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High salicylic acid concentration alters the electron flow associated with photosystem II in barley

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ABSTRACT

In this study, the effects of exogenously applied salicylic acid (0.5 and 5 mM SA) on the rates of photosystem II (PSII) activity was analysed in 4-week-old barley (*Hordeum vulgare*'Bahman') seedlings using chlorophyll (Chl) *a* fluorescence transient (*OJIP*) measurements. No evident changes in Chl and carotenoid contents as well as chlorophyll fluorescence transient curves were observed in either of the studied concentrations after 24 h of SA application. After 5 d, low SA concentration (0.5 mM) increased PSII activity, Chl *b* and carotenoid contents in barley seedlings. In contrary, 5 days after 5 mM SA treatment, the maximal quantum efficiency of PSII (F_v/F_m) and the Performance Index (PI_{ABS}), as an indicator of PSII structure and functioning, were significantly decreased. This lower F_v/F_m and PI_{ABS} coupled with lower levels of Chl *b* and carotenoids, and lower values of photosynthetic electron transport chain components including the electron transport flux (φE_o) and the inferred oxygen evolving complex activity (F_v/F_o). By monitoring the chlorophyll *a* fluorescence rise kinetics, from the initial "*O*" level to the "*P*" (the peak) level, a dramatic increase in "*OJ*" phase was detected, which coincides with an increased photo-reduction of Q_A as a result of blockage of electron flow. This study provided the evidence that the high concentration of SA induced damage to different sites of the PSII.

Key words: photosynthetic pigments; photosynthetic electron flow; *Hordeum vulgare* 'Bahman'; *OJIP* transient flourescence; salicylic acid

IZVLEČEK

VELIKA KONCENTRACIJA SALICILNE KISLINE SPREMINJA PRI JEČMENU FOTOSINTEZNI, S FOTOSISTEMOM II POVEZAN ELEKTRONSKI PRETOK

V raziskavi so bili preučevani učinki dodajanja salicilne kisline (0.5 in 5 mM) na aktivnost fotosistema II (PSII) pri 4-tedne starih kalicah ječmena (*Hordeum vulgare*'Bahman') z meritvami fluorescence (*OJIP*) klorofila a (Chla). Nobenih sprememb v vsebnosti klorofila in karotenoidov kot tudi ne sprememb v fluorescenci ni bilo opaznih po 24 urah dodajanja obeh koncentracij salicilne kisline. Po petih dneh so se v kalicah ječmena pri dodani manjši koncentraciji salicilne kisline (0.5 mM) povečali aktivnost PSII, vsebnost Chl b in karotenoidov. Nasprotno sta se pet dni po obravnavanju s 5 mM salicilno kislino značilno zmanjšala učinkovitost PSII (F_v/F_m) in PI_{ABS} indeks kot indikatorja zgradbe in delovanja PSII. Zmanjšanje F_v/F_m in PI_{ABS} je bilo povezano z zmanjšanjem vsebnosti klorofila b in karotenoidov ter z manjšimi vrednostimi komponent fotosintezne elektronske verige, vključno s elektronskim pretokom (φE_o) in z njim povezano aktivnostjo kompleksa, ki sprošča kisik (F_v/F_o). Pri spremljanju povečanja fluorescence klorofila a od začetne "*O*" na največjo vrednost "*P*" je bilo opazno njeno dramatično povečanje v fazi "*OJ*", kar je soupadalo s povečano fotoredukcijo Q_A kot posledica blokade fotosintezenega elektronskega pretoka. Raziskava dokazuje, da večja koncentracija salicilne kisline povzroči poškodbe na večih mestih PSII.

Ključne besede: fotosintezna barvila; fotosintezni elektronski pretok; *Hordeum vulgare* 'Bahman', *OJIP* prehodna fluorescenca; salicilna kislina

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1 INTRODUCTION

Salicylic acid (SA), as a common plant-produced phenolic compound, plays an important role in plant growth and development as well as in tolerance to biotic and abiotic stresses (Li et al., 2014; Khan et al., 2014; Janda and Ruelland, 2015). In recent years the involvement of SA in the plant growth and yield (Javaheri et al., 2012), and the regulation of some photosynthetic reactions (Arfan et al., 2007; Li et al., 2014) has widely been studied.

