PHOTOSYNTHETICA 43 (2): 237-246, 2005

Isolation of chestnut chloroplasts: Membrane potentials of chestnut and spinach thylakoids

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Abstract

Typical chestnut thylakoid extracts isolated by mechanical disruption of leaf tissues had an equivalent of 0.28 kg m^{-3} chlorophyll (Chl) which is six times less than in thylakoids obtained from spinach, although Chl content in leaves was only half as small. According to optical microscopy, the vesicles showed a good integrity, exhibiting at 21 ºC a high capacity of photon-induced potential membrane generation, which was demonstrated by the almost full 9-amino-6-chloro-2-methoxyacridine fluorescence quenching in a hyper-saline medium containing 150 mM KCl and having osmotic potential of –1.5 MPa. The half-time of the thylakoid potential generation was 11.7 s with the time of dissipation around 8.9 s. In such conditions, spinach thylakoids showed an increased swelling and also differences in the half-time generation which was almost four times faster than was observed in chestnut. However, when spinach thylakoids were incubated in a typical hypo-saline medium without KCl with osmotic potential –0.8 MPa, no additional swelling was observed. Consequently the half-time of potential dissipation was 35 s. Studies with nigericin suggested a chestnut thylakoid ΔpH significantly smaller than that observed in spinach, which was confirmed by the measurements of the ATP driven pumping activity.

Additional key words: 9-amino-6-chloro-2-methoxyacridine; *Castanea sativa*; fluorescence; osmotic potential; photosynthesis; *Spinacia oleracea*; temperature.

Introduction

The chestnut (*Castanea sativa* Mill.) has been a major world nut crop for many years (Crawford 1995). The largest producers are China, Korea, Italy, Turkey, France, Spain, Portugal, and Greece. In Portugal, chestnut orchards are mainly located in the Trás-os-Montes Region (Cortizo *et al.* 1996). Because of the importance of this species, there is extensive research material on the chestnut, but no reported research of chestnut chloroplasts. Chloroplast studies are common in plants such as spinach, lettuce, pea, and maize, but not in tree species, where bioenergetic studies of chloroplasts are rare, as is the case with chestnut.

For species such as spinach and pea, wide media have been used successfully when mechanical disruption of tissues is employed. This is a procedure with many advantages such as its rapidity, economy, and high yields of stable chloroplasts (Leegood and Malkin 1986). However, its application is restricted to just a few species (see above). One important consideration is that the osmotic potential of cell sap must be close to that of the leaves, which is near –1.0 MPa for spinach leaves grown hydroponically (Walker *et al.* 1997).

In chloroplasts, thylakoid membranes are the places where photons are absorbed and converted into chemical

Received 16 February 2004, *accepted* 13 January 2005.
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Abreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; EDTA, disodium ethylenediamine tetraacetic acid; F, ACMA fluorescence quenching under dark conditions, MV, methyl viologen; PS, photosystem; Q, light-induced ACMA fluorescence quenching; R, ACMA fluorescence recuperation; $t_{0.5}$l, half-time fluorescence quenching; $t_{0.5}$d, half-time fluorescence recuperation; Tricine, N-tris (hydroxymethyl) methylglycine; Δp, electric membrane potential; ΔpH, electrochemical potential difference of protons; Δψ, transmembrane potential; Ψ_π, osmotic potential; Ψ_w, water potential.

Acknowledgements: The authors kindly acknowledge support for this research from Project PAMAF 2091 and Project Agro 499.

energy in photosynthesis. Irradiation causes an increase in the negative charge at the outer surface of thylakoid membranes as a consequence of the electron transfer chain (Torres-Pereira *et al.* 1974a, Barber 1982, 1986, Kraayenhof *et al.* 1984). The surface charge changes of the inner and outer side of the thylakoid membrane changes with opposite sign upon a photon switch, with the lumen side becoming positive and the stroma side negative (Witt 1979). Part of the surface charge increase is attributed to conformational changes observed in the globular proteins (at the level of acid groups from aspartic and glutamic amino acids) as a consequence of the electron transfer chain (Torres-Pereira *et al.* 1974b, Nakatani *et al.* 1978, Barber 1982). Under these negative charges, positive ions (counter ions) in the medium interact to form a diffuse double-layer in close vicinity to the membrane surface, which can be studied by cationic probes such as fluorescent 9-amino-6-chloro-2-methoxyacridine, ACMA (Packer *et al.* 1975, Kraayenhof *et al.* 1984).

One of the main objectives of this investigation was to develop a procedure to isolate competent chestnut thylakoids by mechanical disruption of tissues, in order to provide material for photosynthesis research.

Materials and methods

Mature and healthy leaves from chestnut plants (cv. Aveleira) were selected from around the north side of the canopy (Gomes-Laranjo *et al.* 2002) at the end of September and during the first fortnight of October, between 1998 and 2001. The trees were eight years old and from a well watered orchard in the UTAD field in Vila Real (Portugal). For spinach chloroplasts (*Spinacea oleracea* L. cv. Giant Viroflay) isolations, seedlings were regularly planted in the field near to the chestnut trees. The spinach plants were regularly watered.

