by several research studied [14, 29, 11]. In order to find out more detail in the matter, some physiological and

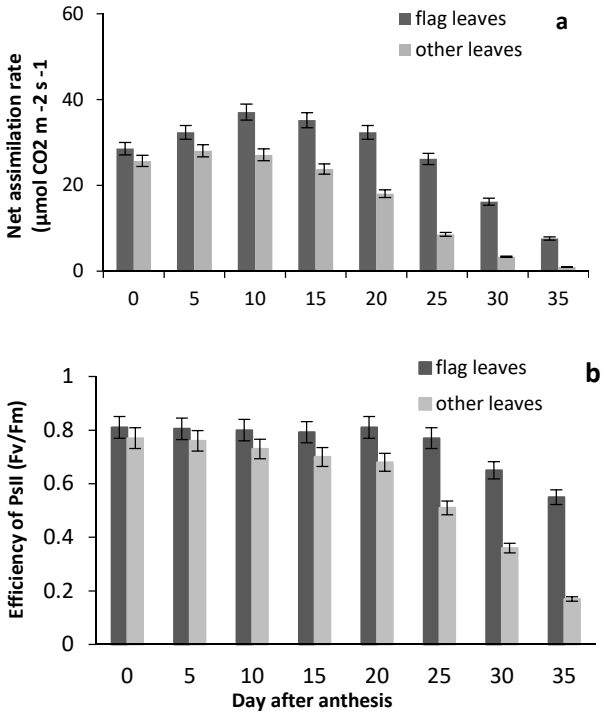

Figure 3. Changes in net CO_2 assimilation (a) and maximal efficiency of PSII (b) photochemistry on a basis of leaf area during leaf senescence of field-grown barley plants. Data are means ±SE of 3 independent measurements.

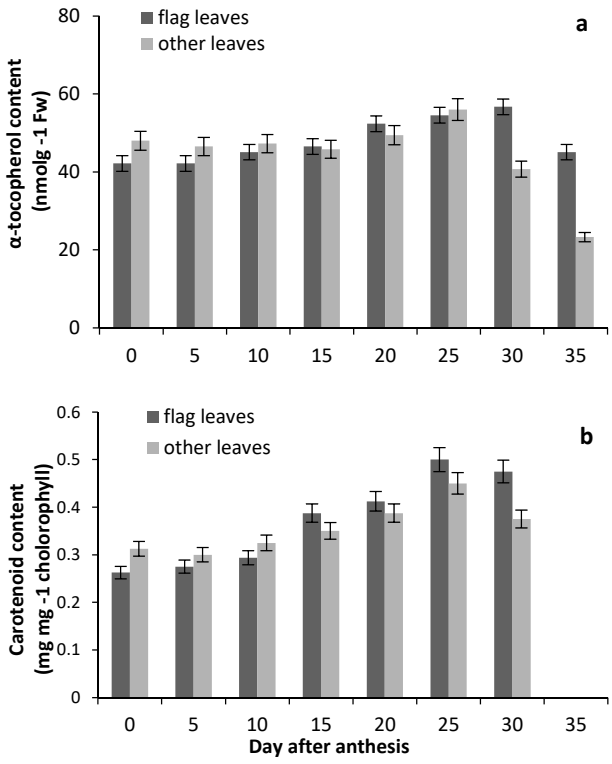

Figure 4. Changes in α-Tocopherol (a) and Carotenoid (b) in field-grown barley plants leaves. Data are means ±SE of 3 independent measurements.

biochemical measurements have been checked out. The results of chlorophyll and protein determination showed a substantial decrease in content of these traits during leaf senescence (Fig.2). However, in all periodic sample times the amount of protein and particularly chlorophyll of flag leaf was greater than other leaves. This could be a possible good reason to justify the efficiency of flag leaf in photosynthetic gain and resulting more grain yield, in attached flag leaves barley plants (Hollman et al., 2014). There was a significant positive correlation (r = 0.76) between chlorophyll content and photosynthetic capacity, indicating high chlorophyll content in leaves particularly in flag leaf is considered as a favorable trait in crop production. Similar result has been reported by other research groups [11,31].

The results of the present study showed a substantial decrease in the maximal efficiency of PSII photochemistry (Fv/Fm) during senescence of other leaves (Fig.3b). Whereas, the Fv/Fm ratio showed little change during flag leaf senescence (Fig.3b). This suggests that PSII apparatus remains functional in senescence flag leaf and that there is a continued efficient use of light captured by the remaining PSII apparatus. The fact that net CO_2 assimilation rate in flag leaves showed greater amount than its amount in other leaves could be because of relatively constant amount of Fv/Fm ratio in flag leaves (Fig. 3a, b).

The relative constant PSII function observed in senescent flag leaves of field-grown barley plants in this study are contrary to the results that PSII activity, measured it in isolated chloroplasts or thylakoids, decreased markedly in senescent leaves [9, 11]. The constant PSII function observed in senescent flag leaves of barley may suggest that it measurement of PSII activity using isolated chloroplasts or thylakoid membranes may not always reflect PSII photochemical activity in vivo. In addition, it should be mentioned that in barley plants grown in the field, senescence occurred under natural conditions. Thus, the discrepancy between result of present study and other reports may be due to differences in growth condition or in the methods for inducing senescence e.g. natural senescence versus dark-induced senescence [23].

