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# Improvement of plant heat tolerance by modification of xanthophyll cycle activity

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**ABSTRACT**

Plants are sessile organisms hence environmental factors such as excessive light and high air temperature lead to significant reductions of their productivity and quality of gained yield. In fact, scientific and agriculture hubs make lots of efforts to improve crop tolerance to elevated temperature, selecting more tolerant varieties. We analyzed less expensive and highly efficient method to improve resistance of well-known cultivars of crop plant by reversible modification of xanthophyll cycle. It functions as a safety valve to adjust energy transfer and protects fragile structures of photosynthetic machinery from excessive light, especially accompanied by heat or water stress. Efficiency of modified xanthophyll cycle activity was measured after pre-treatment with four, chemically different regulators, with or without light illumination. Analyses were carried out on barley (*Hordeum vulgare* L.) cv. Zenek treated with ascorbic acid (AsA), dithiothreitol (DTT), putrescine (Put) and calcium ions ($Ca^{2+}$). To measure the scale of thermal energy dissipation we traced energy transfer absorbed by PSII with PAM chlorophyll fluorescence technique. Results showed clear correlation between AsA (activator of violaxanthin de-epoxidase) treatment and stimulation of the Φ(NPQ) at increased temperature. DTT (inhibitor of violaxanthin de-epoxidase) decreased the cycle activity at 45 °C at the same time increasing its value at 35°C, caused by interaction with other enzymes. Action of Put (hydrogen ions buffer) concerned mainly a non-regulated Φ(NO) energy quenching. We noticed that application of $Ca(NO_3)_2$ ($Ca^{2+}$ source for enzyme activity) reduced the Φ(NPQ) at 45 °C and stimulated it at 25 °C. Obtained results confirmed postulated possibility of creating new type of plant protection products (PPPs) able to precisely manage natural mechanisms of heat resistance.

***Keywords*:** ascorbic acid (AsA);,calcium ions ($Ca^{2+}$), dithiothreitol (DTT), heat stress, plant protection products (PPPs), putrescine (Put), xanthophyll cycle

## 1. INTRODUCTION

High temperature is one of the main abiotic stresses limiting the growth and productivity of plants, mainly through the negative impact on the photosynthesis considered to be the most sensitive aspect of their functioning (Trojak et al., 2016a). Evolution of higher plants in oxygen-rich atmosphere, forced development of numerous protective strategies to avoid photooxidative damage of the photosynthetic apparatus. Plants evolved mechanisms allow reducing formation of reactive oxygen species (ROS) or detoxifying of already formed ones (Yang et al., 2013). There are two major pathways of ROS formation in chloroplast. The first one, connected with disturbed electron transfer of photosynthetic electron transfer chain, resulted in single-electron donation to molecular oxygen at the acceptor side of photosystem I (PSI) or photosystem II (PSII). Reaction is the main source of superoxide anion ($O_2^{\cdot -}$) converted, at subsequent reactions, to hydrogen peroxide ($H_2O_2$) or hydroxyl radical ($^{\cdot}OH$). The second source is the energy transfer from long-lived triplet form of chlorophyll ($^3Chl^*$) to molecular oxygen, leading to the formation of singlet oxygen ($^1O_2^*$) (Jahns and Holzwarth, 2012). The most important mechanism of balancing the absorbed light energy and thereby preventing the photosynthetic apparatus photoinhibition is the xanthophyll cycle-dependent thermal energy dissipation, measured as the non-photochemical quenching (NPQ) of chlorophyll fluorescence (Li et al., 2005). In xanthophyll cycle zeaxanthin (*Z*) is formed by de-epoxidation of violaxanthin (*V*) through the intermediate antheraxanthin (*A*) (Xiong et al., 2012) (Figure 1).

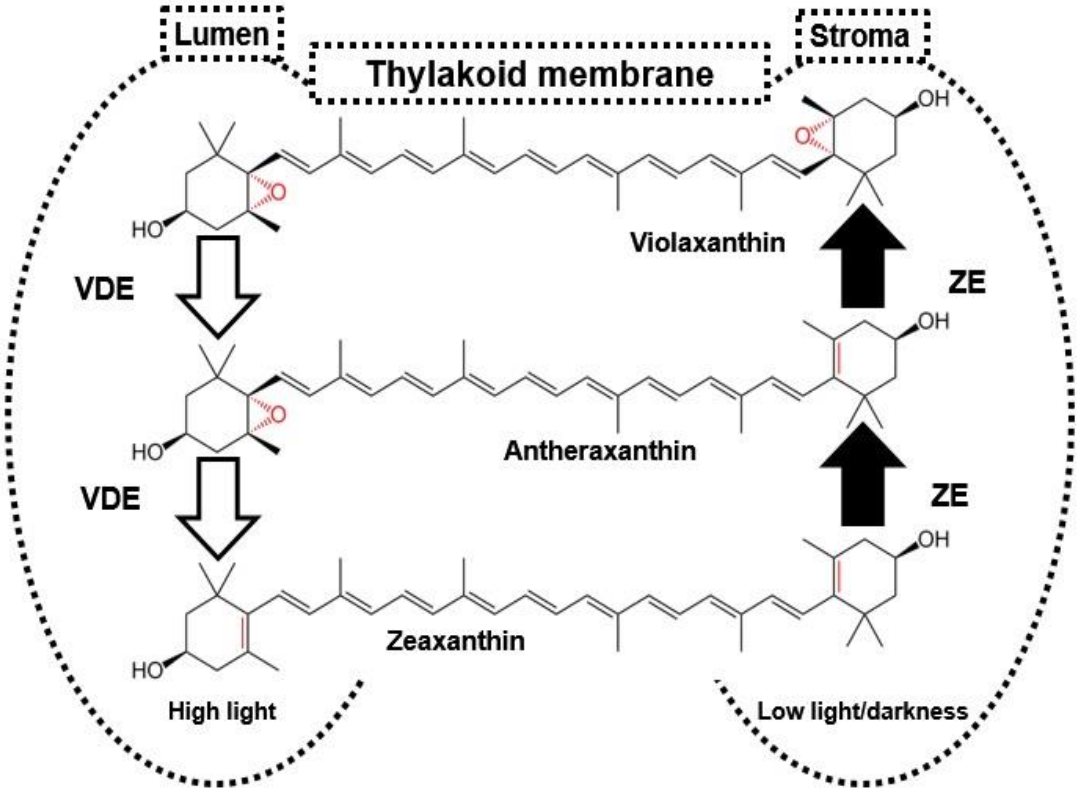


**Figure 1.** Reactions of the xanthophyll cycle typical for higher plants.
VDE - violaxanthin de-epoxidase, ZE - zeaxanthin epoxidase

Conversion of *V* to *Z* takes place when plants are illuminated with high light intensity, while low light and darkness stimulate the opposite reaction. Two enzymes, localized on opposite sides of the thylakoid membrane, are engaged in this process: violaxanthin de-epoxidase (VDE), situated on the thylakoid lumen-side of the membrane, catalysing the de-epoxidation of *V* to *Z* and stromal-side enzyme zeaxanthin epoxidase (ZE), carrying out the reverse, epoxidation reaction of *Z* to *V*. *A* as well as *Z* protects plants from photoinhibition by dissipating the excess light energy as heat (Vaz and Sharma, 2011). Activity of the xanthophyll cycle affects the physical properties of the thylakoid membrane. Gruszecki et al. (1996) observed that VDE could cause the change of the fluidity of the thylakoid membrane and Havaux et al. (1991) showed that decreased fluidity was closely related to the increased *Z* concentration after high light treatment. Besides they showed that *Z*-enriched thylakoid membrane has enhanced stability under strong light and high temperature conditions (Xiong et al., 2012).

