Available online at www.worldscientificnews.com

World Scientific News

WSN 83 (2017) 168-181 EISSN 2392-2192

Non-invasive technique of crop heat-stress resistance estimation

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ABSTRACT

Sessile organisms like plants have drastically reduce possibility to avoid harmful influence of the natural habitat. Evolution of first land plants allowed adapting to harsh conditions, yet despite broad range of tolerance factors such as excessive light and temperature impose limitation for biological activity because viability and in the case of crops the productivity is limited. In fact, physiologists make lots of efforts to increase stress tolerance searching and selecting new cultivars of known crops. As in most cases the exact mechanism of reached improvement is still missing selection is occupied by immense amount of time, energy and funds. Proposed in paper technique of stress resistance estimation is inexpensive and highly reproducible solution for crop selection even at early stage of development. It is based on well-known fluorescent assessment (PAM) of plant photosynthetic machinery expose to factor of interest. We tested two barley (*Hordeum vulgare* L.) cultivars spring Carina and winter Lomerit exposed to thermal stress and estimated maximal (Fv/Fm) and effective photosystem II quantum yield precisely specifying the way absorbed energy is utilized by photochemical (ΦPSII) or regulated (ΦNPQ) and non-regulated (ΦNO) non-photochemical manner. Studies have confirmed various heat-resistance of tested barley cultivars and proved PAM-technique utility.

***Keywords*:** barley, heat resistance, heat stress, modulated fluorescence (PAM), photosystem II (PSII)

1. INTRODUCTION

Oxygenic photosynthesis is the most important biochemical process on Earth and there is no exaggeration to say that its appearance had changed the course of life on our planet. Atmosphere composition and its oxidative character have initiated evolution of almost all known life forms existing allowing to more completely utilization of captured sun energy accumulated in biomass of photosynthetic organisms. Photosynthesis has absolutely fundamental role for organic compounds production and function as the very first link of food chain from microscopic algal cells to giant redwoods, as well as in the case of crop plants, essential process for mankind existence. At the same time, plants are sessile organisms constantly exposed to environmental factors such as heat stress, disturbing cells integrity, physiological activity and the structure of molecules finally reducing the photosynthetic efficiency. Disturbed sink/source balance of assimilates accelerates aging process of plant structures initiating the premature death (Skowron et al., 2016). In addition, according to expertises presented by climatologists global average temperatures are going to rise and this trend will be preserve till new, higher energy equilibrium even after introduction of greenhouse gases zero emission policy (IPCC, 2013). Hence, there is a real need to evaluate economic, rapid and reproducible methods for the selection of tolerant plant cultivars, crucial for food production, especially in perspective of the overpopulation threat.

We evaluated non-invasive measurement of the plant photosynthetic apparatus state, to identify cultivars best adapted to the rapidly changing climatic conditions based on well-known Pulse-Modulated Technique for measuring the fluorescence (PAM) of chlorophyll *a*. Estimation of photosynthetic efficiency provided by analysis of chlorophyll fluorescence within PSII is useful tool to assess plant response and tolerance (Willits and Peet, 2001; Gorbe and Calatayud, 2012). PAM estimation concerns light-dependent photosynthesis phase taking place in grana structure of plant chloroplast, where most of PSII reaction centres (RCs) are located. Photons of light captured by chlorophyll-protein complexes organized in antenna-like structures allow gathering energy from peripheral areas and transfer it to RCs.

Absorption of energy is realized by an extensive system of proteins named light harvesting complexes (LHCs) organized to be photoactive pigments complexed with proteins very efficiently funnelling energy to RCs. Depending on the physiological condition and PSII activity the energy is directed to the three major pathways and utilized photochemically or disperse in controlled (NPQ) or passive way (NO) (Schreiber et al., 1986). Yet, only in the case of photochemical quenching, energy is absorbed by active pigments allowing plant to produce assimilates while non-photochemical, regardless of form it takes, may be concerned as kind of energy waste. Energy may be disperse with several ways as most of solar radiation reaching plants is useless to photosynthesis resulted from inadequate intensity of incidental light or non-adapted photosynthetic machinery. What is more, the spectrum of solar radiation contains wavelengths absorbed by water, anthocyanins and other non-active dyes or simply reflected by protective layers, epidermal waxes and scattered by tomentose. Energy loss is also managed by fluorescence emitted with chlorophyll molecules untenable to transfer absorbed energy, observed after dark adaptation or overreduced and disintegrated plants photomachinery.