It has been suggested that the effects of SA on plant physiological and biochemical processes depends on the concentration of the applied SA (Miura and Tada, 2014). At low concentrations (0.1–0.5 mM for most plants), it enhances the efficiency of the antioxidant system and the efficiency of PSII photochemistry (Chen et al., 2016), whereas at higher concentrations (1-10 mM for most plants) it increases oxidative damage (Hara et al., 2012; Miura and Tada, 2014). Although negative effect of SA is probably correlated with an imbalance in antioxidant metabolism (Hasanuzzaman et al., 2013), the specific mechanisms of SA-mediated damages remain elusive. It is assumed that, plants respond to high SA depend on PSII response to this stress (Chen et al. 2016). To address this issue, the chlorophyll (Chl) *a* fluorescence transient (OJIP) measurements were used to study photosynthetic apparatus functioning in response to various SA concentrations and incubation times in this study.

Chl *a* fluorescence induction (*OJIP*, where *O* (or F_o) is the minimum fluorescence when all Q_A (the primary quinone acceptor of PSII) are in the oxidized state, *P* (or F_{max}) is the maximal fluorescence when all Q_A is in the reduced state (QA^-)) has been studied extensively in photosynthesis physiology research (Jee, 1995; Kalaji et al., 2011; Hamdani et al., 2015). The reduction of Q_A by PSII causes chlorophyll *a* fluorescence to rise from its minimal fluorescence level "*O*" to a "*J*" level (or F_J). Fluorescence rise from "*J*" level to the "*I*" level (or F_I) is related to the filling up of the plastoquinone pool. Finally, a traffic jam of electrons on the electron acceptor side photosystem I generates a fluorescence rise from the "*I*" level to the "*P*" level. The analysis of chlorophyll *a* fluorescence signals using '*JIP*-test', explores the information about the structure and function of the photosynthetic apparatus mostly related to PSII (Strasser et al., 2000; Bussotti et al., 2007) as well as some parameters due to energy fluxes for light absorption (ABS), trapping (TR) of excitation energy and electron transport (ET) per reaction center (RC) or per sample area called cross-section (CS) (Strasser et al., 2000).

As a noted above, the exact mechanisms by which SA affects photochemistry remain obscure. The present paper is the first report on the SA-mediated changes in specific chlorophyll fluorescence parameters. In order to improve our knowledge of barley photosynthetic apparatus in response to SA treatment, the *OJIP* fluorescence transient was measured in barley plants in responses to different concentrations of SA.

2 MATERIALS AND METHODS

2.1 Plant material and harvest

The randomly selected healthy seeds of barley (*Hordeum vulgare* 'Bahman') were sterilized with 5 % sodium hypo-chlorite solution for five minutes prior to sowing. Seeds were then sown on filter paper moistened with distilled water. Ten-day-old seedlings were transferred to modified Hoagland nutrient solution (Johnson et al. 1957) containing 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM H_2MoO_4 and 0.02 mM $FeSO_4$-EDTA for 15 days prior to the start of treatments. The pH of the nutrient medium was adjusted to 5.5–5.7. The seedlings were grown in a controlled growth room under a 16/8 light/dark cycle and a photosynthetically active radiation (PAR) of 200 ± 30 µmol m^{-2} s^{-1} and an average day/night temperature of 25 ± 1/18 ± 1 °C. Salicylic acid (SA) was dissolved in absolute ethanol then added drop wise to water (ethanol/water: 1/1000 v/v, pH was adjusted to 5.7) (Williams et al. 2003). At 25 days after germination, the foliar application of SA was carried out in the morning (between 08:00 and 10:00) with a compression sprayer of 1 L capacity. Non-SA applied plants were sprayed with ethanol/water (1/1000 v/v). At 1 and 5 days after treatment, the plants were harvested and the recent fully expanded and mature leaves were used for measurement of chlorophyll fluorescence and other analysis.

2.2 Chlorophyll *a* fluorescence measurements

Chlorophyll *a* fluorescence transients (*OJIP* transients) were measured with a Packet-PEA chlorophyll fluorimeter (Plant Efficiency Analyser, Hansatech Instruments Ltd., King's Lynn, Norfolk, PE 32 1JL, England) in dark-adapted (for at least 20 min) leaves of barley. We used the *JIP*-test (Strasser and Strasser,

1995; Strasser et al., 2004) to analyse chlorophyll a fluorescence rises. The measured and calculated parameters are described in Tab 1. Specific parameters were calculated from energy fluxes for light absorption (ABS), trapping (TR) of excitation energy and electron transport (ETR) per reaction center (RC) or per sample area called cross-section (CS).