Thylakoids were isolated according to the basic procedure (Torres-Pereira 1974, Torres-Pereira *et al.* 1974b) and Packer *et al.* (1975) for spinach, with modifications. The grinding medium was composed of 20 mM sorbitol, 10 mM tricine-NaOH (pH 8.4), 30 mM KCl, 5 mM $MgCl_2$, 0.75 mM EDTA, 0.1 % (m/v) bovine serum albumin (BSA), and 1 % (m/v) ascorbic acid. 0.4 % polyvinylpyrrolidone were added just before the isolation. The final osmotic potential (Ψ_π) of the grinding medium was –0.51 MPa. The composition of the storage medium was 165 mM sorbitol, 10 mM Tricine-NaOH (pH 8.4), 5 mM $MgCl_2$, and 1 % ascorbic acid giving the Ψ_π of the medium –0.65 MPa.

To determine Ψ, 100 mm^3 of cell sap or media were measured in an osmometer (model *3*, *Advanced Instruments*, Needham Heights, MA, USA) and values were converted to MPa according to the Van't Hoff equation (Salisbury and Ross 1992). To obtain 100 mm^3 of cell sap, 2 g of leaves were pressed in a laboratory built press.

Leaves were collected early in the morning, washed, and left in water for 4 h. Under these conditions, the leaves had a Ψ_π in the range of –0.5 to –0.3 MPa, which is close to the Ψ_π of the grinding medium and would therefore prevent any osmotic shock (Walker *et al.* 1997). After that, 35 g of leaves without midribs were weighed. The leaves were homogenized in a *Waring blender* at maximum speed in 4 pulses of 5 s with 5 s break time. The homogenized material was rapidly filtered through eight layers of cheesecloth (20–50 μm, *Ref[a] Monodur PA50N*, Holland) and centrifuged for 40 s at 560×*g* in a refrigerated centrifuge. The pellet was discarded and the supernatant was centrifuged at 1 500×*g* for 60 s. The pellet of the thylakoid membranes was re-suspended in the washing medium and a second centrifugation (1 500×*g* for 60 s) was carried out. The final pellet was re-suspended to 0.5 kg(Chl) m^{-3} in the storage medium containing 500 μM butylated hydroxytoluene (BHT) (Kraayenhof *et al.* 1984, Torres-Pereira *et al.* 1974b, 1984).

The content of photosynthetic pigments was calculated following the equations of Lichtenthaler (1987). For thylakoid extracts, 100 mm^3 of extract were added to 10 cm^3 of 80 % (m/v) acetone (pH 7.5, buffered by 25 mM Hepes). After 10 min, the extracts were filtered for readings in a *Pye Unicam* (Cambridge, UK) spectrophotometer model *SP-8-100*. Extraction of pigments was made from 6 leaf disks (8 mm diameter) suspended in 10 cm^3 of 80 % (m/v) acetone during 48 h.

The fluorescence emission spectrum was measured in a spectrofluorimeter model *Jasco FP-777*, using a 440 nm excitation radiation of 110 μmol m^{-2} s^{-1} and the fluorescence emission in the range of 650–800 nm with a 10 nm bandwidth (Hipkins and Baker 1986). Thylakoids were incubated in a medium containing 200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 μM MV, 5 μM ACMA, 15 μM DCMU, and 25 g(Chl) m^{-3}.

Changes in thylakoid membrane potential ($\Delta\psi$) were observed with fluorescent cationic probe such as 9-amino-6-chloro-2-methoxyacridine (ACMA) (Schuldiner *et al.* 1972, Packer *et al.* 1975, Kraayenhof *et al.* 1996, Rottenberg 1997). The fluorescence intensity was measured with a fluorimeter built in the Plant Physiology Laboratory of the Free University, Amsterdam (Schuurmans *et al.* 1982), with minor modifications. In short, excitation beam was emitted by an actinic irradiator (25 mW cm^{-2}) at 420 nm (filter *J43-107*) and led to the cuvette (2 cm^3) by a bifurcated light guide (front-face configuration, 0º), after which it was recorded at 500 nm (filter *J43-117*) in the detector. The cuvette was thermostabilized with a Peltier system and irradiated from the bottom with 250 W m^{-2}, which was filtered above 610 nm with a broadband filter (*J30-792*) and a red filter

(600 nm, *J46-160*). All the filters used were interference filters (±5 nm) made by *Edmund Scientific Company* (NJ, USA). The fluorescence quenching induced by the red radiation was characterised by the percentage of quenching (Q) and the half-time of quenching ($t_{0.5}$l) and the recovery of fluorescence after the red radiation was off was measured by R (percentage of recovery) and half-time of recovery ($t_{0.5}$d) (Fig. 1). A data-acquisition plaque (*PLC818*) achieved the acquisition of data for PC. Additionally, a software program in *C-ANSI* language was developed that allowed a systematised form of data reading (*ASCII* format) and was transferable to the *MATLAB* programme workspace. A set of routines was built to offer the possibility of extraction and analysis of the data. ACMA was synthesized in the Plant Physiology Laboratory of the Amsterdam Free University according to the recipes of Albert (1966).