Mechanism of NPQ energy dissipation includes a state transition (qT), which balances energy between PSII and PSI, the energy- or pH-dependent mechanism (qE), zeaxanthin-dependent NPQ (qZ) and quenching associated with closed reactive centres due to photoinhibition (qI). The first one balances energy between photosystems by reversible modification of light harvesting complexes (LHCs) to final adjustment of absorbed light energy. State transition component, allows separating LHCs from PSII and reducing the amount of excitation energy absorbed by this photosystem. The qE mechanism represents the major NPQ component activated under moderate light condition. It requires a thylakoid lumen pH below 6, de-epoxidased xanthophylls and the PsbS protein (Ware et al., 2015) and relaxes within seconds to minutes after illumination. The qI component is related to photoinhibition and seems to be rather the result of not the response to prolonged excessive light treatment. Explanation of qI genesis is reduced D1 protein turnover, therefore it shows very slow relaxation kinetics in the range of hours. qZ NPQ form is often considered to be part of qE, because it is based on *Z* mechanism of energy quenching. Nonetheless, *Z* accumulation in inner chloroplast membranes caused its independence of lumen pH once *Z* is already formed, hence it relaxes in tens of minutes (Müller et al., 2001; Brooks et al., 2013).

Ascorbate (AsA) is known to be a major non-enzymatic antioxidant, managing both the assimilation and photoprotection process in the hydrophilic and hydrophobic compartments of the chloroplast. AsA is also a cofactor for the antioxidant enzymes, scavenging ROS, produced by PSI in the Mehler-peroxidase reaction (water-water cycle, WWC) (Leipner et al., 2000; Müller-Moulé et al., 2002). WWC is defined as a photoreduction of oxygen to a superoxide anion radical, followed by its dismutation by superoxide dismutase to hydrogen peroxide and oxygen. Ascorbate peroxidase (APX) reduced hydrogen peroxide to water, followed by the regeneration of AsA by direct reduction of monodehydroascorbate with PSI or by the NADPH-dependent monodehydroascorbate reductase. This reaction results in electron flow from PSII to PSI with no net oxygen evolution. The proton gradient generated by this "pseudocyclic" electron flow has been shown to be important for *Z* formation and qE, under $CO_2$-fixation limiting conditions (Neubauer and Yamamoto, 1994; Müller-Moulé et al., 2002). On the other hand, the Mehler-peroxidase reaction competes with VDE for AsA, decreasing its availability for VDE. Decreased concentration of active (fully reduced) form of AsA resulted in qE limitation and the modulation of PSII activity (Calatayud et al., 1999; Leipner et al., 2000).

Dithiothreitol (DTT), also known as Cleland's reagent, is a compound widely used as reducing agent for reduction of disulfide bonds between cysteine residues and protection of sulfhydryl groups from oxidation. Additionally, the mechanism of DTT action depends on the structure of the protein, hence it reacts also with protein domains with no cysteine residues (Alliegro et al., 2000). DTT chelates metal cations, efficiently inhibiting enzymes activity or interacts with the protein surface, limiting or even completely preventing spatial fit of the ligand to the defined active site (Rockholm and Yamamoto, 1996). In study DTT was used to block the conversion of *V* into *Z* as is the potent inhibitor of the VDE (Zeiger and Zhu, 1998; Song et al., 2011).

Putrescine (Put) with spermidine and spermine are the major plants polyamines (PAs). PAs are essential for cell growth and stress tolerance. Exogenous application of PAs has been shown to protect against various stress conditions such as heat stress or salinity. PAs protect plants against oxidative damage, lipid peroxidation, stabilize photosynthetic reaction centres and modify the integrity of internal chloroplast membranes (Ali, 2000; Sobieszczuk-Nowicka et al., 2009, Shu et al., 2012). Explanation of Put protective action is based on the model of "biological weak base" (BWB), proposed by Ioannidis et al. (2012). BWBs, such as Put, balance both components of the proton-motive force (*pmf*), namely the proton gradient ΔpH and electrochemical potential Δψ (Ioannidis et al., 2012). Mechanism of Put action is similar to that by which amines dissipate the ΔpH component of *pmf* in isolated thylakoids. Amines, such as Put, function in the protonated or free base form. Positively charged amines, trapped inside thylakoid vesicles, determined equilibrium between free external and protonated luminal forms. Excess captured energy is dissipated in controlled manner by increased proton gradient, which results in acidification of the thylakoid lumen and the initiation of xanthophyll cycle. Put, acting as a buffer, reduces the concentration of free hydrogen protons (ΔpH drops), while has no influence on electrical component (Δψ), allowing the ATP production (Skowron et al., 2016a; Trojak et al., 2016a).

Furthermore, Put is well known for its positive effects on photosynthetic efficiency under various stress conditions (Yuan et al., 2015). Because of its acid-neutralizing and antioxidant properties, Put initiates photoprotection of the photosynthetic reaction centres through energy-dependent non-photochemical quenching (qE), the process that dissipates excess absorbed light energy as heat, thus protecting the photosynthetic apparatus from photodamage (Ioannidis et al., 2006; Ioannidis et al., 2012). Published studies showed that application of exogenous Put may protect plants against oxidative damage and lipid peroxidation, increase active reaction centre population, energize the cell by ATP production (Ioannidis et al., 2007) and improve the photosynthetic capacity of PSII under heat stress (Shu et al., 2012).

Calcium ($Ca^{2+}$) is known to act as a regulator of many physiological and biochemical processes in response to abiotic stress in plants. Increased level of intracellular free $Ca^{2+}$, stimulates gene expression and activates a series of biochemical responses (Liang et al., 2009). Increased free $Ca^{2+}$ can be detected in plants in response to various stresses, such as high temperature, cold injury, drought and salt stress (Zhao and Tan, 2005). The fact, that $Ca^{2+}$ improves plant resistance is related to a higher photosynthetic rate under stresses. The light-induced $Ca^{2+}$ influx into chloroplasts not only influences the cytosolic concentration of free $Ca^{2+}$ but also regulates the enzymatic processes inside the chloroplast (Yang et al., 2013). Several studies have showed that exogenous $Ca^{2+}$ improves the net photosynthetic rate (*Pn*), carboxylation efficiency and relative quantum yield in response to high temperature stress

(Yang et al., 2013). $Ca^{2+}$ ions have an impact on the PSII reaction centres stability, enhance the activity of antioxidant enzymes and limit ROS accumulation. Reduced intracellular ROS concertation alleviates the photodamage of PSII and increases the rate of D1 protein synthesis and turnover (Yang et al., 2015).