Table 1: Some groups of measured and calculated parameters using the *JIP*-test (Yusuf et al., 2010)

Nomenclature	Explanation
Data extracted from the recorded fluorescence transient OJIP	
Area	Total complementary area between F_o and F_m (reflecting the size of the plastoquinone pool)
F_J	Fluorescence intensity at the *J*-step (2 ms) of *OJIP*
F_I	Fluorescence intensity at the *I*-step (30 ms) of *OJIP*
Fluorescence parameters derived from the extracted data	
F_m or F_{max}	Maximal chlorophyll fluorescence intensity measured when all photosystem II (PSII) reaction centers are closed
F_o	Minimal fluorescence (all PSII RCs are assumed to be open)
F_v	Variable chlorophyll fluorescence ($F_m - F_o$)
V_j	Relative variable fluorescence at time *J* (relative variable fluorescence at phase *J* of the fluorescence induction curve
The specific energy fluxes (per reaction center, RC)	
ABS/RC	Light absorption flux (for PSII antenna chlorophylls) per RC
DI/RC	Dissipation energy flux per RC
ET/RC	Maximum electron transport flux (further than Q_A^-) per RC
TR/RC	Trapped (maximum) energy flux (leading to Q_A reduction) per RC
The phenomenological energy fluxes (per excited cross-section of leaf, CS)	
ABS/CS	Absorbed photon flux per cross section
TR/CS	Maximum trapped excitation flux per cross section
ET/CS	Electron transport flux from Q_A to Q_B per cross section
DI/CS	Dissipation energy flux per cross section
Quantum yields and efficiencies	
φE_o or phi (E_o)=ET_o/ABS	Quantum yield for electron transport (ET)
De-excitation rate constants of PSII antenna	
k_N	Non-photochemical de-excitation rate constant
k_P	Photochemical de-excitation rate constant
Performance index	
PI_{ABS}	The performance index that is calculated as: $(RC/ABS)\times(\varphi_{Po}/(1-\varphi_{Po}))\times(\psi_o/(1-\psi_o))$, where, RC is for reaction center; ABS is for absorption flux; φ_{Po} is for maximal quantum yield for primary photochemistry; and ψ_o is for the quantum yield for electron transport

2.3 Determination of total carotenoids and chlorophylls *a* and *b*

Leaf concentrations of chlorophylls and carotenoids were measured after extraction of pigments in the methanol according to Lichtenthaler and Wellburn (1985). The weighed samples were homogenized with homogenizer at 1000 rpm for one minute. The homogenate was filtered, and was centrifuged at 2500 rpm for 15 minutes. The supernatant was separated and the absorbance was read at 400-700 nm on spectrophotometer. Leaf concentrations of chlorophylls and carotenoids were calculated as:

$$\text{Chl } a = 15.65\ A_{666} - 7.340\ A_{653} \quad \text{(Eq.1)}$$

$$\text{Chl } b = 27.05\ A_{653} - 11.21\ A_{666} \quad \text{(Eq.2)}$$

$$\text{Total carotenoids} = 1000\ A_{470} - 2.860\ \text{Chl}a - 129.2\ \text{Chl}b/245 \quad \text{(Eq.3)}$$

2.4 Statistical analysis

Experiments were performed in complete randomized block design. All data satisfied the assumption for ANOVA for normal distribution and homogeneity of variance. Chlorophyll fluorescence were done on 20 plants from each treatment, and 3 replicates for each plant (n = 60) while the other measurements were performed on 4 plants from each treatment and we had one replicate for each plant (n = 4). Statistical analysis was carried out using Sigma Stat (3.5) with Tukey test ($P<0.05$). Correlation analysis using Spearman Rank Order Correlation in Sigma Stat was done to determine the relationship between PI_{ABS} and leaf carotenoids.

3 RESULTS AND DISCUSSION

We found that the low concentration of SA (0.5 mM) caused a significant increase in Chl *b* and carotenoid content compared with the control (Fig. 1). Our results are in line with the findings of Singh and Usha (2003), and Javaheri et al. (2012), who observed that the treatment with low SA increases photosynthetic pigment contents in some plants under normal or stress conditions. In the present study, treatment with higher concentration of SA (5 mM) resulted in a lower total Chl and carotenoid content as compared to the control. In agreement with our results, Chandra and Bhatt (1998), Moharekar et al. (2003) and Hayat et al. (2010) reported that high SA concentrations (1–5 mM) induced a reduction of chlorophyll contents in wheat and *Arabidopsis*.