Different conditions of reaction medium such as pH, salt (KCl and $MgCl_2$), sugar (sorbitol), and temperature effects were tested in order to find changes in the membrane potential of the thylakoids. The study was done in the above order, starting (on the pH curve) with a basic reaction medium composed of 300 mM sorbitol, 2 mM tricine-NaOH (pH 8.0), 20 mM $MgCl_2$, 200 mM KCl, 25 g(Chl) m^{-3}, 30 µM methylviologen (MV), and 5 µM ACMA.

Oxygen evolution of isolated thylakoid membranes of both spinach and chestnut was measured polarographically using a Clark-type oxygen electrode connected to a suitable recorder. Assays were run in the above-described thermo-stabilized cuvette at 20 ºC, the "actinic light" being provided from the bottom. An equivalent of 25 mg(Chl) m^{-3} of thylakoid membrane extract was incubated in 200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 µM MV, and 5 mM sodium azide. Calculations were done assuming an oxygen concentration at 20 °C of 0.276 nM O_2 in the experimental medium.

The action of the uncoupler nigericin on the membrane potential was also assayed (Giersch *et al.* 1980, Mills 1986, Brock *et al.* 1995). Under the same conditions as described above, 25 g(Chl) m^{-3} were incubated with 200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, and 30 µM MV. After 2-min stabilization, 5 µM ACMA was added, and after another 540 s thylakoids were excited by red radiation (>600 nm). Nigericin was applied after 200-s energization, inducing a recovery in ACMA fluorescence (R) (Fig. 1). The nigericin was prepared according to Mills (1986).

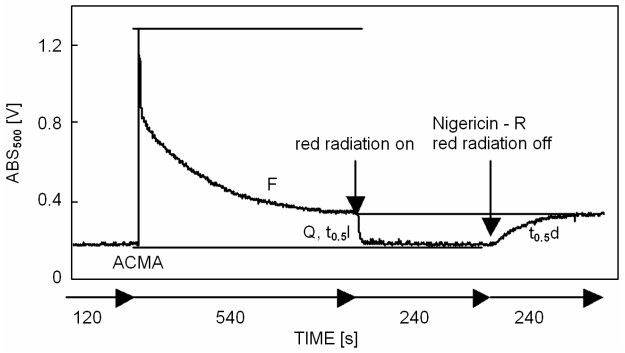

Fig. 1. Typical ACMA fluorescence assay in isolated spinach chloroplasts. Chloroplasts at an equivalent concentration of 25 g(Chl) m^{-3} were put into the cuvette with the reaction medium, incubated for 120 s, then 5 µM ACMA was added and a further 540 s were needed during which time the ACMA's molecules were adsorbed to the free negative charges of the medium inducing independent light-induced decay on the fluorescence signal (F). For ACMA, the excitation wavelength was 420 nm and the emission wavelength was 500 nm. As a result of the thylakoidal energization by red radiation for 240 s, a quenching on ACMA fluorescence was obtained (characterised by Q and $t_{0.5}$l), after which recuperation on ACMA fluorescence was induced by removing the red radiation (characterised by $t_{0.5}$d) or by the addition of nigericin (R).

ATP driven pumping activity was measured at 20 ºC in the above described cuvette with a combination of a pH electrode and a pH meter built by the Chemistry Department of University of Trás-os-Montes and Alto Douro. The reaction medium contained 200 mM sorbitol, 0.5 mM tricine-NaOH (pH 8.4), 2 mM Na_2HPO_4, 4 mM $MgCl_2$, 150 mM KCl, and 30 µM MV. Thylakoids equal to 25 g(Chl) m^{-3} were added to the cuvette. After 2 min of incubation, the thylakoids were irradiated during 3 min with the saturating red radiation, after which 50 µM ADP was added. Proton transfer through F_0 was blocked after 3 min more with 0.225 mg m^{-3} oligomycin (Ewy and Dilley 2000).

All the reactants used in the experiments were of analytical grade.

Results

Extracts of chloroplasts from chestnut and spinach were observed by optical microscopy. According to Fig. 2, chestnut chloroplasts maintained their structure at a good intact level, as did spinach, and thus they could display photosynthetic activity *in vitro*.

The used method allowed the isolation of chestnut chloroplast extracts with an equivalent of 0.3 kg(Chl) m^{-3} which corresponds to 16 % of Chl (*a*+*b*) content in spinach extracts (Table 1). However, when the results were compared to those obtained from leaf extraction, chestnut had only less than 52 % of Chl (*a*+*b*) than spinach. The Chl *a*/*b* ratio of spinach extracts was about 3.1 and that of chestnut extracts was about 3.6. These values are smaller than those obtained from leaf extracts

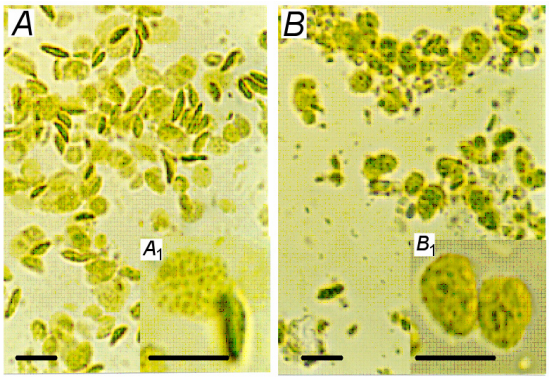

Fig. 2. Optical microscopy images of spinach (*A*) and chestnut (*B*) chloroplasts. In the *inserts* (A_1 and B_1), chloroplast images from 100× objective are shown. *Inserted bars* correspond to 5 µm.

