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Australian Journal of Plant Physiology

Volume 27, 2000
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AJPP

An international journal of plant function

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the Australian Academy of Science

Aust. J. Plant Physiol., 2000, **27**, 1085–1091

Electrokinetic properties of thylakoids in *in vitro* cultured *Gypsophila paniculata* plants

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Abstract. *In vitro* cultured *Gypsophila paniculata* L. plants were used as a model to evaluate the effect of some cytokinins and anticytokinins on thylakoid surface charge. Influence of the cytokinins *N*-6-furfurylaminopurine (kinetin) and N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (4-PU-30), cytokinin antagonists 2-chloro-4-cyclobutylamino-6-ethylamino-1,3,5-triazine and *N*-(4-pyridyl)-*O*-(4-chlorophenyl) carbamate on the pigment content, surface charge density (σ), fluorescence induction kinetics and millisecond-delayed light emission was studied. Our results showed that the chlorophyll (a+b) content significantly decreased after the 1st and the 2nd month of *G. paniculata* growth in the presence of the cytokinins kinetin and 4-PU-30. In our model system, cytokinins enhanced the number of open lateral buds and, as a consequence, more shoots per explant. Hence, chlorophyll synthesis was not inhibited but so-called 'dilution of the pigments' was available. Anticytokinins inhibited the formation of more than one shoot, and the chlorophyll content was not influenced significantly. The phenylurea cytokinin 4-PU-30 and anticytokinins increased the electrophoretic mobility, zeta potential and surface charge density of thylakoids after a longer time of treatment. Making thylakoid membranes more negatively charged, phenylurea cytokinin and anticytokinins increased the aggregation of the complexes and the energization of the membrane. Our results showed that plant growth regulators decreased the primary photochemical activity of photosystem II (estimated by the ratio F_v/F_m) and delayed fluorescence intensity in the 1st month. However, no significant changes were observed in these parameters in the 2nd month.

Keywords: cytokinin, cytokinin antagonist, electrophoretic mobility, *Gypsophila paniculata*, prompt and delayed fluorescence, surface charge density, zeta potential.

Introduction

The membrane surface electric charge is a characteristic of cell metabolism. It modulates ion exchange between the cell and its extracellular medium. The existence of electrical charges on the surface of chloroplast thylakoid membranes has important implications (Barber 1980). Electrical properties of thylakoid membranes control a number of photosynthetic phenomena including thylakoid stacking, membrane conformational changes and electron transport (Barber 1982).

The net negative electrical charge on the outer surface of the thylakoid membrane is mainly due to the carboxyl groups of glutamic and aspartic acid residues of exposed portions of integral proteins. Other components, giving little or no contribution from the head groups of the minor acidic lipids, are negatively charged phosphatidylglycerol and sulfolipid sulfoquinovosyldiacylglycerol (Berg *et al.* 1974; Nakatani *et al.* 1978; Nakatani and Barber 1980). At neutral pH, thylakoid membrane surfaces carry excess negative electrical charge. Below pH 4.3–4.5 the surface becomes positively charged, which results from the guanidine group of exposed

Abbreviations used: ACK-1, 2-chloro-4-cyclobutylamino-6-ethylamino-1,3,5-triazine; ACK-2, *N*-(4-pyridyl)-*O*-(4-chlorophenyl) carbamate; Chl, chlorophyll; $(a+b)/(x+c)$, weight ratio of chlorophylls to carotenoids; D, minimum of the delayed fluorescence induction kinetics; DF, delayed fluorescence; EDTA, ethylenediaminetetraacetic acid; EPM, electrophoretic mobility; F_o , initial, F_v, variable and F_m, maximum chlorophyll fluorescence; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); I, maximum of the delayed fluorescence intensity; kinetin, *N*-6-furfurylaminopurine; P, maximum of the intersystem's electron transport connected to the delta pH gradient formation delta pH gradient formation; PS I and II, photosystem I and II; 4-PU-30, N_1-(2-chloro-4-pyridyl)-N_2-phenylurea; Q_A and Q_B, the primary and secondary plastoquinone acceptors of PS II; s.e., standard error of mean; $\boldsymbol{u}$, mean of EPM, ζ, zeta potential; σ, surface charge density.