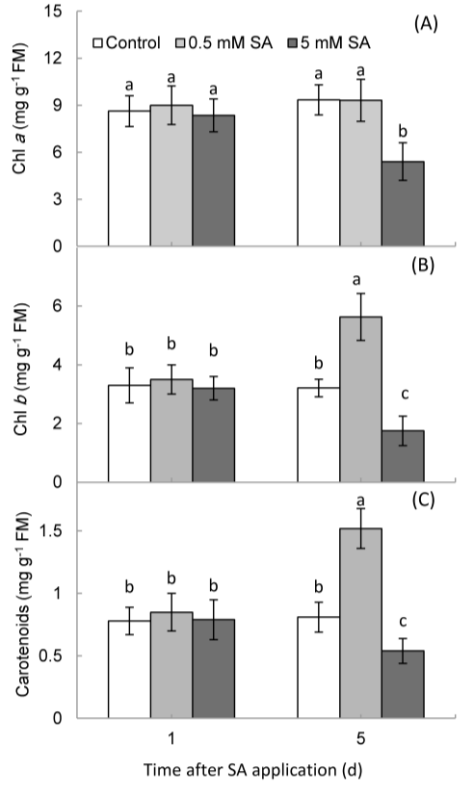

Figure 1: Effects of SA concentrations (0.5 and 5 mM) on the concentration of chlorophyll *a* (A), chlorophyll *b* (B) and total carotenoids (C) at different time intervals after treatment in barley plants. Bars indicated with the same letter are not significantly different ($p < 0.05$). Values are the mean ± SD ($n = 4$)

In present experiment, chlorophyll *a* fluorescence signals were measured by using the '*JIP*-test' (Strasser et al., 2000, 2004), in order to analyse the responses of the photosynthetic apparatus and energy flow among PSII in response to SA treatment. After 24 h of SA treatment, a slight decrease in the *IP* phase was noticed (Fig. 2). 5 days after 0.5 mM SA treatment, a much slower fluorescence rise from level "*I*" to a "*P*" level (or F_m) was observed, which coincided with a large increase in F_o fluorescence (Fig. 3), due to the structural damage leading to decreased excitation energy transfer from the antenna to the reaction center (Kalaji et al., 2011). Under these conditions, an upregulation of *OJ* phase was detected. This higher *OJ* phase rise is closely related to the increased photo-reduction of Q_A in the active PSII centers (Stirbet and Jee, 2011, 2012), mainly because of a blockage of electron flow.

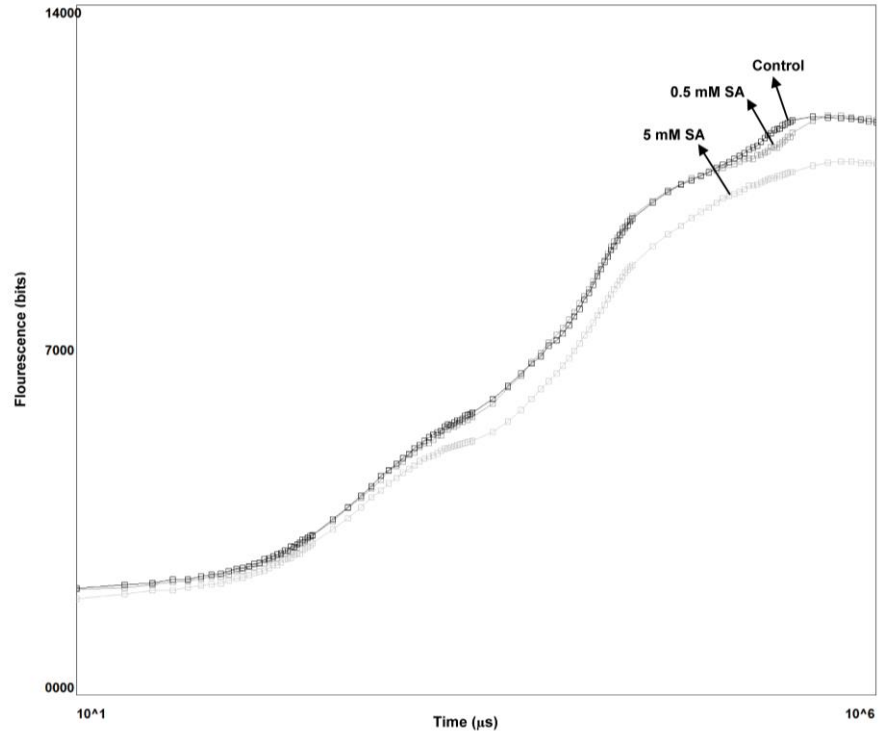

Figure 2: Chlorophyll *a* fluorescence induction curve of barley seedlings grown under 0 mM (Control), 0.5 mM and 5 mM SA for 24 h