(3.5 and 3.8, respectively). The ratio Chl/Car was similar in both types of chloroplasts, at 4.7.

The thylakoid fluorescence emission spectrum was characterized at 20 ºC. It showed a typical maximum at about 680–685 nm which is associated with the antennae Chls of photosystem (PS) 2 and the related light-harvesting complex (Fig. 3). A shoulder was noted at 740 nm, the attributed band for PS1 (Hipkins and Baker 1987).

According to the O_2 evolution and membrane potential generation, chestnut chloroplasts isolated and stored in the conditions described above preserved their activity at a level higher than 90 % in the first 4 h (Fig. 4).

The generation of thylakoid membrane potential was studied under different pH and different KCl, $MgCl_2$, and sorbitol concentrations in the medium (Fig. 5). Chestnut thylakoids showed the highest activity

Table 1. Amounts of chlorophyll (Chl) and carotenoids (Car) [kg m^{-3}] in extracts of chestnut and spinach thylakoids (n = 22, n = 8) and leaves (n = 6).

		Chl *a*	Chl *b*	Chl (*a*+*b*)	Chl *a*/*b*	Car	Chl/Car	Chl/total
Thylakoids	Chestnut	0.215±0.019	0.060±0.005	0.275±0.023	3.60±0.10	0.058±0.005	4.70±0.80	
	Spinach	1.309±0.074	0.425±0.023	1.734±0.096	3.08±0.08	0.378±0.026	4.68±0.70	
Leaves	Chestnut	90.71±3.70	24.00±1.30	114.7±4.9	3.78±0.02	25.00±1.60	4.60±0.12	379.8±16.0
	Spinach	169.40±3.5	49.00±1.20	219.3±4.8	3.45±0.03	48.66±1.50	4.51±0.11	726.1±14.0

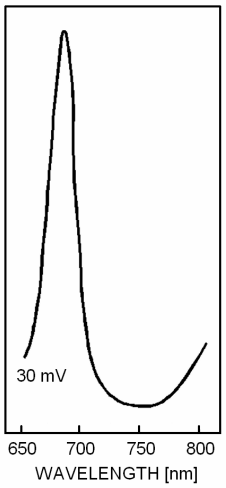

Fig. 3. Fluorescence emission spectra of isolated chestnut thylakoids at 20 ºC. The thylakoids were suspended in a medium containing 200 mM sorbitol, 2 mM Tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 µM MV, 5 µM ACMA, 15 µM DCMU, and 25 g(Chl) m^{-3}. The thylakoids were excited with a photon flux density of 100 µmol m^{-2} s^{-1} of 440 nm radiation with a 10 nm bandwidth.

at pH 8.4 (Q of 82 %) (Fig. 5*A*). In relation to KCl, optimal concentration was found at 150 mM KCl (Fig. 5*B*) with Q 82 %, $t_{0,5}$l 13.3 s, and $t_{0,5}$d 11.5 s. 4 mM $MgCl_2$ allowed a quenching of Q 89 %, $t_{0,5}$l 10.6 s, and $t_{0,5}$d 6.7 s (Fig. 5*C*), and for sorbitol, 200 mM induced Q 96 %, $t_{0,5}$l and $t_{0,5}$d of about 11.5 s and 9.2 s, respectively (Fig. 5*D*). Final composition of the optimised reaction medium was found with 200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, and 150 mM KCl corresponding to Ψ_π of about –1.5 MPa. Concerning the effect of temperature (Fig. 6), according to ACMA parameters chestnut chloroplasts showed the best results in the range of 17–23 ºC, whereas for spinach the interval was 19–27 ºC. Alternatively, considering the temperature dependence of Q to contain two rather than three phases, 21 and 24 ºC are the temperatures that allow the maximal energization

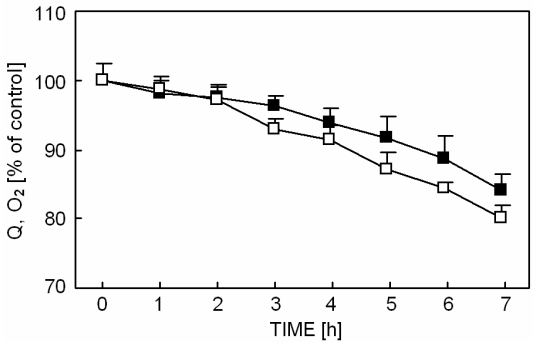

Fig. 4. Preservation activity in 4 ºC-stocked chestnut thylakoids, simultaneously measured by ACMA parameters (■) and O_2 evolution (□). Thylakoids were assayed at 20 ºC in a medium containing 200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 µM MV, 5 µM ACMA, 5 mM sodium azide, and 25 g(Chl) m^{-3} (n = 3).

in chestnut and spinach thylakoids, respectively.