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10.1071/PP99042 0310-7841/00/111085

arginine residues (Nakatani and Barber 1980; Goltsev *et al.* 1983; Doltchinkova 1990). The average surface charge density (σ) may vary depending on growth conditions that alter the relative levels of appressed and non-appressed membranes (Barber 1986).

The behavior of cells in an electric field allows measurement of electrophoretic mobility and characterization of the surface properties (Bauer and Golovanov 1999). Zeta potential (ζ), the electrostatic potential at the hydrodynamic plane of shear, is calculated from the measured value of electrophoretic mobility. Zeta potential is determined by the genotype of the cell and is a constant physiological parameter (Bauer and Golovanov 1999). It could be used to estimate the stability and electrokinetic properties of colloidal-dispersed systems.

The purpose of this study was two-fold: firstly, we wanted to investigate the changes in electrokinetic properties of thylakoid membranes isolated from *in vitro* cultured *Gypsophila paniculata* L. plants in the presence of cytokinins and anticytokinins. These classes of chemicals, as well as many pharmacologically active drugs such as antibiotics and steroids, interact with the lipid components of the membrane and change their physical properties (Bloom *et al.* 1991). This is because the lipid components could be involved in zeta potential formation due to interaction between the foreign molecules and the various parts of the membrane. Secondly, since the surface electrical properties of thylakoid membranes influence a number of photosynthetic characteristics, we were also looking for a relationship between the surface charge and the photosynthetic activity of thylakoid membranes. Therefore, the effect of cytokinins and anticytokinins on photosynthetic activity, as estimated by the parameters of prompt and delayed chlorophyll fluorescence, was also studied. The ratios F_v/F_m and F_v/F_o are considered to be a measure of photosystem II (PS II) effectiveness in the primary photochemical reactions (Butler 1977; Krause and Weis 1991). Delayed chlorophyll fluorescence registered in the millisecond time-range may be used as an informative parameter of photosynthetic activity, and originates in plants from the recombination of primary separated charges in the reaction center of PS II (Lavorel 1975). The initial rapid rise of millisecond-delayed fluorescence (DF) of thylakoids is related to the electric potential difference (Δψ) and the secondary slow rise to the proton concentration difference (ΔpH) generated across the membranes (Itoh *et al.* 1971; Wraight and Crofts 1971).

There is no experimental data available concerning the influence of plant growth regulators on thylakoid surface charge. Since the surface electrical properties of thylakoid membranes influence the number of photosynthetic characteristics, we are studying thylakoid surface charge modification in relation to functional parameters of thylakoid membrane activity after 1 and 2 months of *in vitro* cultivation of *G. paniculata* plants.

Materials and methods

Plant material

Gypsophila paniculata L. cv. 'Bristol Fairy' plants were cultured *in vitro* on standard full-strength MS medium (Murashige and Skoog 1962), with 2.0% sucrose and 0.8% agar. The medium was supplemented with cytokinins [*N*-6-furfurylaminopurine (kinetin) — a cytokinin of the purine type, and N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (4-PU-30) — a cytokinin of phenylurea type)] and anticytokinins [triazine anticytokinin, 2-chloro-4-cyclobutylamino-6-ethylamino-1,3,5-triazine (ACK-1) and the carbamate anticytokinin, *N*-(4-pyridyl)-*O*-(4-chlorophenyl) carbamate (ACK-2)], at a concentration of 10^{-6} M, pH 5.7 (KOH) before autoclaving. Autoclaving does not affect the activity of these organic molecules. Cultures were grown in a growth chamber at 20°C, with a 16-h photoperiod and 60 μmol m^{-2} s^{-1} photosynthetically active radiation, for 1 and 2 months.

Methods

Chloroplasts were isolated from the 1- and 2-month-old *in vitro* cultivated *G. paniculata* plants according to the method of Cerovic and Plesnicar (1984) with modifications. Harvested *G. paniculata* shoots were blended in a low-cation medium containing 343 mM sorbitol, 0.4 mM KCl, 0.04 mM ethylenediaminetetraacetic acid (EDTA), 2 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes) (KOH) buffer, pH 7.8. Plant material was ground with 20 mL of low-cation medium at 0° C. The resulting slurry was filtered through eight layers of muslin. The filtrate was centrifuged in a K24 'Janetski' type laboratory bench-top centrifuge (VEB Kombinat Medizin und Labortechnik, Leipzig, Germany) at 200 *g* for 60 s. Chloroplasts were quickly separated from the supernatant by centrifugation at 2000 *g* for 100 s. The remaining pellet was washed in medium containing 25 mM Hepes (KOH) buffer, pH 7.5, 300 mM sucrose and 10 mM NaCl.