## 2. METHODOLOGY

### 2. 1. Plant material and treatment

At the initial stage we selected two varieties of the winter barley (*Hordeum vulgare* L.): Malwinta and Zenek (from seventeen tested), based on their heat resistance (data unpublished). Next step was the selection of proper cultivar for xanthophyll cycle research. We have tested Malwinta and Zenek and selected the latter one as highly tolerant, taking into account that tolerance of Zenek seems to be xanthophyll cycle-dependent (Trojak et al., 2016b). Barley seeds, sterilized with antifungal powder (T 75 DS/WS) and germinated on wet paper for three days in a complete darkness, were sown in P9 containers filled with universal substrate (white peat, black peat, perlite and NPK 9:5:10). 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 16/8 h per day. Relative humidity was 50-60%, with temperature varied in the range 22-23 °C/18 °C day/night. Three weeks after sowing, plants were divided into control and four groups treated with 10 mM AsA, 3 mM DTT, 10mM Put and 6mM $Ca(NO_3)_2$, respectively. Collected plant material were 3 mm diameter discs, cut out from the second true leaf of 25-day-old barley plants, transferred immediately to Petri dishes filled with a wet filter paper. Leaf discs were left for 25 minutes in the complete 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). Then all discs were soaked with AsA, DTT, Put and $Ca^{2+}$ and incubated for next 5 minutes in thermal block (Eppendorf® ThermoStat Plus, Hamburg, Germany) at the proper temperature (25, 35 or 45 °C), simultaneously exposed to 500 µmol $m^{-2}$ $s^{-1}$ RBG LED light to activate xanthophyll cycle.

### 2. 2. Estimation of photosynthetic and xanthophyll cycle activity

Evaluation of the photosynthetic activity of PSII was done with Pulse Amplitude Modulation (PAM) technique. It is based on modulated signal analysis to obtain spectrofluorometric measurements of the kinetics and light response (light curves) of PSII chlorophyll molecules. 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 excitation energy between photochemical utilization Φ(PSII) or regulated Φ(NPQ) and non-regulated heat dissipation Φ(NO) (Table 1). Measurements were carried out between 7.00 to 11.00 a.m. CET, during maximal photosynthetic activity of plants. Parameters were estimated with the LED-Array Illumination Unit IMAG-MAX/L features with royal-blue (450 nm) LED-lamps equipped with collimating optics, provides the pulse-modulated blue excitation light for actinic illumination and saturation pulses. Light curves were estimated during 400 second run with sixteen, gradually increasing actinic light intensities (0, 0, 1, 21, 56, 111, 186, 281, 336, 396, 461, 531, 611, 701, 926, 1251 µmol $m^{-2}$ $s^{-1}$, 20 second each) started with

saturation pulse, used for quenching analysis, given repetitively at defined clock-intervals. Recorded light curves showed at figure 3-5, illustrate run in the 0-396 μmol $m^{-2}$ $s^{-1}$ range, to avoid misinterpretation between temperature- and light-caused inhibition of PSII. Measurements were made on dark adapted (30 minutes) leaf discs incubated on a wet paper.

**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 |
| Φ(PSII) | effective PSII quantum yield | Φ(PSII) = (Fm' - F)/Fm' |
| Φ(NPQ) | quantum yield of regulated energy dissipation | Φ(NPQ) = 1 - Φ(PSII) - 1/(NPQ + 1 + qL(Fm/Fo -1)) |
| Φ(NO) | quantum yield of non-regulated energy dissipation | Φ(NO) = 1/(NPQ + 1 + qL(Fm/Fo - 1)) |

where: Φ(PSII) + Φ(NPQ) + Φ(NO) = 1; Fv - variable fluorescence; Fm - maximal fluorescence yield in dark adapted samples; Fm' - maximum of fluorescence yield in illuminated samples; F - fluorescence yield; Fo - dark fluorescence yield; qL - coefficient of photochemical quenching; NPQ – non-photochemical quenching

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### 2. 3. Statistical analysis

All statistical analyses were performed using STATISTICA 12.0 software (StatSoft Inc., Oklahoma, USA). Normally of the random variables distribution was verified with Shapiro-Wilk's test and to assess the equality of variances for variables we used Levene's test, both at the 0.05 significance level. The one-way analysis of variance (ANOVA) was used to determine difference in the values of the evaluated parameters in all tested temperatures. 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

Previous study (Trojak et al., 2016a) showed that in the absence of light DTT has limited xanthophyll cycle activity approximately by 20% at 25-35°C and 30% at 45 °C. At the same time illuminated samples showed 20% higher Φ(NPQ) value at 35 °C and more than 20% decrease at the highest, tested temperature. In this study we decided to characterize xanthophyll cycle activity with light curves, to precisely document the influence of chemical regulators on xanthophyll cycle activity and consequently their role in the plant heat tolerance improvement.

DTT-dependent inhibition of VDE, obtained with longer and more severe light (range 0-396 µmol $m^{-2}$ $s^{-1}$) is more parallel to natural conditions than illumination, previously used for kinetics assessment (56 µmol $m^{-2}$ $s^{-1}$). Samples, without the initial light exposure, did not present any Φ(NPQ) limitation relative to control. We demonstrated that in the range of 100-396 µmol $m^{-2}$ $s^{-1}$ at 35 °C DTT application significantly increased the NPQ value, indicating that DTT-dependent inhibition of enzyme activity at elevated temperature is mitigated by van't Hoff rule of temperature dependent rate of enzymatic reaction. We proposed that observed DTT-effect may concern to a greater extent enzyme such as APX, than VDE, which is the uppermost competitor for AsA utilization (Neubauer, 1993).

To simplify, we postulated that final VDE activity is a result of the AsA availability, temperature and functionality of enzymes which used the same cosubstrate. Without light exposure the most probable explanation is the leverage effect of DTT, which simultaneously inhibited the wider group of AsA utilizing enzymes, especially at higher (35 °C), optimal for enzymatic activity, temperature. Almost identical NPQ light response curves were obtained after 500 µmol $m^{-2}$ $s^{-1}$ light irradiation. Results confirmed role of DTT as a cycle inhibitor at 45 °C due to reduced *Z* accumulation before heat stress exposure. At the same time the supressed VDE activity reduced *V* conversion. Previous PSII analyses, carried out without light pre-treatment, showed slight difference only at 35°C. However, at 45 °C we noticed reduction of Fv/Fm value (Figure 2).

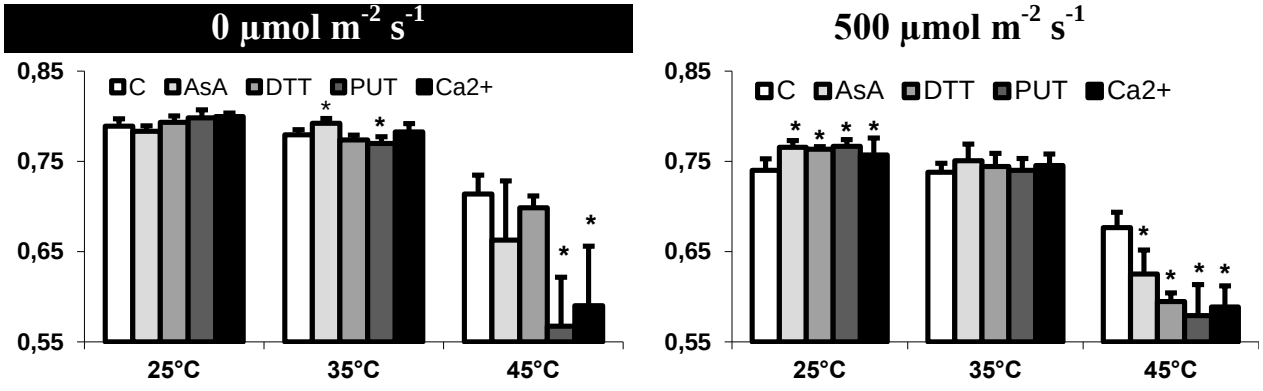


**Figure 2.** Maximal PSII quantum yield (Fv/Fm) assessed with dark adapted samples (30 minutes) exposed to saturation pulse of blue light (450 nm). Bars present means ±SE of two different experiments (0 and 500 µmol $m^{-2}$ $s^{-1}$). Asterisks state for statistically significant differences between the means (n = 6) ($p < 0.05$, Tukey's HSD test).