The rate of O_2 evolution in chestnut thylakoid membranes was about 1.61 pM(O_2) s^{-1}, while in spinach thylakoids it was 2.15 pM O_2 s^{-1}.

The influence of the optimised medium [200 mM sorbitol, 2 mM tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl; Ψ_π –1.5 MPa] in the irradiation-induced energization was tested in spinach thylakoids, a widely

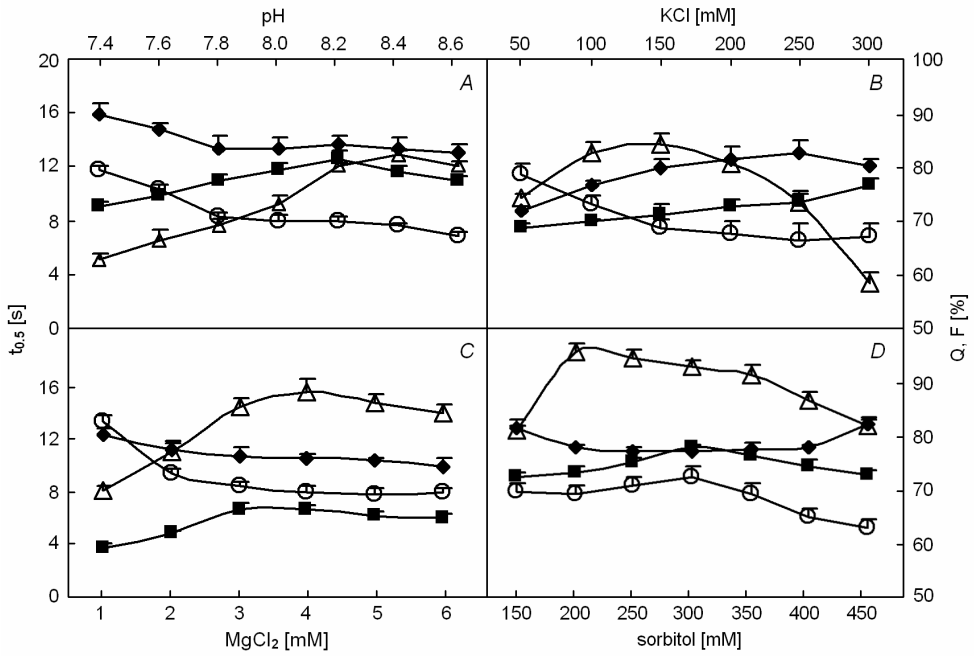

Fig. 5. Effects of pH (*A*), KCl (*B*), $MgCl_2$ (*C*), and sorbitol (*D*) on energization capacity in chestnut thylakoids measured by ACMA fluorescence parameters, Q (Δ), F (○), $t_{0.5}$l (♦), and $t_{0.5}$d (■). For more details see Fig. 1 (n = 3).

used photosynthetic material (Fig. 7*A*,*B*). In such reaction conditions, the light-induced quenching (Q) was about 120 %, $t_{0.5}$l was now 1.5 s, and dark-induced ACMA fluorescence quenching (F) about 63 %, giving similar values for $t_{0.5}$d (11.5 s). Comparison of these results with those obtained using a typical hypo-saline medium [345 mM sorbitol, 2 mM Tricine-NaOH (pH 8.4), and 2 mM $MgCl_2$; Ψ_π –0.8 MPa] (Fig. 7*B*, control) showed significant differences in all parameters, which were now Q 100 %, F 84.9 %, $t_{0.5}$l 2.5 s, and $t_{0.5}$d 34.9 s. In order to better interpret these differences, the influence of KCl and $MgCl_2$ was studied. Under successive increases in KCl concentration, fluorescence quenching of ACMA over 100 % was observed, with maximal values around 110 %, when KCl changed from 0 to 150 mM (Fig. 8*A*). Important changes were also noted in $t_{0.5}$d, which diminished from 38 to 19 s. In relation to F parameter, a decline from 72 to 64 % was observed. This parameter reflects the induced ACMA fluorescence quenching induced by the negative free electrical charges at the membrane surface, which is a consequence of the level of thylakoid stacking. Maximal excitation, Q 100 %, was attained when the content of $MgCl_2$ was changed from 0 to 1 mM (Fig. 7*B*), when also $t_{0.5}$d increased from 25 to 32 s. Evolution of the F parameter indicated a transition point at 3 mM $MgCl_2$ with 73 %, whereas in the absence of $MgCl_2$ the value was 87 %.

The ionophore nigericin catalyses an electroneutral H^+ efflux compensated by K^+ fluxes across the thylakoid and hence dissipates ΔpH without affecting Δψ, since no net charge crosses the membrane (Mills 1986). Dissipation of ΔpH was measured in excited spinach and chestnut thylakoids by determination of ACMA fluorescence recuperation under photon excitation (R, see Fig. 1). Results indicate a concentration of about 168 and 939 μM nigericin as a concentration inducing maximal recuperation of fluorescence; the value for spinach was six to seven times higher than that for chestnut (Fig. 9). These results are partially consistent with ATP driven pumping activity, measured by ΔpH of the reaction medium, which indicates a proton uptake in spinach thylakoids (Fig. 10) significantly higher than that observed in chestnut thylakoids. According to this experiment oligomycin did not completely stop the transfer of protons between two sides of the membranes in both spinach and chestnut.