The clean final pellet of chloroplasts was resuspended in 0.2 mL of resuspending medium (containing 25 mM Hepes (KOH) buffer, pH 7.5, 300 mM sucrose and 10 mM NaCl). Prior to use, thylakoids were diluted with an appropriate buffered medium (25 mM Hepes (KOH), pH 7.5, 10 mM NaCl) to a chlorophyll concentration of 4 μg chlorophyll (Chl) mL^{-1}. The transfer of isolated chloroplasts into a low salt medium (i.e. 10 mM NaCl) not only led to a loss of stacked membrane regions but also caused a complete intermixing of all intramembrane protein complexes (Staehelin 1986). 100 μL of the sample was further diluted into 20-mL (final volume) tubes of the low ionic strength medium.

Chlorophyll content, chlorophyll *a* to *b* ratios and the weight ratio of chlorophylls to carotenoids [(*a*+*b*)/(*x*+*c*)] were determined in 80% acetone according to Lichtenthaler (1987). The chlorophyll concentration was approximately 1 mg mL^{-1}.

Electrophoretic mobility studies were performed using a cytopherometer (OPTON, Feintechnik Ges, m.b.H., Wien, Austria). The OPTON cytopherometer was equipped with a television monitor, which allowed different types of thylakoid membranes to be observed, and migration by 20–40 particles to be timed. The observation light with an intensity of 15 μmol m^{-2} s^{-1} was used for electrophoretic mobility (EPM) measurements. The particles were observed under a light microscope connected to a video camera (Video Camera head CH-1400 CE, Sony Corporation, Japan) providing 800× magnification. Electrophoretic migration was timed for both forward and backward (reversed field) runs over a known distance of 32 μm. The s.e values of the electrophoretic mobility (***u***) were 2–6%. Usually the value of the EPM of stripped thylakoids without an outer envelope membrane is expressed in units of 10^{-8} m^2 V^{-1} s^{-1}.

Zeta potential was calculated from the measured value of the EPM by the Helmholtz–Smoluchowski equation (Overbeek and Wiersema 1967; Nakatani *et al.* 1978):

$$\zeta = \eta \cdot u/\varepsilon_r \cdot \varepsilon_o\,,$$

where ε_r is the dielectric constant of the aqueous phase (ε_r = 78.5 at 25°C), ε_o is the permitivity of free space ($\varepsilon_o = 8.8542 \times 10^{-12}$ F m^{-1}), and η is the viscosity of the aqueous phase (η = 0.000880 Pa s at 25°C).

When the thylakoid membrane surface is bathed in a medium containing a monovalent : monovalent electrolyte such as NaCl, and temperature = 298 K, the surface charge density is given by:

$$\sigma = 0.1174\ (C_{i\alpha})^{1/2} \sinh\ (Z\ F\ \psi_o/51.7)\,,$$

where σ is in C m^{-2}, $C_{i\alpha}$ is the concentration of the *i*th ion at an infinite distance from the membrane (i.e. in the bulk) in mol L^{-1}, F is the Faraday constant and ψ_o is the electrical potential at the surface (i.e. x = 0) in mV (Barber 1989). The electrical conductance and viscosity of the different media containing the thylakoids were measured with a Radelkis OK-104 conductometer (Hungary) and a Rheo viscosimeter (Germany), respectively. Assuming that ψ_o is equal to ζ, we can estimate the electrokinetic charge density of the observed membrane particles. The values of surface charge density are almost certainly underestimated because of the assumption that $\zeta \cong \psi_o$ (Barber 1980). Also, the technique of particle microelectrophoresis tends to give an average potential and does not give information about localized charge densities resulting from the heterogeneity of the surface charge distribution (Mansfield *et al.* 1982).