Optimal condition reduces the amount of energy emitted in that way and fluorescence mostly is not exceed 2% of the total radiation absorbed by plants (Maxwell and Johnson,

2000). Yet, fluorescence phenomenon is successfully used to analyse photosynthetic efficiency. Another process of energy transfer is related to the thermal dissipation based on the xanthophyll-associated cycle, protecting PSII against damage caused by excessive light radiation or imbalanced absorption and utilization of energy (Holt et al., 2005). Prolonged exposure to stress factors, acting mostly synergistically (such thermal and radiation stress), results in state of inactivated or reduced electron transport between photosystems named photoinhibition. At the same time, plants are not able to uncouple energy absorption with LHCs exacerbating disturbed equilibrium. Uncontrolled state of chlorophyll excitation generates reactive oxygen species (ROS) mainly due to electron leak to oxygen evolves from water. Hence, the mechanism of controlled LHCs-RCs separation as well as conversion of violaxanthin (V) to zeaxanthin (Z) are crucial to reduce the risk of oxygen stress.

Influence of increased ROS concentration is alleviated with antioxidants both enzymatic and non-enzymatic, which allow restring cell redox homeostasis. Unfortunately, the main compound used for oxygen protection – the ascorbic acid (AsA) is also essential for xanthophyll cycle activity as rate-limiting enzyme violaxanthin de-epoxidase (VDE) requires AsA as co-substrate to initiate xanthophyll conversion. Decreased VDE activity leads to inefficient dissipation of excess light energy, destabilization of PSII structures and once again ROS generation (Fernández-Marín et al., 2009; Trojak and Skowron, 2017).

According to Hakala et al. (2005) photoinhibition is defined as a light-induced damaged of PSII, mainly the D1 protein of the core part, which is repaired via *de novo* synthesis as quick as possible. Yet, the mechanism of D1 replacement requires ATP-energy, which as PSII is deconstructed, is produced mainly by photosystem I (PSI) cyclic electron transport (CET). Previous studies of Marutani et al. (2012) proved that PSI is less sensitive to high temperature than the PSII, fully inactivated by prolonged thermal stress.

The mechanism of CEF-dependent ATP production is based on electron transfer and requires light to sustain proton movement across thylakoid membrane (Zhang et al., 2009). High temperatures negatively affect photosynthetic CO_2 fixation because reduced stomata openness decreases CO_2 availability for Rubisco. At the same time light-dependent water decomposition produces O_2 competing for enzyme active site. When photorespiration is active CO_2 fixation requires more ATP because of Rubisco activase resulted in whole cell higher energy demands (Rumeau et al., 2007).

Two pathways of CET around PSI are active. The main, mediated by the PGR5/PGRL1 complex, interacting with PSI and minor one depends on chloroplast NDH complex (NADH dehydrogenase-like) forming the supercomplex with PSI. In addition another type of electron transport, named pseudo-cyclic, also alleviates overeduction with alternative electron sink sustaining transport. It is realized with water-water cycle (WWC) producing superoxide radical immediately scavenged by superoxide dismutase (SOD) and ascorbate peroxidase (APX). The second is related to flavodiiron protein (Flv) and mediates direct reduction of O_2 to H_2O (Shikanai and Yamamoto, 2017).

In this paper we measured PSII quantum efficiency in barley cultivars exposed to stress and estimated the physiological condition of the plant which is exceptionally useful for the assessment and further selection of new heat-stress resistance cultivars. The aim of study was to estimate the suitability of PAM as a quick and reliable tool for selection high temperature tolerance barley cultivars.