The main consequence of reduced xanthophyll cycle activity and simultaneous action of light was a significant increase in the value of Φ(NO), indicating the uncontrolled mechanism of energy dissipation and explaining the Fv/Fm drop (Trojak et al., 2016a).

Light curves (Figure 3-5) allowed for more accurate analysis of DTT impact on PSII photosynthetic efficiency and significance of its components, illustrating destination of absorbed energy. We concluded that mechanism of DTT-dependent inhibition of NPQ is not directly related to the damaged components of photosystem with reducing agent but seems to be result of limited both the qE and *Z* accumulation.

Incubation of leaf discs with AsA resulted in a significant Φ(NPQ) stimulation. Photosynthetic response in the light range 0-396 μmol $m^{-2}$ $s^{-1}$ (Figure 4a, 4b) showed precisely, that AsA induced non-photochemical light quenching in regulated manner. Obtained result demonstrates the overriding role of light and increased temperature in the de-epoxidation process. Nonetheless, statistical analysis was proved only for irradiated samples incubated at 35 °C. Analyses of Φ(PSII) (Figure 3a, 3b) have showed that after AsA treatment absorbed energy was transferred from photochemical to non-photochemical regulated quenching mechanism, affecting the yield of assimilates production, which in relatively stable conditions is less preferable. The protective action of the AsA includes its role as a key compound in ROS detoxification system, as well as xanthophyll cycle activator, mainly noted in the case of pre-illuminated samples at 45 °C.

Previous results (Trojak et al., 2016a) have showed that Put is a potent regulator of the cycle, especially after light exposure activating it nearly by 15% and 27% at 25 °C and 35 °C, respectively. Therefore, it was a bit confusing that Put has almost no effect on light curves shape. We noted that Put had no statistically significant impact on the Φ(NPQ), as well as almost no effect was documented for Φ(PSII). According to proposed mechanism of Put action and noted influence on the Φ(NO) (Figure 5a, 5b), we concluded that Put regulates mainly the non-regulated form of absorbed energy quenching which may, in some circumstances, be beneficial for PSII protection, like AsA limitation or at conditions of elevated, enzyme deactivating temperature.

Previously detected increase of xanthophyll cycle activity in illuminated samples, treated with $Ca(NO_3)_2$ was approximately 22% and 30% at 25 °C and 35 °C, respectively. The stimulatory effect of $Ca^{2+}$ to Φ(NPQ) value, similar to that observed for AsA, was noted only at 25 °C. Despite well documented role of $Ca^{2+}$ in membrane stabilization and the integrity of oxygen evolving complex (OEC), there was no increase of Φ(PSII) after $Ca^{2+}$ application. Nonetheless, $Ca^{2+}$ has an impact on non-regulated energy dissipation Φ(NO) at 45 °C (for both light conditions), probably due to limited NPQ energy dissipation, caused by reduced ΔpH gradient, as increased $Ca^{2+}$ concentration within chloroplast stimulates $Ca^{2+}/H^{+}$ antiporter, allowing the transport of calcium into the thylakoid lumen (Höhner et al., 2016).

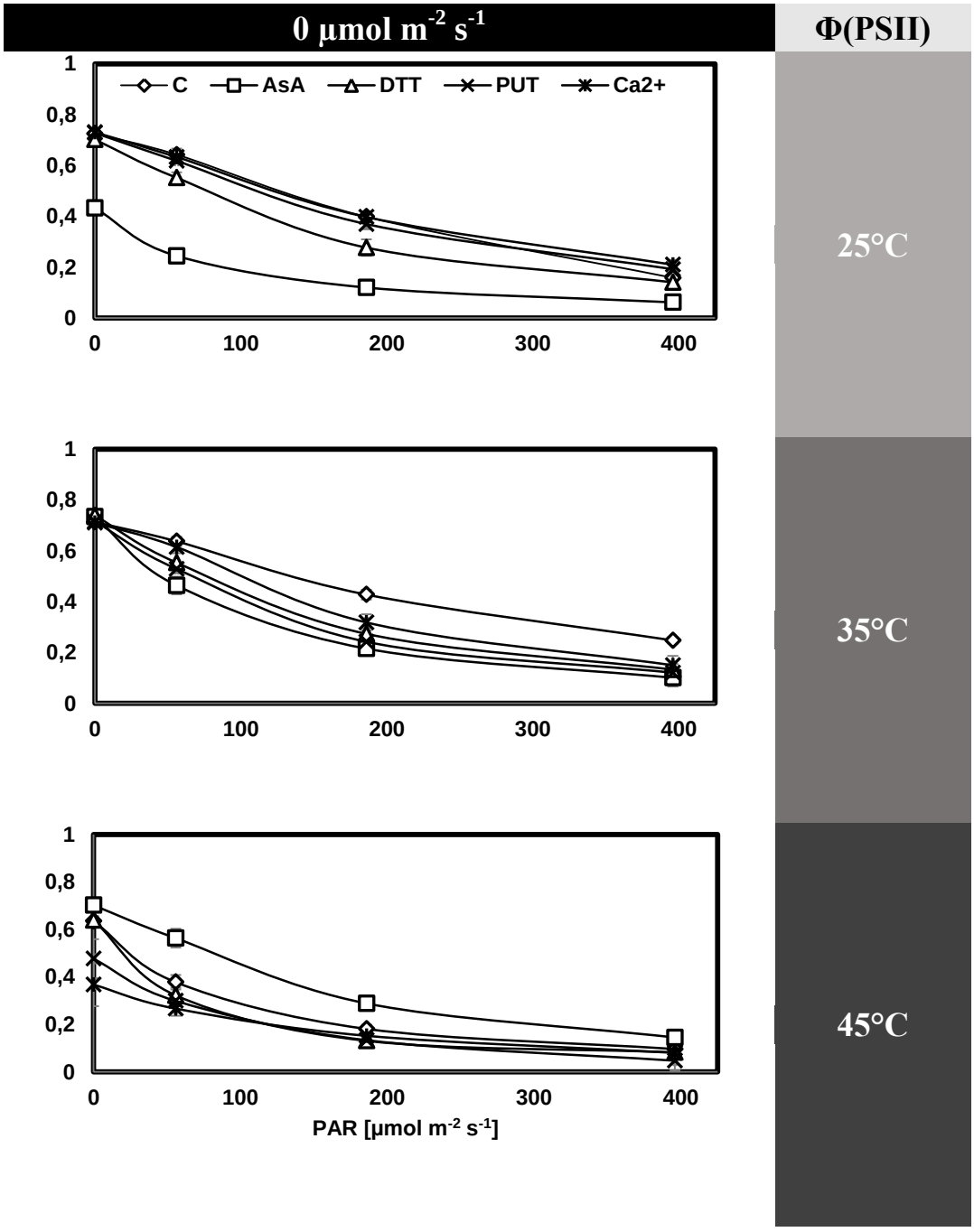


**Figure 3a.** Light curves for the photochemical quenching Φ(PSII), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n = 6). SE is indicated by bars when larger than the symbol.