Millisecond-DF was measured in a quasi-steady-state regime by means of a conventional Becquerel-type phosphoroscope consisting of a pair of rotating disks with appropriately spaced holes (Matorin *et al.* 1976). The illumination time was 3.5 ms and the delayed fluorescence was measured 1.0–4.5 ms after illumination. The induction kinetics of DF were recorded after 3 min dark adaptation of the sample. The thylakoids suspended in a fused-silica cell were illuminated with red light, provided by a tungsten–iodine lamp. The mean irradiance of the white excitation light at the level of the sample was 250 µmol m^{-2} s^{-1}. Prompt chlorophyll fluorescence was detected in suspending medium and the F_o, F_v, F_m parameters and the F_v/F_m and F_v/F_o ratios were measured by a pulse amplitude modulated fluorometer (PAM 101-103, Heinz Walz, Effeltrich, Germany). Chlorophyll fluorescence was induced by illumination with an irradiance of about 250 µmol m^{-2} s^{-1}. The chlorophyll concentration was 50 µg mL^{-1} and the sample volume was 1 mL. Values of s.e. were calculated using Student’s *t*-distribution, each experiment including three replicates.

Results

Effect of cytokinins and anticytokinins on the pigment content of G. paniculata *plants*

Our results showed that the chlorophyll (*a*+*b*) content in *G. paniculata* decreased significantly after the 1st and the 2nd months of growth in the presence of the cytokinins kinetin and 4-PU-30 (Table 1). This was due to the reduction of both chlorophyll *a* and *b* contents. In contrast, the chlorophyll content of anticytokinin-treated explants was not significantly altered compared to the control. Normally, cytokinins stimulate chlorophyll synthesis and have an anti-senescence effect when cut leaves and cotyledons are used as explants. In our model system, cytokinins enhanced the number of open lateral buds and, as a consequence, more shoots per explant and higher fresh and dry weights were observed. Hence, chlorophyll synthesis was not inhibited, but so-called ‘dilution of the pigments’ was available. Anticytokinins inhibited the formation of more than one shoot, and decreased the fresh and dry weights, but the chlorophyll content was not significantly influenced.

The chlorophyll *a/b* ratio and (*a*+*b*)/(*x*+*c*) were not influenced by the cytokinin and anticytokinin treatment (Table 1).

Influence of cytokinins and anticytokinins on the electrokinetic properties of G. paniculata *thylakoids*

Data on the influence of the cytokinins and anticytokinins on the electrokinetic properties of *G. paniculata* thylakoids, evaluated by electrophoretic mobility, zeta potential and surface charge density are presented in Table 2. It was found that kinetin and 4-PU-30 did not change greatly the surface charge of the membrane. However, there was a tendency for enhancement of the net negative surface charge density of thylakoids, isolated from *G. paniculata* plants after 2 months growth in the presence of 4-PU-30 (Table 2). The electrophoretic studies indicated that ACK-1 increased EPM, zeta potential and surface charge density by approximately 15% in the 1st and 2nd months. The increase in electrokinetic charge upon treatment of the thylakoid membranes corresponded to an increase in electric polarizability. In this case, the electric polarazability was determined by the mobile electrokinetic charge. No specific conductance change in the thylakoid suspensions in time was registered (specific conductance varied from 1.98 to 2.15 mS cm^{-1}). Enhanced σ was also found for ACK-2, but only in the 2nd month.

Influence of cytokinins and anticytokinins on the chlorophyll fluorescence parameters of G. paniculata *thylakoids*

According to Crofts *et al.* (1971), delayed fluorescence may be affected by the membrane potential. After illumination of the dark-adapted photosynthesizing objects, the intensity of the millisecond-DF undergoes characteristic changes (OIDP), which reflects the transition of the photosynthetic apparatus from the dark to the light state (Ganeva *et al.* 1988). The induction kinetics of the DF in isolated thylakoids showed two pronounced maxima. The maximum of the delayed fluorescence intensity (**I**) is proportional to the effectiveness of the dark-adapted reaction centre luminescence. The DF intensity reflects the electron transfer dynamics in the acceptor side of PS II from Q_A^- via Q_B to plastoquinone. The fast phase **I** could be related to rapid dissipation of the photoinduced transmembrane electric gradient (Wraight and Crofts 1971; Schmidt and Schneckenburger 1995). ‘**I-D**’ decay (where **D** is the minimum of the delayed fluorescence induction kinetics) correlates to the transition of one part of the PS II reaction centres into the *closed* state during illumination (Goltsev 1979) as a result of the reduction of primary acceptor Q. The slow phase **D-P** (where **P** is the maximum of the inter-

system's electron transport connected to the delta pH gradient formation) reflects the increase of the DF yield caused by photoinduced energization on the thylakoid membrane (Barber and Kraan 1970), i.e. **D-P** is connected with proton gradient formation.