2. METHODOLOGY

2. 1. Plant material and treatment

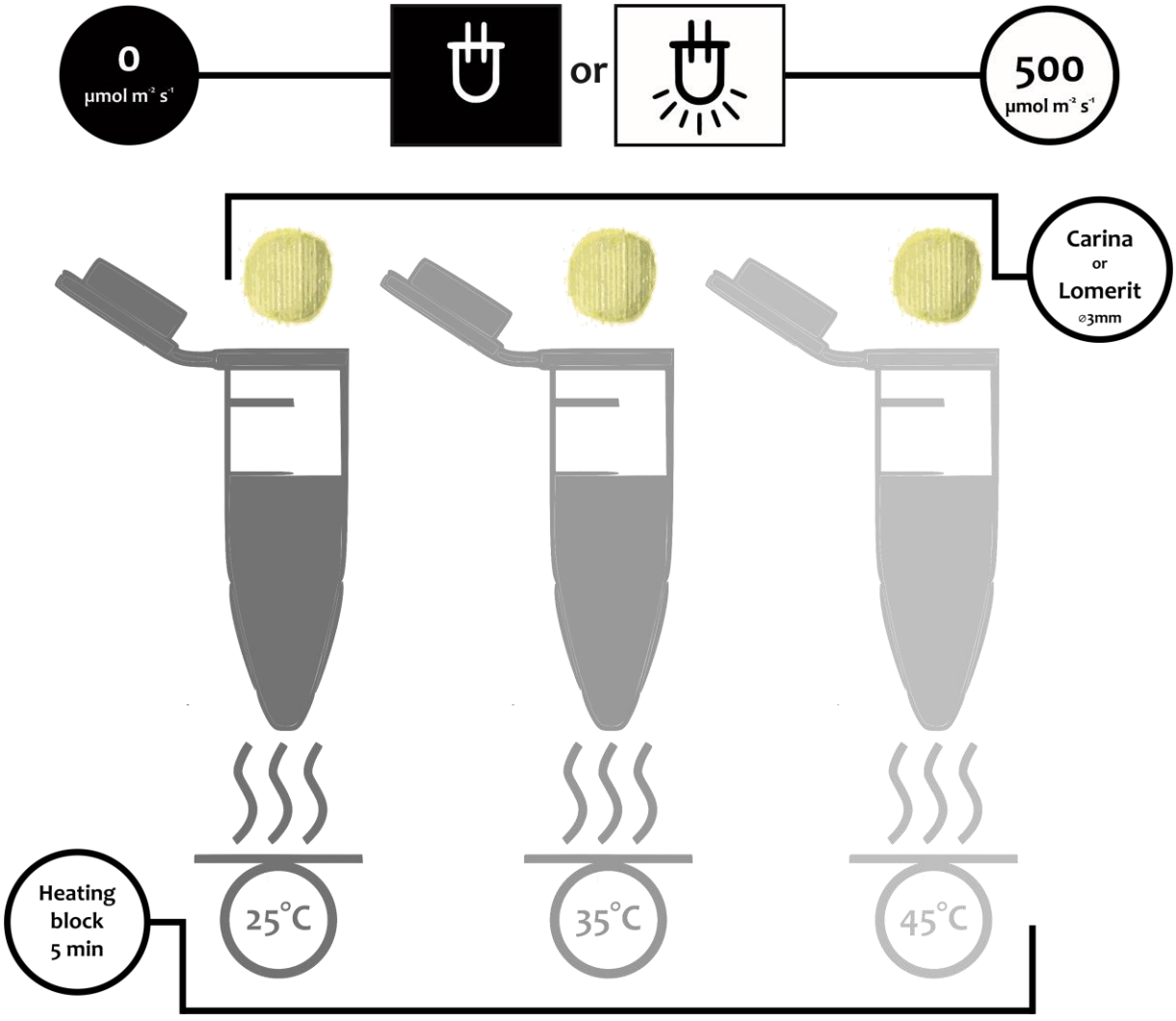

Figure 1. Scheme of heat stress induction with (500 µmol m $^{-2}$ s $^{-1}$) or without (0 µmol m $^{-2}$ s $^{-1}$) light exposition. Leaf discs of barley Carina or Lomerit cultivars cut out from second true leaf were incubated with heating block for 5 min suspended in distilled water at 25, 35 or 45 ºC (n = 12).

We selected two barley (*Hordeum vulgare* L.) cultivars: spring Carina and winter Lomerit based on previous analyses of their varied senescence mechanism and drought resistance (Krupinska et al., 2012; Skowron et al., 2016). Barley considers to be cereal model plant successfully used for our previous research on the xanthophyll cycle activity (Trojak et al., 2016a).

Barley seeds, sterilized with antifungal powder (T 75 DS/WS) and germinated on wet paper for three days in darkness, were sown in P9 containers filled with universal substrate. Plants were grown in a growth chamber at average light intensity of 130 µmol m^{-2} s^{-1} (Px256 PxCrop RGB LED, 671:524:438 nm; PXM, Podłęże, Poland) and photoperiod of 16/8h per day. Relative humidity was 50-60%, with temperature varied in the range 22-23°C/18°C day/night. Four weeks plants were selected for measurements. 3 mm diameter discs were cut out from fully developed second true leaf and immediately transferred to Petri dishes filled with wet filter paper. For light adaptation leaf discs were left for next 25 min in the darkness or illuminated with 500 µmol m^{-2} s^{-1} (LED Light Source SL 3500-C, 627:530:447 nm; Photon Systems Instruments, Brno, Czech Republic) to distinguish between light-deprived and enriched mechanism of D1 protein turnover. Next discs were transferred to tubes and incubated for 5 min at increasing temperature (25, 35 and 45 °C) (Fig. 1) according to procedure used in previous research (Trojak and Skowron, 2017). Temperatures and maximal light intensity used for radiation were estimated with light-curve analyses (Trojak et al., 2016b).

2. 2. Pulse-Amplitude-Modulation (PAM)

All measurements were performed at RT (25 °C) with IMAGING-PAM (Heinz Walz, Effeltrich, Germany) fluorometer, determining parameters related to the condition (Fv/Fm) and the quantum yield of PSII, to characterized the partitioning of absorbed energy between photochemical utilization (ΦPSII) or regulated (ΦNPQ) and non-regulated dissipation (ΦNO) (tab. 1). The experiment was carried out on second true leaves obtained from plants during most photosynthetically active day period (between 8.30 and 11.00) using 450 nm actinic light (56 µmol m^{-2} s^{-1}), after at least 30 min of dark adaptation.