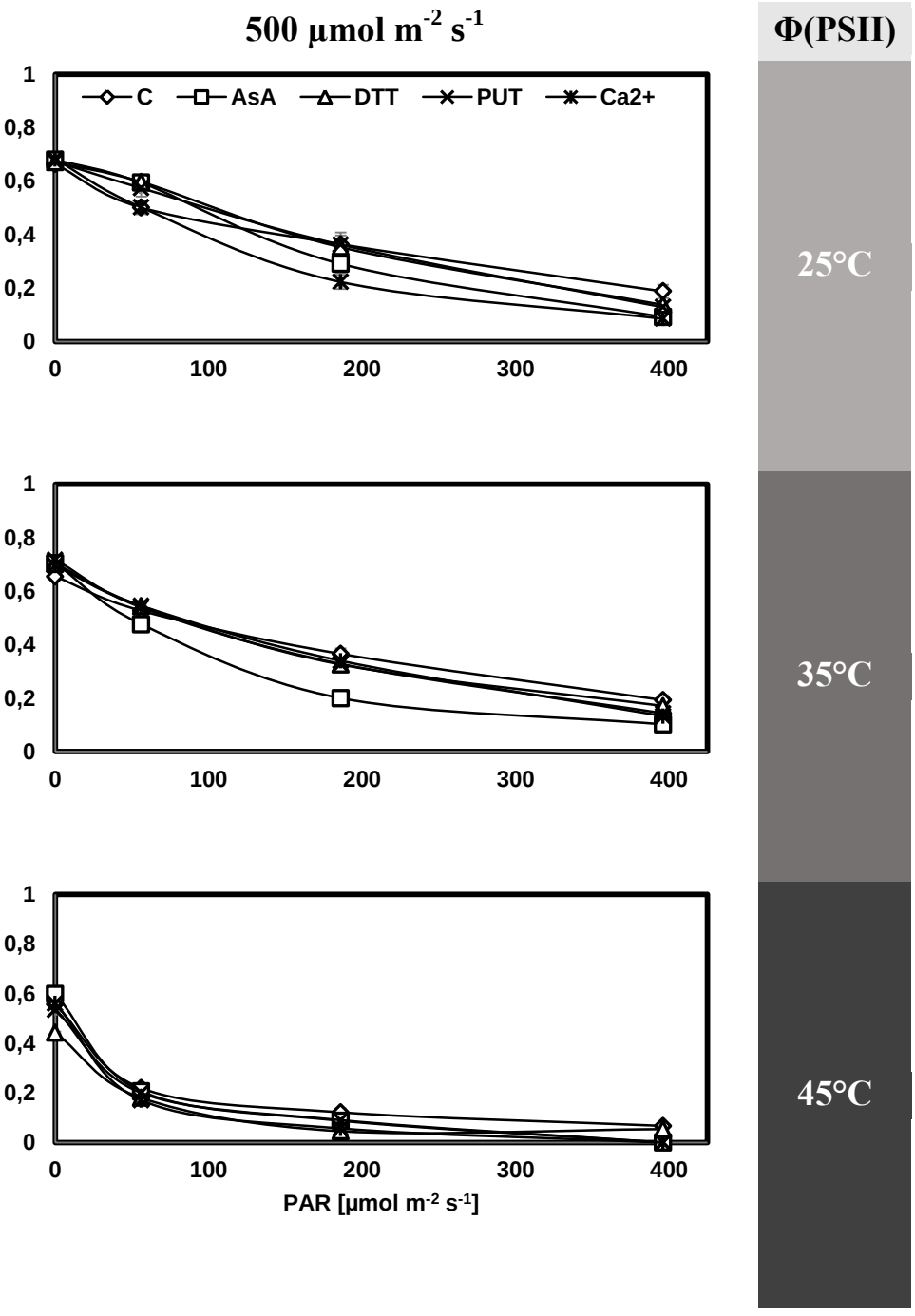


**Figure 3b.** Light curves for the photochemical quenching Φ(PSII), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n = 6). SE is indicated by bars when larger than the symbol.