Discussion

According to Fig. 2, chestnut thylakoids preserved a good intact structure, similar to spinach, a widely used model, with no significant alterations in energization capacity in the first four hours (Fig. 4). Chestnut thylakoids showed a darker green colour than spinach thylakoids, suggesting the existence of a greater stacking degree in chestnut. The Chl content of the chestnut extracts was six times less than that of the spinach extracts, which could be due to the more cellulose characteristic of chestnut leaves, since total content in chestnut leaves was only almost half (Table 1). This gave the chestnut thylakoid extracts a higher Chl *a*/*b* ratio (Table 1) than in spinach. However, both extracts had similar Chl/Car, which indicated a similar photoprotection activity in both types of chloroplasts.

The energization of thylakoids lead to an extra increase of the net charge at their stroma surfaces. Changes produced in the adjacent, diffuse double layer of the thylakoid membranes occurred as a consequence of potential membrane generation in the internal plane of the membrane and could be followed by monoamine molecules such as ACMA (Schuldiner *et al.* 1972, Torres-Pereira *et al.* 1974a,b, Kraayenhof 1980). Our results suggest that chestnut thylakoids need to be incubated in a medium with higher osmolarity and salinity, 150 mM KCl and 200 mM sorbitol (Fig. 5*B,D*), Ψ_π = –1.5 MPa, than is the medium widely used for spinach (Ψ_π = –0.8 MPa) where osmolarity is generally obtained by 345 mM sorbitol (Fig. 7) (Walker *et al.* 1997). Under such conditions, chestnut thylakoids show high rates of photosynthetic activity expressed by 96 % of ACMA fluorescence

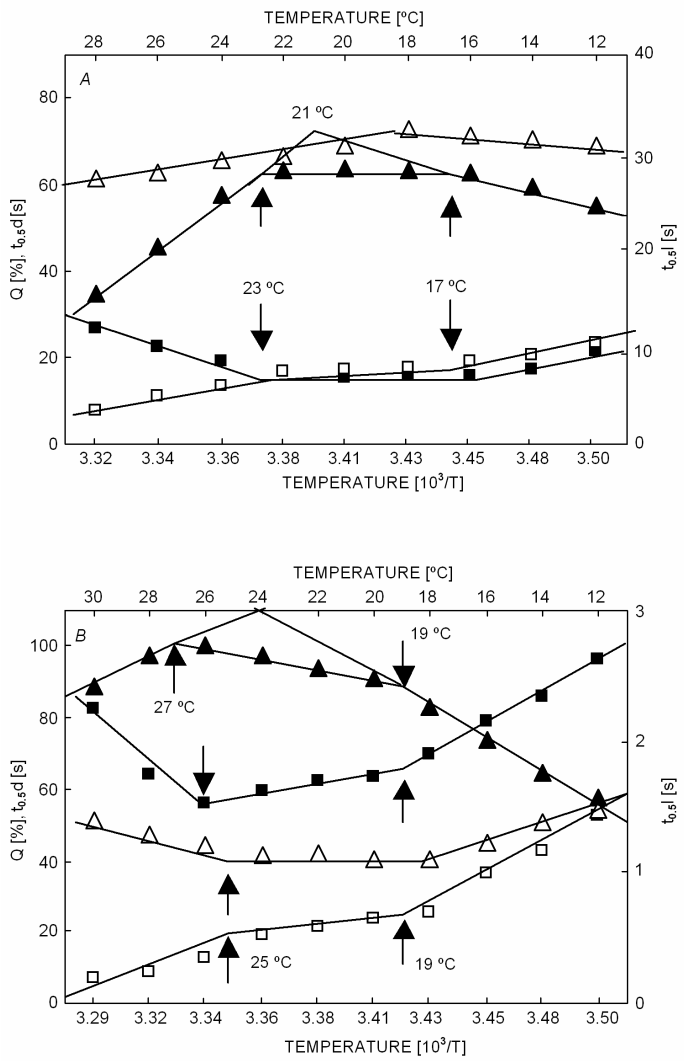

Fig. 6. Variation of the ACMA fluorescence parameters, Q (▲), F (Δ), $t_{0.5}$l (■), and $t_{0.5}$d (□) in thylakoids of chestnut (*A*) and spinach (*B*) as a function of temperature. Thylakoids equivalent to 25 g(Chl) m^{-3} were assayed in 200 mM sorbitol, 2 mM Tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 μM MV, and 5 μM ACMA. *Arrows* are inserted at the transition points. Results from a single assay, but similar results were seen in three different experiments.