Table 1. Photosynthetic efficiency characterized by maximal Fv/Fm (dark adapted) or effective (light exposed) components of quantum yield of PSII

Parameter	Description	Formula
Fv/Fm	maximal PSII quantum yield	Fv/Fm=(Fm-Fo)/Fm
ΦPSII	effective PSII quantum yield	ΦPSII=(Fm'-F)/Fm'
ΦNPQ	quantum yield of regulated energy dissipation	ΦNPQ=1-ΦPSII-1/(NPQ+1+qL(Fm/Fo-1))
ΦNO	quantum yield of non-regulated energy dissipation	ΦNO=1/(NPQ+1+qL (Fm/Fo-1))

where: ΦPSII+ΦNPQ+ΦNO=1;
Fv - variable fluorescence; Fm - maximal fluorescence yield in dark adapted samples; Fo - dark fluorescence yield; Fm' - maximum of fluorescence yield in illuminated samples; F - fluorescence yield; NPQ - non-photochemical quenching; qL - coefficient of photochemical quenching;

2. 3. Statistical analysis

All statistical analyses were performed using STATISTICA 12.0 software (StatSoft Inc., Oklahoma, USA). Firstly the normally of the random variables distribution was verified with Shapiro-Wilk's test at the 0.05 significance level. Assessment of the equality of variances for variables were done with Levene's test and the one-way analysis of variance (ANOVA) was used to determine difference in the values of the evaluated parameters in all tested temperatures both at the 0.05 significance level. Finally post-hoc analyses with Tukey's multiple range test were done to test differences among more than two means at the 0.05 significance level.

3. RESULTS

Comparison of selected barley cultivars showed temperature-dependent differences in estimated fluorescence parameters. We have noted increase of the effective quantum yield for both cultivars at 25 to 35 °C shift. Yet, the essential discrepancy was related to light exposition as for Carina 10% ΦPSII activity increase was noted only at not irradiated (0 μmol m^{-2} s^{-1}; NL) samples (Fig. 2a), while Lomerit was characterized by 25% ΦPSII advance occurred in the irradiated samples (500 μmol m^{-2} s^{-1}; HL) (Fig. 2b).

Higher temperature, optimal for enzymatic activity erased differences in the effective PSII yield between cultivars noted previously at 25 °C. Yet, analyses of Fv/Fm at 35 °C after light exposition showed approximately 10% decrease of the maximal PSII yield only for Lomerit indicating its higher thermal sensitivity (Fig. 3). A bit confusing results were obtained for HL samples as we noted lower Fv/Fm values in both cultivars indicating light exposition resulted in the radiation stress.

Previous analyses with light curves (Trojak et al., 2016b) did not indicate any decrease at 35 °C. Compared to the control Fv/Fm at 35 °C was 3% (NL) and 4% (HL) lower for Carina and 7.5% (NL) and nearly 17% (HL) for Lomerit. We expected that light might alleviate heat stress, as D1 protein regeneration is ATP-dependent. Despite that, expedient influence of light pre-adaptation was noted for the xanthophyll cycle activity measured with ΦNPQ at 45 °C. In the case of NL samples excess energy was dissipated mostly with non-regulated way.

The energy loss directly related to fluorescence (ΦNO) in Carina showed no statistically significant changes in temperature range 25-35 °C while 45 °C triggered a significant increase in the ΦNO value. It was resulted from thermal stress inactivating PSII activity. After light exposition at 45 °C energy was dissipated mostly with non-photochemical regulated quenching managed by VDE. Nonetheless, we observed approximately 60% decrease in ΦPSII value in both cultivars for NL and HL samples, indicating that protective mechanism was insufficient to sustain photosynthetic activity. Yet, more efficient NPQ energy dissipation may prevent further stress-related implications like ROS generation and lipid peroxidation as energy is lost in controlled manner.