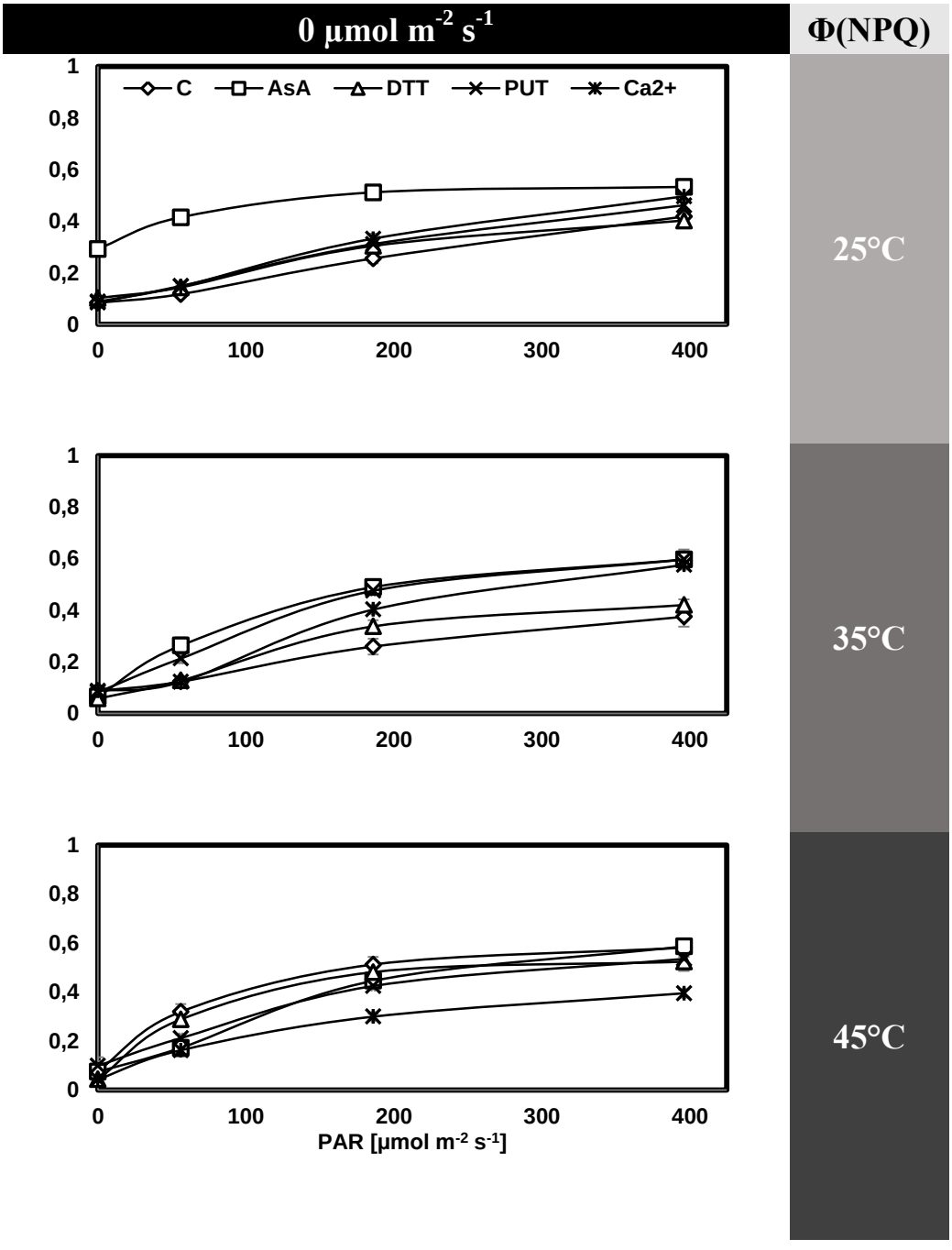


**Figure 4a.** Light curves for the regulated non-photochemical quenching Φ(NPQ), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n = 6). SE is indicated by bars when larger than the symbol.

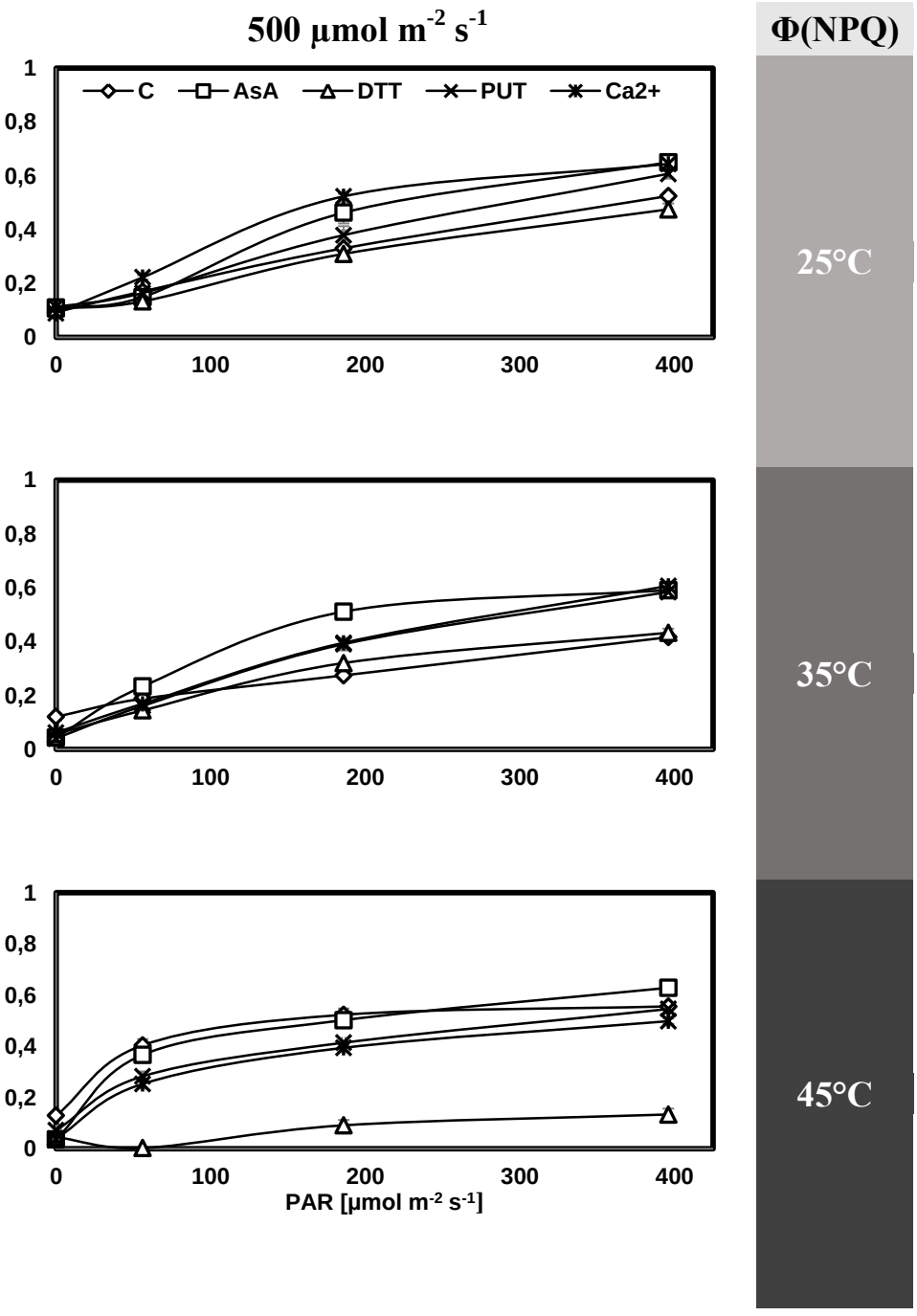


**Figure 4b.** Light curves for the regulated non-photochemical quenching Φ(NPQ), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n=6). SE is indicated by bars when larger than the symbol.