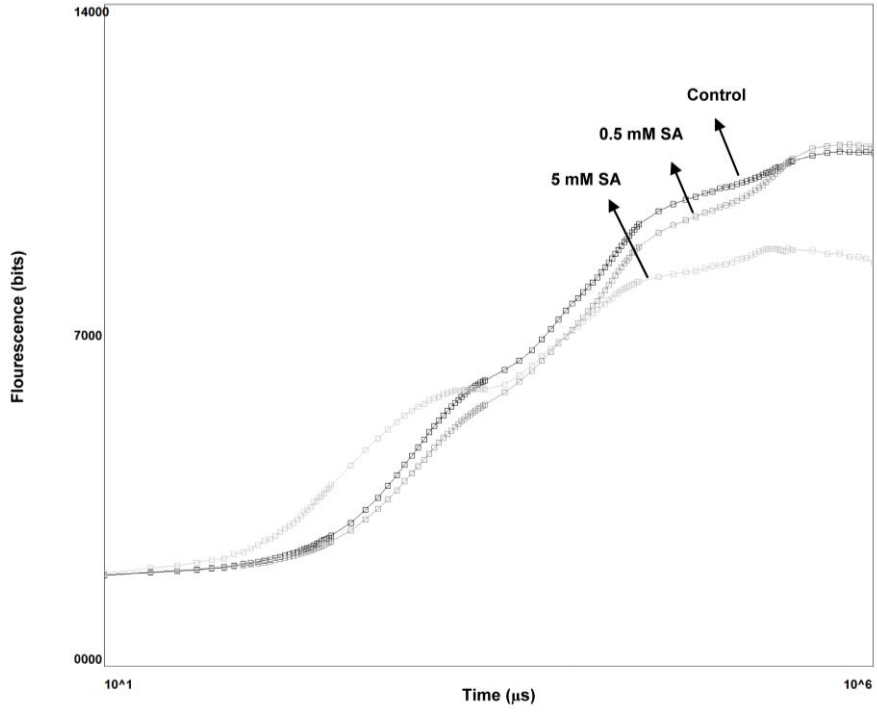

Figure 3: Chlorophyll *a* fluorescence induction curve of barley seedlings grown under 0 mM (Control), 0.5 mM and 5 mM SA for 5 days

After 24 h of SA treatment, the values of the maximal quantum efficiency of PSII (F_v/F_m) and the efficiency of the water-splitting complex on the donor side of PSII (as inferred from F_v/F_o) were similar to those of control plants (Fig. 4 and 5). But after 5 days of 0.5 mM SA treatment, an increase in the PSII function, as estimated by a large increase in performance index (PI_{ABS}), was obtained (Fig. 4 and 6). Carotenoids play an important role in photosynthesis and photoprotection (Cazzonelli and Pogson, 2010; Habibi and Ajory, 2015). Accordingly, we suggest that the increase in PI_{ABS}, after 5 days of treatment at 0.5 mM SA, was associated with the increased Chl *b* and carotenoid levels. Indeed, the accumulation of carotenoids by 0.5 mM SA foliar spray in barley plants; helped them to maintain higher rates of photosynthesis and photosystem II activity (Dong et al., 2013; Habibi and Ajory, 2015).