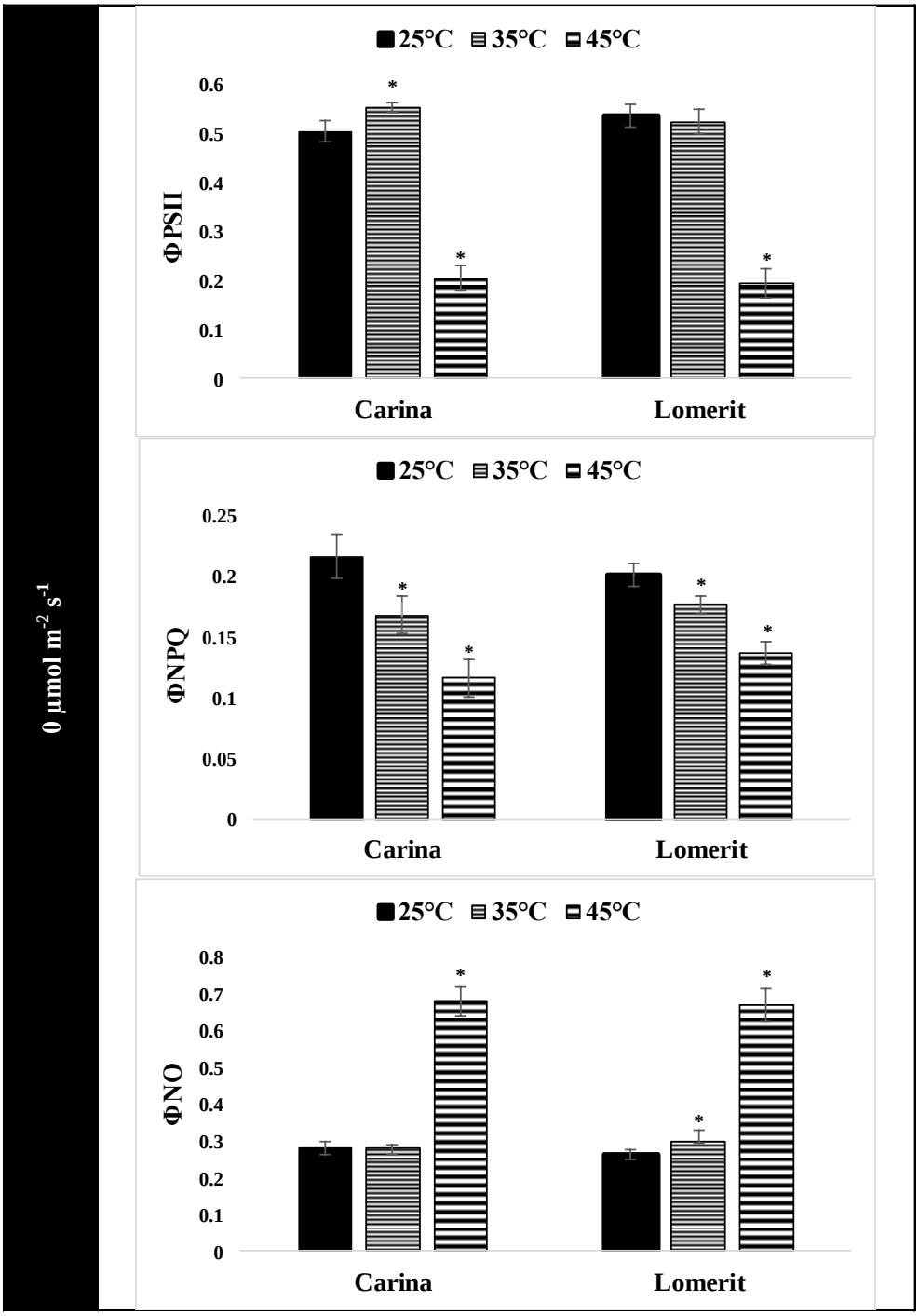

Figure 2a. Effective PSII quantum yield (ΦPSII), quantum yield of regulated and non-regulated energy dissipation (ΦNPQ, ΦNO) of two cultivars of the barley (*Hordeum vulgare* L.) at 25, 35 and 45 °C, assessed with dark adapted samples (30 min) exposed to saturation pulse of blue light (450 nm). Bars present means ±SE of two different experiments (0 and 500 µmol m^{-2} s^{-1}). Asterisks state for statistically significant differences between means (n=12) ($p<0.05$, Tukey's HSD test).