Like the parameter connected with the level of proton gradient, the degree of luminescent enhancement through the slow phase **(P-D)/D** was used. The maximum **P** is connected with the appearance of the intersystem electron transport, related to ΔpH, which will define the level of **P**, as well as a generation of charges in the reaction centres of PS I and II (Satoh *et al.* 1983).

The exogenously added cytokinins and anticytokinins influenced the induction kinetic in different ways. The cytokinins slightly altered the first phase **I** of the induction kinetics of DF in both months (Fig. 1). The anticytokinins decreased the phase **I** by about 30% compared to the control in the 1st month ($P < 0.05$) but they did not significantly influence DF intensity in the 2nd month.

Data about the influence of cytokinins and anticytokinins on the formation of the proton gradient across the thylakoid membrane **(P-D)/D** are presented in Fig. 2. The results show that the treatment of *G. paniculata* plants with plant growth regulators caused a pronounced enhancement of the ΔpH gradient. The effect of anticytokinins on the formation of the ΔpH gradient was significantly higher than that of cytokinins in the 1st month. As already observed for surface charge density, the effect of 4-PU-30 on the **(P-D)/D** ratio was similar to that of ACK-1 and ACK-2 in the 2nd month.

In order to determine whether phytohormones influence PS II activity, we recorded the chlorophyll fluorescence induction kinetics. This allowed us not only to measure the parameters of prompt chlorophyll fluorescence (F_o, F_m, F_v

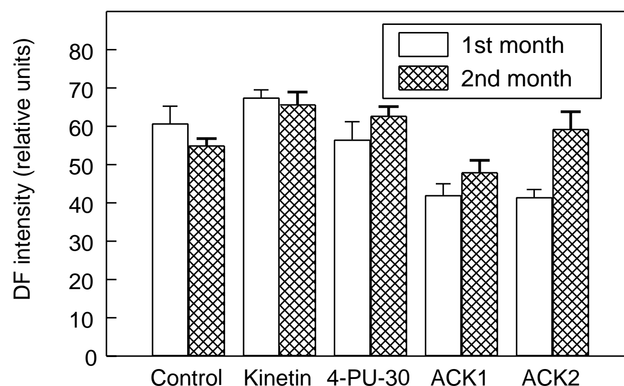

Fig. 1. Influence of cytokinins and anticytokinins on the delayed fluorescence intensity (phase **I**) in thylakoids isolated from *in vitro* cultured *G. paniculata* plants. Thylakoids were suspended in a medium of 25 mM Hepes (KOH) buffer, pH 7.5 and 10 mM NaCl, to a chlorophyll concentration of 4 μg mL^{-1}.

Table 1. Effect of cytokinins and anticytokinins on the chlorophyll content (μg g^{-1} FW), chlorophyll *a*/*b* ratio and weight ratio of chlorophylls to carotenoids [(*a*+*b*)/(*x*+*c*)] of *in vitro* cultured *Gypsophila paniculata*
Data are means (± s.e.) of three replicates from three independent experiments (n = 9). Significant difference from control plants is indicated by * ($P < 0.05$) and ** ($P < 0.01$)

Treatment	1st month			2nd month		
	Chl content	*a*/*b*	(*a*+*b*)/(*x*+*c*)	Chl content	*a*/*b*	(*a*+*b*)/(*x*+*c*)
Control	554.5 ± 35	2.5 ± 0.06	8.9 ± 0.32	538.0 ± 33	2.7 ± 0.08	9.4 ± 0.40
Kinetin	394.6 ± 25*	2.5 ± 0.10	8.0 ± 0.35	319.3 ± 25**	2.5 ± 0.06	7.3 ± 0.37
4-PU-30	260.0 ± 19**	2.3 ± 0.08	8.6 ± 0.40	259.3 ± 24**	2.6 ± 0.10	6.5 ± 0.38
ACK-1	506.0 ± 31	2.4 ± 0.12	6.7 ± 0.28	501.4 ± 30	2.6 ± 0.09	6.6 ± 0.42
ACK-2	569.0 ± 34	2.5 ± 0.10	7.0 ±0.30	473.0 ± 28	2.5 ± 0.11	6.3 ± 0.35