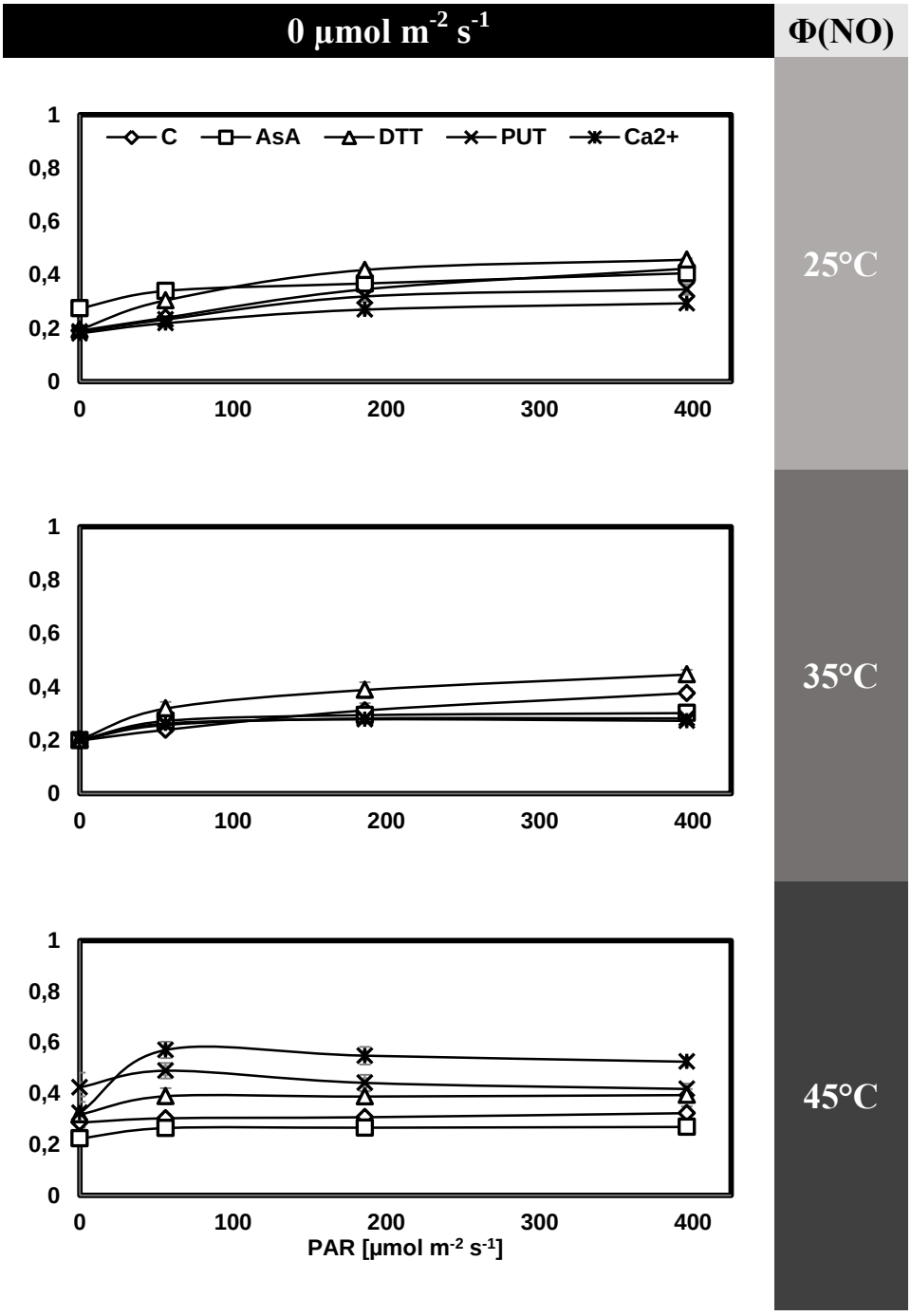


**Figure 5a.** Light curves for the non-regulated non-photochemical quenching Φ(NO), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n = 6). SE is indicated by bars when larger than the symbol.

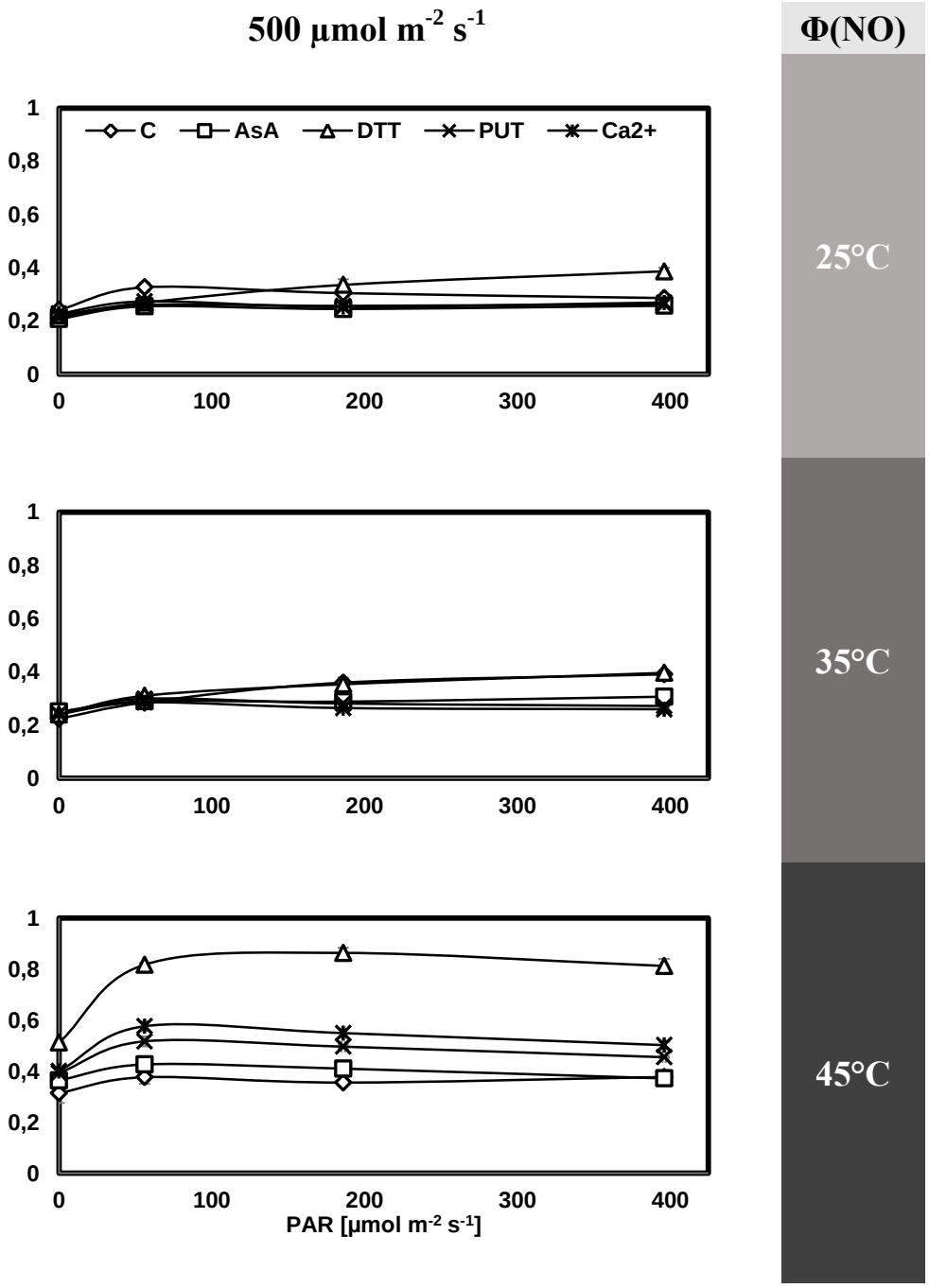


**Figure 5b.** Light curves for the non-regulated non-photochemical quenching Φ(NO), recorded with sixteen actinic light steps (0-1250 µmol $m^{-2}$ $s^{-1}$). Presented light curves are reduced to 0-396 µmol $m^{-2}$ $s^{-1}$ range, to avoid high light-dependent inhibition. Values are mean ±SE (n = 6). SE is indicated by bars when larger than the symbol.

## 4. DISCUSSION

In this paper we assessed the ability of four potential xanthophyll cycle regulators to modify its activity with or without light exposure to distinguish time-dependent effects. Activation of the cycle could be regulated in fast or slow manner and be result of quick ΔpH modification and LHCs redistribution as well as long-term *Z* accumulation in chloroplast inner membranes. According to previous study efficient activation of xanthophyll cycle in C3 plants requires approximately 500 μmol $m^{-2}$ $s^{-1}$ of light to saturate photosynthesis and at the same time to avoid radiation stress (Zhang et al., 2009; Essemine et al., 2012). Light pre-treatment is necessary to activate the photosynthetic electron transfer and produce electrochemical gradient across the thylakoid membrane. As a result of decreased pH inside thylakoid vesicle to optimal pH = 5.0, VDE is activated and converts *V* to *Z*. ΔpH also has an impact on PsbS protein as well as ATP production (Jahns et al., 2009). PsbS, a subunit S of PSII, is a 22-kDa protein, protonated at a low pH in the thylakoid lumen. It plays a key role in qE and with VDE-meditated *Z* accumulation is essential for energy transfer from excited chlorophyll molecule to *Z*, which can return to the ground state via thermal decay (Li et al., 2004). Regardless of carotenoids role in the xanthophyll cycle, they play crucial role in an inactivation of $^3Chl^*$ and $^1O_2{}^*$ within the PSII reducing the ROS (Müller-Moulé et al., 2002; Jahns and Holzwarth, 2012). As well as PSII also PSI is involved in ROS generation due to the mechanism named Mehler reaction, especially after long incubation in dark or in excess light condition.