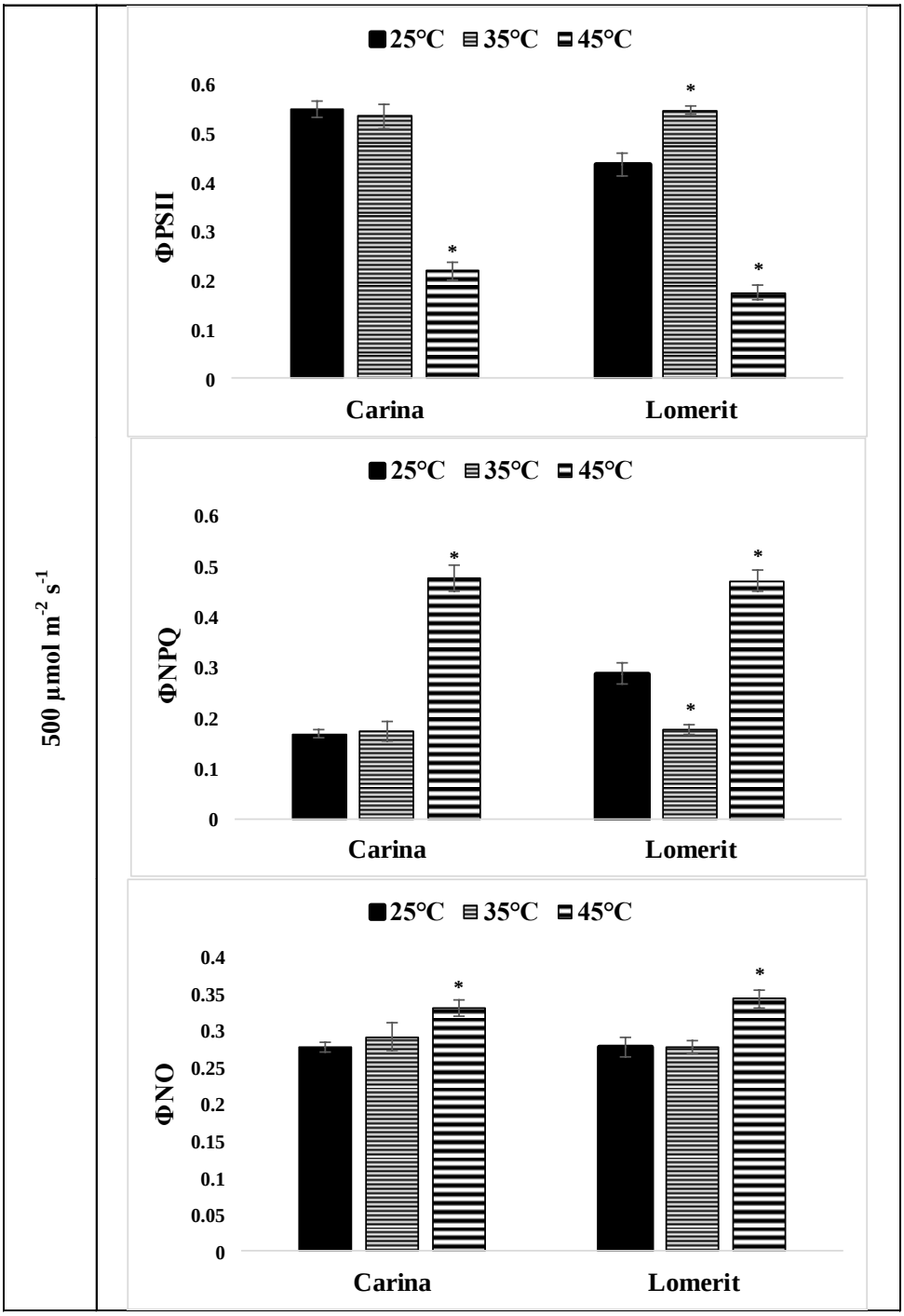

Figure 2b. Effective PSII quantum yield (ΦPSII), quantum yield of regulated and non-regulated energy dissipation (ΦNPQ, ΦNO) of two cultivars of the barley (*Hordeum vulgare* L.) at 25, 35 and 45 °C, assessed with dark adapted samples (30 min) exposed to saturation pulse of blue light (450 nm). Bars present means ±SE of two different experiments (0 and 500 µmol m^{-2} s^{-1}). Asterisks state for statistically significant differences between means (n=12) ($p<0.05$, Tukey's HSD test).