It should be pointed out that the relatively low levels of net CO_2 assimilation rate in other senescent leaves might be associated with the decline in dark reactions of the Calvin cycle [9]. Thus, the decrease in Fv/Fm ratio in other leaves during senescence also may be due to the reduction in the Calvin cycle metabolism activity.

Two main lipid-soluble antioxidant agents including α-Tocopherol and carotenoid did not show significant changes during senescence in both flag leaf and other leaves (Fig.4a, b). However, both antioxidants showed significant increase particularly in flag leaves at late senescence stage (around 25 days after anthesis). It could be a possible reason for more efficient photosynthesis capacity in flag leaves; this has been supported by [11, 33].

Senescence defined as highly genetic regulated of oxidative process. The role of reactive oxygen species (ROS) during senescence showed that high concentrations of ROS can lead to phytotoxicity whereas relatively low levels can be used as signaling molecules in cellular metabolic events such as gene expression and acclimation [5, 20]. Several methods are available for measuring ROS in plants but the technical difficulty associated with quantifying endogenous levels of these very reactive and short-lived species means that over the last year's most of the evidence for measuring ROS has been provided through the study of secondary processes such as lipid peroxidation [7].

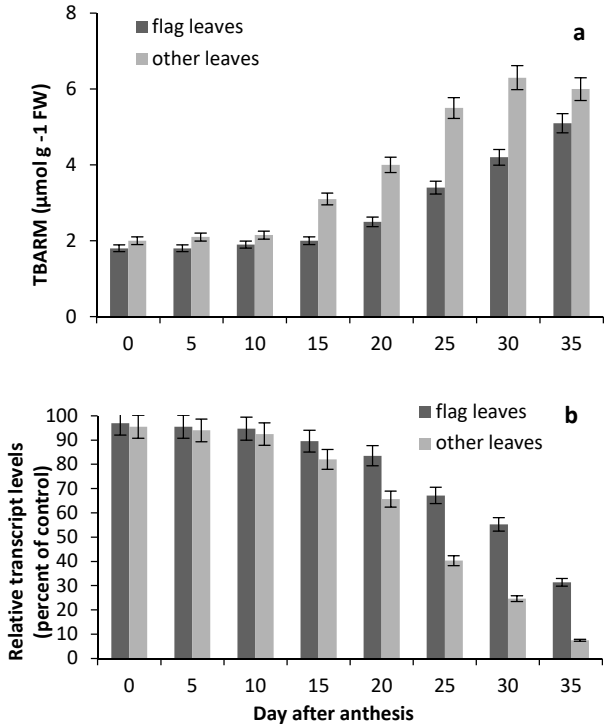

Figure 5. Cellular oxidative levels (TBARM) (a) and photosynthesis gene transcript psbA (b) in field-grown barley leaves during senescence. Data are means ±SE of 3 independent measurements.

Measurement of thiobarbituric acid reactive material (TBARM is a relatively stable end product of lipid and other macromolecular-derived peroxidation reactions).

In this study the amount of TBARM increased gradually during senescence particularly in other leaves (Fig.5a). It seems flag leaves coped with oxidative process more efficient than other leaves. The transcript levels of the chloroplast-encoded psbA decreased during senescence especially in other leaves (Fig.5b). The result showed the expression of this gene in flag leaf was significantly higher than its level in other leaves during senescence. This could be a good reason in favor of flag leaf leading to high level of photosynthetic capacity at the molecular level [8].

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تجزیه و تحلیل بیوشیمیایی و فیزیولوژیکی پیری برگ پرچم در جو زراعی در شرایط مزرعه

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چکیده

ارزیابی فیزیولوزیک و بیوشیمیایی فعل و انفعالات در خلال پیری برگ در برگ پرچم و سایر برگ ها در جو زراعی در شرایط مزرعه مورد بررسی قرار گرفت. صفات مورد بررسی شامل عملکرد دانه، مقدار کلروفیل و پروتئین، میزان تثبیت دی اکسید کربن، مقدار فلورسنت، مقدار آلفا توکوفرول و کاروتنوئید، شاخص اکیداسیون سلولی و مقدار بیان ژن وابسته به فعالیت کلروپلاست بود. نتایج نشان دهنده نقش ممتاز برگ پرچم در میزان عملکرد دانه بود. میزان کلروفیل و پروتئین برگ ها، بویژه برگ پرچم افت چشمگیری در خلال پیری نشان داد. روند کاملا مشابهی برای میزان تثبیت دی اکسید کربن و فلورسنت کلروفیل مشاهده شد، این مسئله مبین تداوم فعالیت کلروپلاست در برگ پرچم بود. دو عامل اصلی و محلول در چربی آنتی اکسیدانی آلفا توکوفرول و کاروتنوئید افزایش معنی داری در خلال پیری برگ پرچم تا مراحل نهایی (۲۵ روز پس از گرده افشانی) نشان داد. مقدار شاخص اکسیداسیون سلولی با ادامه روند پیری برگ پرچم افزایش تدریجی نشان داد. میزان بیان نسبی ژن فتوسنتزی پی اس بی ا کاهش قابل توجه طی پیری بویژه در سایر برگ‌ها نشان داد. تداوم بیان نسبی این ژن در خلال پیری برگ پرچم نتیجه درخور توجهی در پایداری فتوسنتز بود.

کلمات کلیدی: فتوسنتز، پیری، برگ پرچم، فلورسنس، TBARM