Table 2. Influence of some cytokinins and anticytokinins on the electrophoretic mobility (*u*), zeta potential (ζ) and surface charge density (σ) of thylakoids isolated from *in vitro* cultured *Gypsophila paniculata* plants
The suspending medium contained 25 mM Hepes (KOH) buffer, pH 7.5 and 10 mM NaCl. Data are means (± s.e.) of three replicates from three independent experiments. Significant difference from control plants is indicated by * ($P < 0.05$)

Treatment	1st month			2nd month		
	$u \times 10^8$ (m^2 V^{-1} s^{-1})	ζ (mV)	$\sigma \times 10^3$ (C m^{-2})	$u \times 10^8$ (m^2 V^{-1} s^{-1})	ζ (mV)	$\sigma \times 10^3$ (C m^{-2})
Control	–2.15 ± 0.04	–27.3 ± 0.5	–6.48 ± 0.13	–1.89 ± 0.02	–23.8 ± 0.3	–5.61 ± 0.07
Kinetin	–2.27 ± 0.13	–29.8 ± 1.5	–7.16 ± 0.42	–1.92 ± 0.05	–24.2 ± 0.5	–5.70 ± 0.13
4-PU-30	–2.04 ± 0.19	–25.8 ± 2.4	–6.11 ± 0.63	–2.06 ± 0.13	–26.1 ± 1.6	–6.18 ± 0.43
ACK-1	–2.47 ± 0.08	–31.2 ± 1.1	–7.52 ± 0.30	–2.14 ± 0.14	–27.1 ± 1.7	–6.45 ± 0.46
ACK-2	–2.16 ± 0.10	–27.3 ± 0.2	–6.49 ± 0.33	–2.18 ± 0.10	–27.6± 1.2	–6.58 ± 0.32

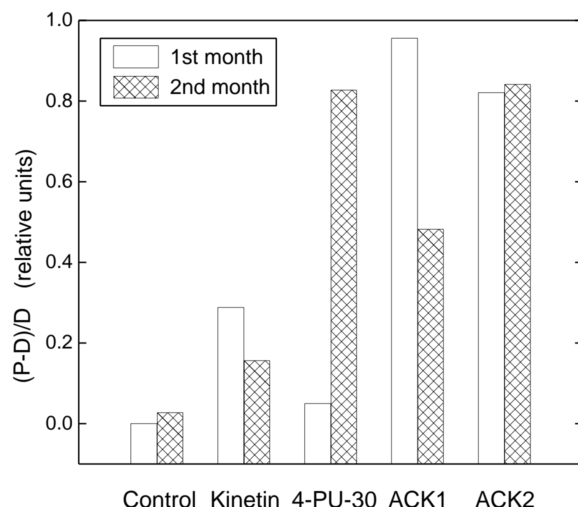

Fig. 2. Influence of cytokinins and anticytokinins on the transmembrane proton gradient, measured by the **(P-D)/D** ratio in thylakoids isolated from *in vitro* cultured *G. paniculata* plants. Thylakoids were suspended in a medium of 25 mM Hepes (KOH) buffer, pH 7.5 and 10 mM NaCl, to a chlorophyll concentration of 4 μg mL^{-1}.

and the ratios F_v/F_o and F_v/F_m) but also to compare the shape of the induction curves. The value of F_v/F_m is very conservative, and could be significantly influenced by strong effectors. In our experiments, the initial value of F_v/F_m was comparatively low because of the low light intensity we used to measure the fluorescence induction kinetics. In addition, in a 'low salt' medium (i.e. 10 mM NaCl), the granal structure disintegrates and the membranes become completely unstacked with a lower variable chlorophyll *a* fluorescence (Telfer *et al.* 1976).

Our results showed that plant growth regulators decreased the functional activity of PS II as measured by the ratios F_v/F_m and F_v/F_o (Table 3) in the 1st month. This effect was better expressed in the plants treated with 4-PU-30 than with kinetin, and was especially so after anticytokinin treatment. No significant changes were detected in PS II activity in the 2nd month in plants treated with kinetin, ACK-1 and ACK-2, whereas the effect of 4-PU-30 was the same as in the 1st month.

Discussion

The surface charge density of biomembranes is an informative parameter for the processes taking place in a single membrane or between adjacent surfaces. The electrophoretic mobility of cells or subcellular structures could provide information on surface charge density dynamics in relation to the structure and functional capability of the studied biosystems and offers possibilities for using this trait as a marker.