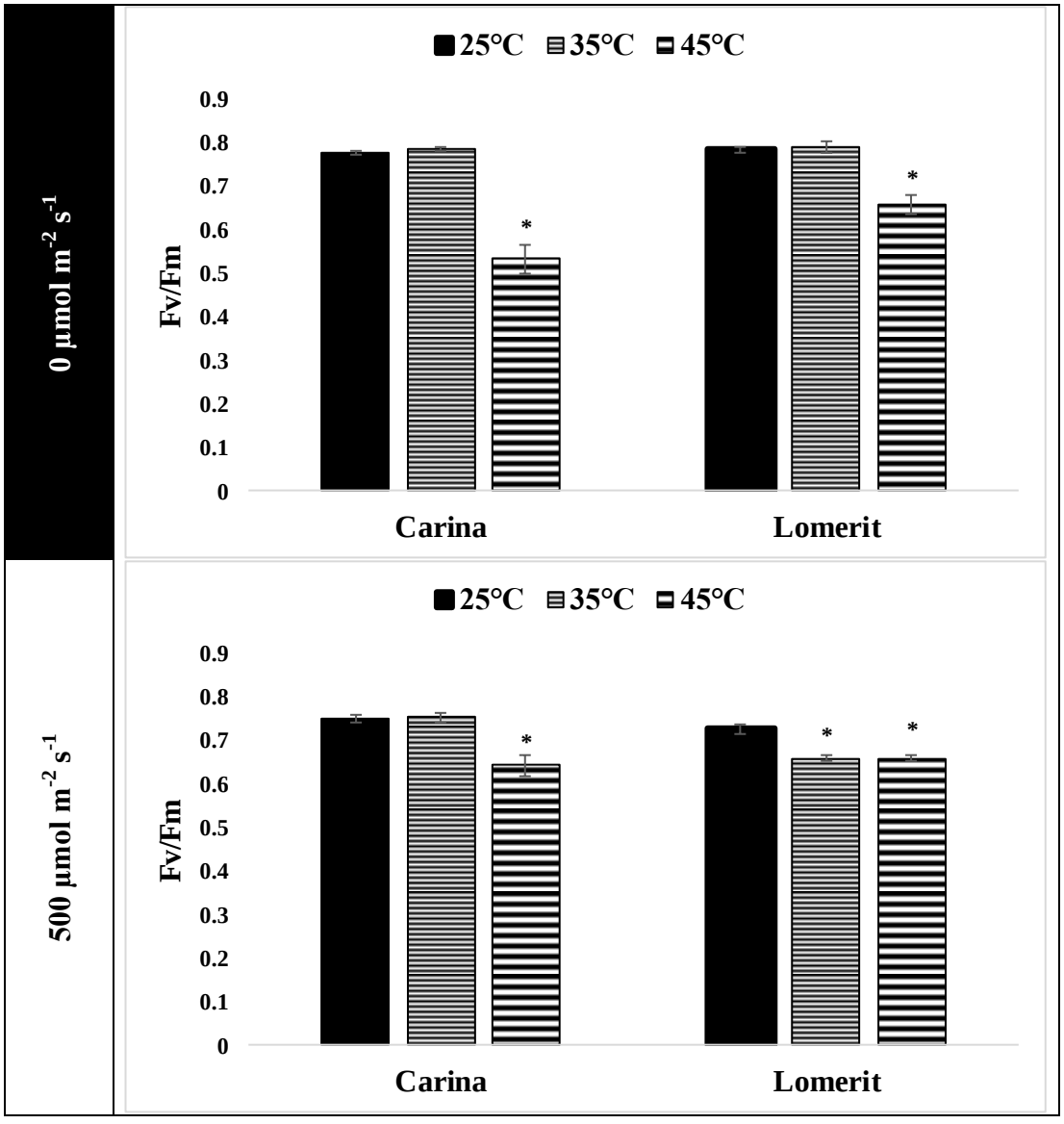

Figure 3. Maximal PSII quantum yield (Fv/Fm) of two cultivars of the barley (*Hordeum vulgare* L.) at 25, 35 and 45 °C, assessed with dark adapted samples (30 min) exposed to saturation pulse of blue light (450 nm). Bars present means ±SE of two different experiments (0 and 500 µmol m^{-2} s^{-1}). Asterisks state for statistically significant differences between means (n=12) ($p<0.05$, Tukey's HSD test).

4. DISCUSSION

Global climate changes and global warming, especially noticeable in the last decade, have prompted efforts to find fast and non-invasive methods for assessing the photosynthetic activity of plants exposed to heat stress and closely related radiation stress. Measurements of the quantum yield of chlorophyll fluorescence *a* allow precise assessment of the radiation transfer direction absorbed by the photosynthetic apparatus (Murchie and Lawson, 2013;

Trojak et al., 2016b). Klughammer and Schreiber (2008) confirmed that the energy absorbed by PSII is used in the photochemical reaction pathway to produce the assimilation force - NADPH and ATP. The efficiency of their course influence on the physiological state of PSII and functionality of the donor and the acceptor side, including the activity of the oxygen evolving complex (OEC), protein complexes of the electron transport chain, PSI functionality and the availability of power reduction, acceptors and precursors of ATP synthesis (Allakhverdiev et al., 2008; Schöttler and Tóth, 2014). Factors limiting the photosynthetic activity in natural conditions include the amount of radiation reaching plants and the temperature which affect the stability and activity of proteins, biological membranes and speed of the enzymatic reaction. Under the influence of PSII damaging factors and disturbance of electrons transport, decreases the value of the effective quantum efficiency ΦPSII. Under these conditions the effectiveness of plants protection mechanisms is determined by the activity of the photoprotection NPQ, measured as ΦNPQ, indicating inability of the plant to protect against radiation and thermal stress. Maintaining high photosynthetic efficiency at elevated temperature is a measure of plant heat resistance, which is characterized by a higher value of ΦPSII, which reduces the loss of light energy, while maintaining a high ratio of ΦNPQ/ΦNO (Klughammer and Schreiber, 2008).

In the evaluation of effects of abiotic stresses, measurement of the maximal quantum yield (Fv/Fm) is also often used to assess the damage of PSII reaction centres (Allakhverdiev et al., 2008). Previous studies (Havaux et al., 1991; Marutani et al., 2012) have shown that the degree of damage to the photosynthetic apparatus is dependent on the simultaneous leaf exposure to light that induces electron flow, allowing for proper redox electron transport and reducing PSII damage. The presence of the radiation also affects the regeneration of the D1 protein which is damaged by thermal stress, necessary for proper functioning of PSII electrons (Marutani et al., 2012).