In contrary, the decreased F_v/F_m and PI_{ABS} in plants treated with 5 mM SA indicated that the high concentration of SA induced damage to photosynthesis, which is agreement with the findings of Chen et al. (2016) in wheat. This decrease in PI_{ABS} is coupled with lower levels of Chl *b* and carotenoids. In confirmation of this, there was a significant correlation ($r = 0.84$, $p<0.01$) between PI_{ABS} and carotenoid level in SA-supplied plants (Fig. 7). Additionally, this down-regulation of F_v/F_m and PI_{ABS} was associated with decreases in electron transport flux per chlorophyll (φE_o) and in efficiency of the water-splitting complex on the donor side of PSII (F_v/F_o) (Fig. 6), which might be related to the photosynthetic electron transport impairment (Pereira et al., 2000). In addition, we suggest that the increased accumulation of inactive reaction centers was due to the significantly higher values of the efficiency of non-photochemical de-excitation processes (K_N) (Kalaji et al., 2011). After 5 days of 5 mM SA application, the specific flux of energy (DIo/RC; dissipative energy flux per reaction center and ABS/RC; the absorption flux per reaction center) parameters were much higher than those determined in control plants (Fig. 6). Thus, the increase in ABS/RC might represent a compensatory mechanism (van Heerden et al., 2007) for maintaining electron transport flux per remaining active reaction centers. The specific rate of the electron transport from Q_A to Q_B, depends on the V_J (relative variable fluorescence at time *J*) value (Stirbet and Jee, 2011), and this parameter was significantly increased after 5 days of 5 mM SA application in the present study.

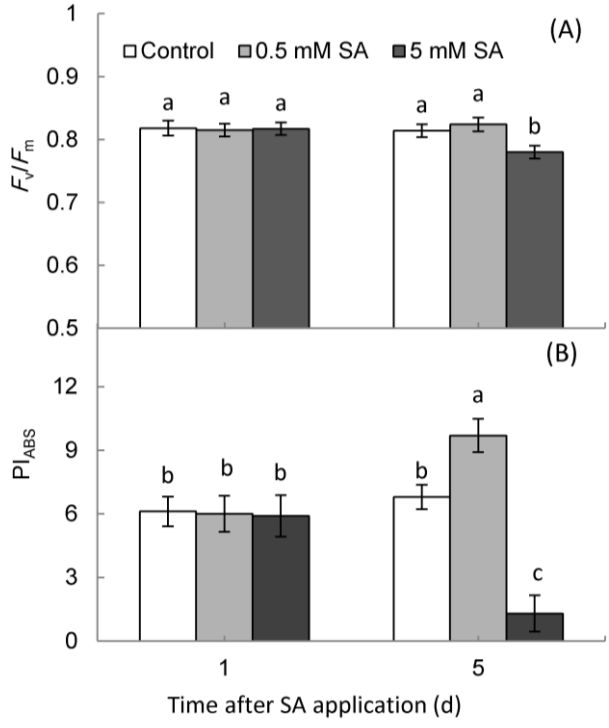

Figure 4: Effects of SA concentrations on the maximum quantum yield of PSII (F_v/F_m) (A) and the Performance Indexes (PI_{ABS}) (B) at different time intervals after treatment in barley plants. Bars indicated with the same letter are not significantly different ($p < 0.05$)

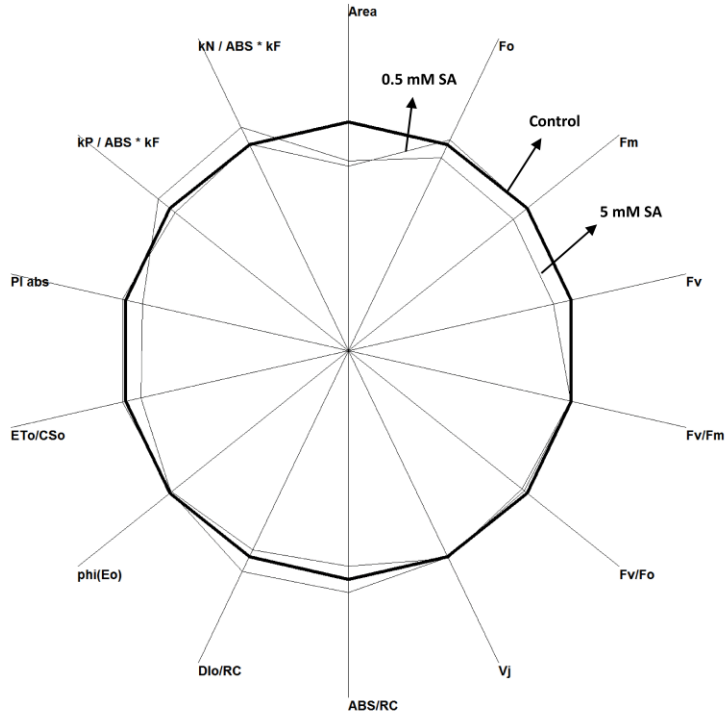

Figure 5: A 'spider plot' of selected parameters characterizing behavior of photosystem II of barley leaves exposed 24 h to 0 mM (Control), 0.5 mM and 5 mM SA (See Tab 1 for the meaning of the parameters)