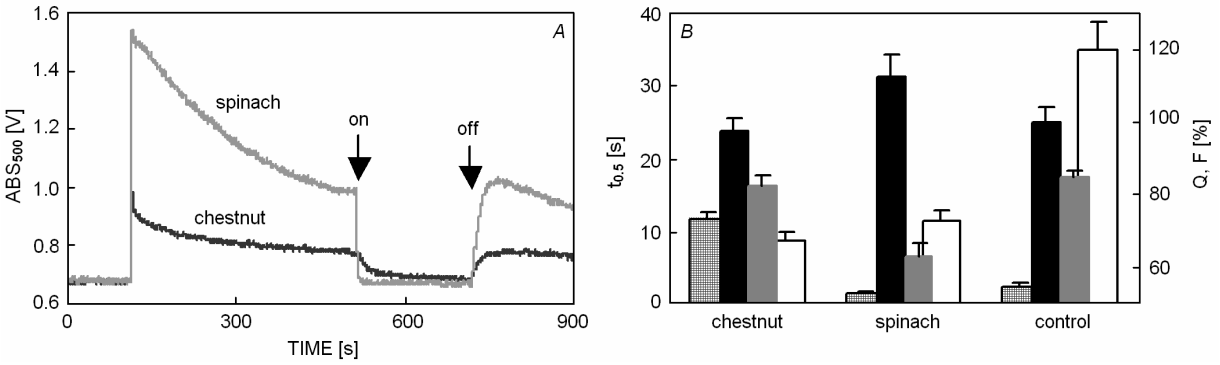

Fig. 7. Typical ACMA fluorescence quenching in chestnut and spinach thylakoids (*A*) incubated in the optimised reaction media composed of 200 mM sorbitol, 2 mM Tricine-NaOH (pH 8.4), 4 mM $MgCl_2$, 150 mM KCl, 30 µM MV, 5 µM ACMA, and thylakoids of 25 g(Chl) m^{-3}. (*B*) Parameters of ACMA fluorescence, Q (*black bars*), F (*grey bars*), $t_{0.5}$l (*cross-lined bars*), and $t_{0.5}$d (*white bars*) obtained with chestnut and spinach thylakoids incubated in the optimised reaction media (spinach thylakoids are control), incubated in a low salt typical media: 345 mM sorbitol, 2 mM Tricine-NaOH (pH 8.0), and 2 mM $MgCl_2$, with 25 g(Chl) m^{-3}, 5 µM ACMA and 30 µM MV. Parameters were measured from the recorder traces of the data shown in *A* and in Fig. 1 for control. Triplicate measurements were made.

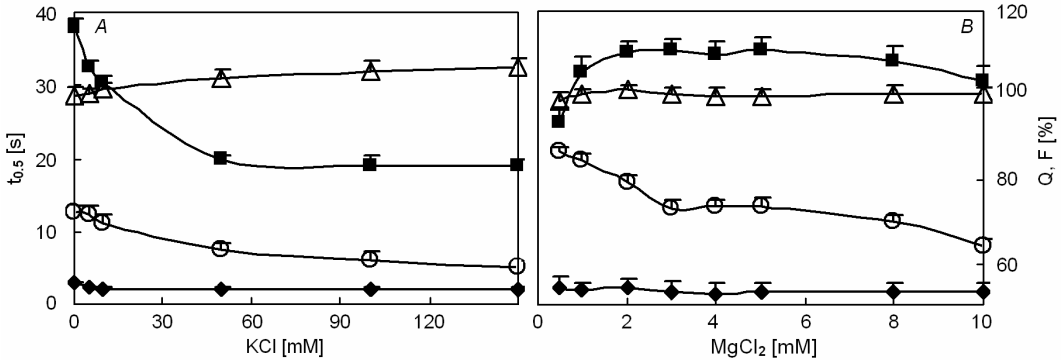

Fig. 8. ACMA fluorescence parameters, Q (Δ), F (○), $t_{0.5}$l (♦), and $t_{0.5}$d (■) in spinach thylakoids under different concentrations of KCl (*A*) and $MgCl_2$ (*B*). Thylakoids at an equivalent of 25 g(Chl) m^{-3} were incubated in 345 mM sorbitol, 2 mM Tricine-NaOH (pH 8.0), 5 µM ACMA, 30 µM MV, and for the experiment of KCl, 2 mM $MgCl_2$ were added (n = 3).

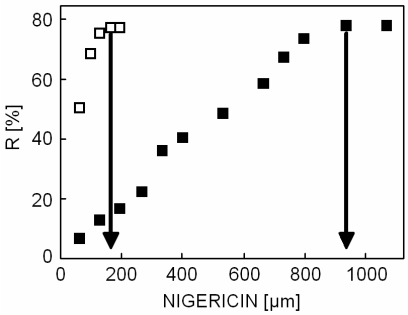

Fig. 9. Characterization of ΔpH in chestnut (Δ) and spinach (■) thylakoids by nigericin action. Results are expressed as function of R parameter, which reflects the fluorescence recuperation of ACMA (see Fig. 1). Triplicate measurements were made at each of the nigericin concentrations employed. Assays were carried out at 21 ºC. Chloroplasts at 25 g(Chl) m^{-3} were incubated in a medium of 200 mM sorbitol, 2 mM Tricine-NaOH (pH 8.4), 150 mM KCl, and 4 mM $MgCl_2$, 30 µM MV, and 5 µM ACMA. Other conditions are described in Materials and methods.

quenching. According to Schuurmans *et al.* (1978), the generation of membrane potential in chestnut thylakoids by the extra need of KCl must be viewed with some caution, because K^+ ions diffuse faster to the lumen space than Cl^- ions. This leads to a balance of charges generating a potential with the same polarity as that originated by the photosynthetic process (Mills 1986, Strotmann and Shavit 1999); hence water diffusion in the same direction leads vesicles to a certain turgescence. According to Berkowitz (1998), these volume changes induced by cell water potential could impose important limitations on the energization capacity of thylakoids.