The presented work shows statistically significant differences in the analysed parameters, both between cultivars and treatments (light, temperature) used to assess their tolerance to thermal stress, which is considered to raise the temperature at 10 to 15°C compared to values in control conditions (25°C) (Allakhverdiev et al., 2008). The increase of ΦPSII value of both cultivars, observed with increasing temperature of 10°C, may be related to the temperature-dependent enzymatic activity of both OEC and ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39), reaching its maximum at 35°C (Cen and Sage, 2005; Marutani et al., 2012). The obtained results suggest that the mechanism underlying the decrease in the efficiency of PSII in studied cultivars varies. This is indicative of a heterogeneous nature, causing the observed changes in PSII. In the case of Lomerit, a decrease of ΦPSII at 25 °C was observed only after irradiation. Light is the activator of the xanthophyll cycle directly affecting the value of ΦNPQ (Trojak and Skowron, 2017).

The temperature at 25 °C is suboptimal for the enzymatic activity of key photosynthetic proteins (Cen and Sage, 2005; Sharkey, 2005). The lower activation threshold of the photoprotective mechanisms in the presence of light at this temperature, for Lomerit, indicated better utilization of the absorbed light energy by Carina. The explanation of this phenomenon, supported by Havaux et al. (1996) is different composition of thylakoid membranes tested barley cultivars. The higher proportion of polyunsaturated fatty acids (PUFAs) in thylakoid membranes of Lomerit determines its higher tolerance to low temperature (unpublished studies) which under thermal stress, raising membranes lability affect the current in the protein components. In addition, increased penetration of some

molecules through the membrane, in particular hydrogen ions, may be responsible for activating the pH-dependent synthesis of Z by the VDE actively participating in the xanthophyll cycle (Routaboul and Fischer, 2000; Sharkey, 2005; Vaz and Sharma, 2011; Trojak and Skowron, 2017). Thermal stress-induced changes in the quantum yield of PSII may also suggest the activation of both cultivars of the cyclic electron transport with the participation of PSI (Agrawal et al., 2016; Egorova and Bukhov, 2002).

Increase in the temperature to 35°C resulted in a decrease in the value of the Fv/Fm parameter of Lomerit, determinant of damage to the PSII reaction centres, changes in its structure (protein D1), and higher thermophilicity of the photosynthetic apparatus (Cui et al., 2006). The absence of identical changes in the dark indicates that the most likely cause of Fv/Fm depression was the photoinhibition phenomenon caused by the synergistic effects of elevated temperature and radiation intensity exceeding the adaptability of PSII of Lomerit. Under these conditions, the reduction of electron acceptors, the increased generation of reactive oxygen species, resulted in oxidative damage (Cui et al., 2006). The sustained in the absence of light the high redox gradient between the plastoquinone pool and the chloroplast stroma is reduced by a further rise in temperature (up to 45°C), and as a result of the sudden exposure of plants to light, cyclic electron transport is activated (Sharkey, 2005).

At 45 °C clearly pointed out the role of earlier exposure to light, measured by the direction of the absorbed energy transfer, which is not used by defective PSII centres. Marutani et al. (2012) reported the reduction damage of PSII because the pathway of regulated energy dissipation has been activated which could further limit the damage to the photosynthetic apparatus. In Carina, the activation of photoprotective mechanisms under conditions caused by thermal stress of photoinhibition allowed for much more effective protection of PSII reaction centres and maintaining the maximum quantum yield at a much higher level only in the presence of light.

5. CONCLUSION

Analysed functionality of the photosynthetic apparatus with chlorophyll *a* fluorescence clearly proved that Carina cultivar is characterized by higher heat resistance. Estimation of the maximal and effective PSII quantum yield as well as ways of energy dissipation allow indicating that protective mechanisms are related to mechanism such as the xanthophyll cycle, modifying NPQ value. Assessment of PSII activity with PAM technique provides almost instant information about the condition of photosynthetic machinery exposed to stress. What is more, analyses is fully repeatable because technique in non-invasive and assessment may be carried out *in situ* to compare time-dependent mechanism of stress-adaptation as well as regeneration. The latter one is especially interesting as our previous research clearly proved that plant resistance to sort of stresses may be increased with regulators modifying physiological conditions of crops. In this paper we have noted that hardiness was mostly related to the xanthophyll cycle activity probably resulted from higher efficiency and stability of the VDE enzyme, especially at elevated temperatures, and the variant-specific content of carotene compounds that are substrates for the xanthophyll cycle. The carotene composition of inner chloroplast membrane has influence on the quenching process of excited chlorophyll molecules as well as the stabilization of thylakoid membranes structures at elevated temperatures (Havaux, 1998). Study proved that PAM fluorescence measurement may be

successfully used to evaluate plant thermosensitivity for selection new crop cultivars and increase agricultural production facing global warming threat.

Acknowledgement

This work was supported by projects of Ministry of Science and Higher Education: 612078 and Polish National Science Centre: UMO-2014/15/N/NZ9/01378.

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(Received 04 August 2017; accepted 20 August 2017)