Electrical charges on the surface of the chloroplast thylakoid membranes dictate its conformational state. Any perturbation of the surface electrical properties therefore brings about a reorganization of the membrane (Barber 1989). Changes in surface charge density reflect the signals transmitted between cells, conveying information about membrane functional activity and the environment of the plant as a whole.

The study of surface charge density as a physicochemical parameter should contribute to elucidation of its role for the structure and function of the thylakoid membrane. We observed that 4-PU-30, ACK-1 and ACK-2 influenced σ in a similar way after a longer time of treatment (2 months). The phenylurea cytokinin, and both the triazine and carbamate anticytokinins, stimulated the enhancement of zeta potential of thylakoids in comparison to the control, possibly due to a direct influence on thylakoid membrane components and an additional negative charges exposure on membrane surface. The conformational rearrangement included changes in protein–lipid interactions, and protein components escaping from the inner surface deeper into the lipid matrix. The transverse motion of the intrinsic protein molecules was associated with the increased exposure of the external surface charge groups, and subsequently increased σ. Making thylakoid membranes more negatively charged, 4-PU-30, ACK-1 and ACK-2 increased the aggregation of the complexes and the energization of the membrane (i.e. a higher level of ΔpH

Table 3. Effect of cytokinins and anticytokinins on the F_v/F_o and F_v/F_m ratios of *Gypsophila paniculata* thylakoids

Data are means (± s.e.) of three replicates from three independent experiments (*n* = 9). Significant difference from control plants is indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$)

Treatment	1st month		2nd month	
	F_v/F_o	F_v/F_m	F_v/F_o	F_v/F_m
Control	1.219 ± 0.021	0.545 ± 0.004	1.214 ± 0.030	0.548 ± 0.006
Kinetin	1.136 ± 0.021*	0.532 ± 0.005	1.167 ± 0.036	0.538 ± 0.008
4-PU-30	1.050 ± 0.015**	0.512 ± 0.004**	0.989 ± 0.038**	0.497 ± 0.031
ACK-1	0.901 ± 0.014***	0.474 ± 0.004***	1.245 ± 0.022	0.554 ± 0.004
ACK-2	0.988 ± 0.051*	0.497 ± 0.013*	1.178 ± 0.069	0.540 ± 0.015

gradient formation across the thylakoid membrane; Fig. 2) after a longer time of treatment (2 months). We related these effects to more extensive stacking by increasing the net space charge density of ions in solution in a plane parallel to the membrane surface at an adequate distance. In this case, more extensive stacking occurs, which is independent of fluidity and which maintains a randomization of complexes (Barber *et al.* 1980).

Our results showed that plant growth regulators decreased the primary photochemical activity of PS II (estimated by the ratio F_v/F_m; Table 3) in the 1st month. The intensity of delayed fluorescence was influenced in the same way (Fig. 1). However, no significant changes were observed in the ratio F_v/F_m and in DF intensity in the 2nd month. This could be related to the additional negative surface charge detected in the electrophoretically accessible thylakoid region.

There were differences in surface charge density changes under the phenylurea cytokinin and anticytokinins after a longer time of treatment. In the case of anticytokinins, the surface charge densities increased by 15% compared to the control, and in the case of a phenylurea-type cytokinin, by 9%, after 2nd months of treatment (Table 2).

The electrokinetic method could be used for the biophysical characterization of *in vitro* cultured plants and their thylakoid membranes. Using the Gouy-Chapman theory (Barber 1980), we investigated the incorporation of phenylurea cytokinin and anticytokinin compounds in the lipid bilayer of thylakoid membranes of *in vitro* cultured *G. paniculata* plants after a longer time of treatment. These compounds changed σ and also affected the molecular arrangement of the lipid bilayer, facilitating proton gradient formation after a longer time of treatment because of the stabilizing of the stress effect of *in vitro* cultivation. Hence, the photosynthetic apparatus of *in vitro* cultured *G. paniculata* plants could be protected by building up a higher proton gradient over the thylakoid membrane after cytokinin and anticytokinin treatment compared to the control plants.

Acknowledgments

We thank Radoslav Sergiev for skilful technical assistance. This study was supported by Bulgarian National Fund ‘Scientific Investigations’ (Project Number B-529/1995).

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Manuscript received 29 March 1999, received in revised form 5 June 2000, accepted 20 June 2000