As a result of acceptor side block, energy gained by chlorophyll *a* within PSI is transferred to oxygen molecules producing highly reactive superoxide anions quickly converted to hydrogen peroxide by chloroplast superoxide dismutase (Skowron et al., 2016b). Next step is the reduction of produced $H_2O_2$, catalysed by APX, using AsA as a reducing agent, at the same time competing with VDE for AsA (Müller-Moulé et al., 2002). Previous study proved that Mehler-peroxidase reaction decreased qE, reducing availability of AsA for VDE (Neubauer and Yamamoto, 1994; Müller-Moulé et al., 2002). Müller-Moulé et al. (2002) and Yin et al. (2010) have proved that limitation of AsA has a direct influence on the xanthophyll cycle key enzyme especially in stress conditions increasing APX activity. Despite the fact, that VDE possess higher affinity for AsA than APX, the latter one is the main competitor for de-epoxidase due to its nearly 30-fold higher concentration in chloroplast and localization in stroma, reducing the amount of free AsA in thylakoid lumen (Neubauer and Yamamoto, 1994). Presented results showed that AsA application clearly increased VDE activity at all tested temperatures. AsA-dependent NPQ stimulation, noted in illuminated samples, presented 1.2 and 1.3-fold higher NPQ value at 25 °C and 35 °C, respectively. It complies with previous studies assuming almost overriding role of light in the rapid generation of pH gradient, initially through the WWC and then with cyclic electron flow (CEF) that have crucial role at the early, unbalanced stage of photosynthetic activation. Interestingly, despite well documented antioxidant properties of AsA (Müller-Moulé et al., 2002), its application at 25 °C and 35 °C has protected PSII only in limited way (Figure 2 and 3a, 3b). At higher temperatures there was no positive effect of AsA application on Fv/Fm, presenting exactly the same reduction as noticed for control conditions. Nonetheless, AsA has a positive influence on PSII condition as well as NPQ activity at highest, tested temperature.

Application of DTT at 45 °C effectively reduced xanthophyll cycle activity regardless of previous light exposure. Different light condition, before DTT application, was used to

assess the scale of NPQ light-dependent activation and distinguish it for this initiated by elevated temperature, as DTT-mediated inhibition of VDE occurred after the initial enzyme activity, stimulating *Z* accumulation. Agrawal et al. (2016) confirmed that temperature higher than 40 °C disrupts the mechanism of controlled energy dissipation with NPQ and as a result excess energy is dissipated by non-regulated energy mechanism assessed with Φ(NO). In fact, we reported the soaring value of Φ(NO) at 45 °C with light which was almost 3-fold higher than control. The molecular mechanism of the xanthophyll cycle inhibition by DTT is a result of VDE characteristic feature – only oxidized form reveals biological activity, connected with six disulfide bonds stabilizing its native structure. At the same time DTT reduces one or more disulfide bond and effectively inactivates VDE (Simionato et al., 2015).

Put, next NPQ regulator, was used based on its postulated role in PSII protection against numerous stresses, which is directly connected with its ability to induce excess energy dissipation as a heat. Nonetheless of Put application, we noted Fv/Fm decrease, suggesting non-regulated mechanism of energy loss from PSII. Moreover, as a result of energy transferred to Φ(NO) the relative quantum efficiency dropped after Put application. Positive effects of exogenous Put treatment were noted for Φ(NPQ). We documented a substantial induction of NPQ at 35° C for both light conditions. A bit confused results were obtained at 25-35 °C as Put application had no effect on Φ(NO), what has been postulated by Ioannidis et al. (2012). They stated that Put regulates the ΔpH proton gradient, delaying the VDE activation, measured by controlled heat loss. However this regulation is strictly temperature dependent as enzymes are labile proteins, inactivated by elevated temperature. We noted that Put stimulated Φ(NO) only at 45 °C, hence it would be consequence of heat-inhibited VDE activity. Amines play an important role in the *in vivo pmf* modulation based on BWB mechanism. Obtained results showed that Put provides an independent mechanism adjusting the qE intensity with *pmf* to balance and optimize energy transfer used as well for photochemical and non-photochemical reactions, especially under stress (Ioannidis et al., 2012).

Intracellular calcium ions ($Ca^{2+}$) regulate numerous processes, therefore their local concentration triggers a wide range of signal transduction pathways via binding proteins such as calmodulin (CaM), activating destination enzymatic and non-enzymatic proteins, forming signal cascade to final adjustment of plant cell metabolism. In addition, $Ca^{2+}$ concentration controls membrane permeability modifying its mobility and compounds exchange between cell and its environment. Zhao and Tan (2005) have found clear correlation between sharp temperature rise and electrolyte leakage, suggesting significant damage of the cell membrane and concluded that pre-treatment with $Ca^{2+}$ significantly mitigated fluid leakage. Moreover, our study revealed that exogenous application of $Ca^{2+}$, in form of $Ca(NO_3)_2$, had at least two positive effects on photosynthetic activity – it stimulated xanthophyll cycle-dependent energy dissipation (NPQ) (at 25°C) and reduced the risk of PSII photoinhibition (Yang et al., 2015). We propose that exogenous $Ca^{2+}$ may protect the subunits of PSII reaction centres against photoinhibition by reducing the generation of ROS. Therefore, exogenous $Ca^{2+}$ seems to play an important role in alleviating stress and oxidative damage of cellular components such as membrane lipids. In plant exposed to thermal stress $Ca^{2+}$ ions may stabilize protein complexes and allow maintaining PSII activity. It is also postulated the $Ca^{2+}$ could alleviate stress connected with ROS generation inducing elongation of *pbsA2* gene product. *pbsA2* encodes D1 protein, fundamental component of PSII, which efficient regeneration is directly connected with its availability (Yang et al., 2015).

## 5. CONCLUSIONS

Concluding, among used compounds AsA seems to be most efficient activator of the xanthophyll cycle due to its influence on VDE activity, limited by AsA concentration in chloroplast compartments, especially in stress condition when AsA utilization by other metabolic pathway reaches the peak. Despite high efficiency in the xanthophyll cycle activation, AsA is also nontoxic, quite cheap and easy available chemical and may be considered as an important component of future sort of plant protecting compounds modifying their response to environmental stresses. A similar, positive effect was also observed for $Ca^{2+}$ application at 25-35°C, stimulating heat dissipation by NPQ. Study proved that $Ca^{2+}$ has protective role exclusively in pre-illuminated samples, which could be consequence of its leverage effect on the D1 protein turnover. Despite Put application may be beneficial at elevated temperature because its reversible impact on energy dissipation, protecting the PSII, it should be made further investigation on its influence on heat stress improvement. DTT, used as a known VDE inhibitor, proved that observed results were directly connected to xanthophyll cycle controlled non-photochemical energy quenching with NPQ mechanism.

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