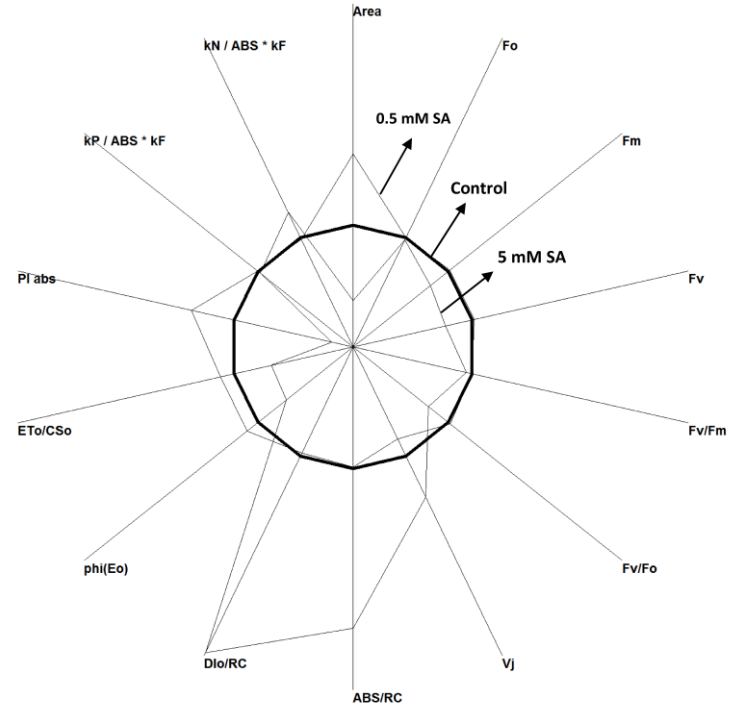

Figure 6: A 'spider plot' of selected parameters characterizing behavior of photosystem II of barley leaves exposed 5 days to 0 mM (Control), 0.5 mM and 5 mM SA (See Tab 1 for the meaning of the parameters)

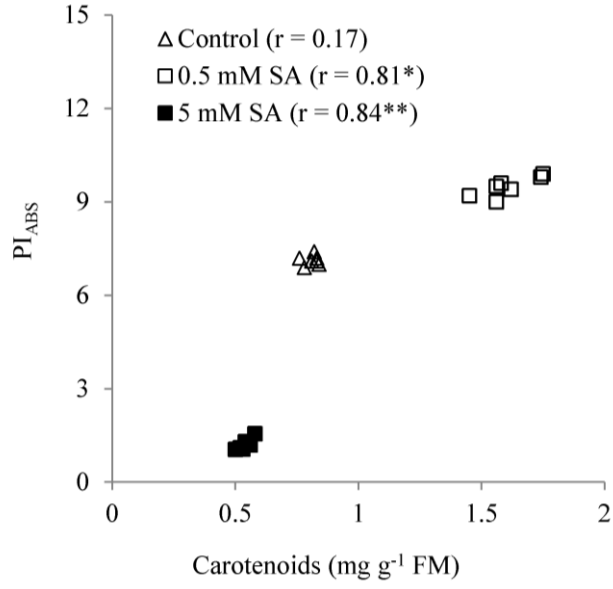

Figure 7: Correlations between the Performance Indexes (PI_{ABS}) and the leaf carotenoids levels in barley plants grown for 5 days under 0 mM (Control), 0.5 mM and 5 mM SA treatment: ns, *, and **: non-significant, significant at the 5 % and 1 % levels of probability, respectively

In conclusion, SA at low concentration improved the efficiency and the yield of energy transfer and primary photochemistry in barley seedlings as related to the higher levels of Chl *b* and carotenoids. In contrary, several parameters related to PSII activity (e.g., the time needed to reach the maximal chlorophyll fluorescence, the variable fluorescence, the inferred oxygen evolving complex activity, the electron transport flux, and the calculated Performance Index) were significantly decreased by SA application at high concentration indicating that the high concentration of SA induced damage to photosynthesis. On the other hand, increasing the photosynthetic activity of barley plants at low SA concentration can help for crop research and practical applications in order to improve crop productivity and increase plant nutritional value for a growing world population.

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