Neutralization of the membrane surface charge, obtained with the help of Mg^{2+}, can favour thylakoid stacking, which only happens when the negative charge density is lower than 300 nm^{-2} (Torres-Pereira *et al.* 1984). According to the values of the Q parameter, an incubation medium with 4 mM $MgCl_2$ (Fig. 5*C*) can be enough to promote an adequate thylakoid stacking and hence adequate spatial separation between PS2 and PS1

complexes which is very important in inhibiting "spill-over" (Trissl and Wilhelm 1993). This value is four times higher than that achieved for spinach (Fig. 8*B*).

For chestnut thylakoids the best activity was achieved between 17 and 23 ºC (Q parameter), which can be treated as 21 ºC if a double phase curve is considered (Fig. 6*A*), and is below the 24 ºC achieved for spinach in such conditions (Fig. 6*B*). Below 17 ºC, interference can occur at the level of plastoquinone mobility in the plane of the membrane, which could be a consequence of a different saturated/unsaturated fatty acid composition (Hurry *et al.* 1998). Under temperatures above the optimal values, a decrease in energization capacity is a consequence of interference in the integrity of the membrane, making membranes permeable to protons (Bakker-Grunwald and Van Dam 1974, Mills 1986). It allows diffusion of protons to the stroma through the thylakoid membrane fissures (Gilmore and Govindjee 1999). The inhibition is complemented by the gradual de-stacking as is shown by the diminution of F for those temperatures.

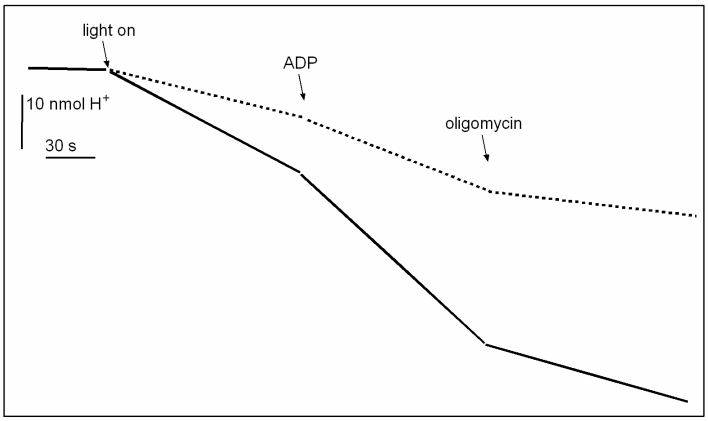

Fig. 10. Characterization of proton uptake measured with pH electrode in chestnut (*dashed line*) and spinach (*black line*). Assays were carried out in triplicate at 21 ºC. Chloroplasts of 25 g(Chl) m^{-3} were incubated in a medium of 200 mM sorbitol, 0.5 mM Tricine-NaOH (pH 8.4), 2 mM Na_2HPO_4, 150 mM KCl, 4 mM $MgCl_2$, and 30 μM MV during 2 min, after which they were irradiated during 3 min with a saturating red radiation. Then, 50 μM ADP was added and after 3 min the proton uptake by ATPase was stopped by adding 0.225 g m^{-3} oligomycin. Other conditions are described in Materials and methods.

Comparing the results obtained with chestnut and spinach thylakoids, half times of ACMA fluorescence, quenching, and recuperation were different (Fig. 7*A,B*). In chestnut the half time of quenching was longer and the recovery was shorter than in spinach. This means that the cationic probe was adsorbed much more slowly in chestnut, indicating the possible presence of pores, increasing the H^+ leakage across the thylakoid membrane by the ATP synthase (Strotmann and Shavit 1999). The high salt content in the optimised media induced a reduction in the percentage of F, since a higher concentration of cations annulled negative charges in membranes and left fewer free places for ACMA (Figs. 7*B* and 8*A*). These hyper-saline conditions were the basis for the unexpected values over 100 % on ACMA fluorescence quenching, which induced swelling as the respective chlorocrit indicates (data not shown), and which in turn lead to a diminution in the transmittance capacity of the extract and to a diminution in the referred $t_{0.5}$d. According to Murakami and Packer (1969) and Wang and Packer (1973), dilatation of membranes can interfere not only with the stacking degree but also, under strong hyper-saline conditions, with the integrity of membranes. This leads to the appearance of pores that in consequence operate as an uncoupler that induces the referred shortfall of $t_{0.5}$d. These alterations in vesicle volume are mainly due to the high KCl concentrations in the reaction medium (Fig. 8*A*), and not to the $MgCl_2$ concentration (Fig. 8*B*).

Photon excitation of thylakoid membranes induces the appearance of charges, deriving from the electric membrane potential (Δp), which is composed of two major components: the fast transmembrane potential (Δψ) and the slow electrochemical potential difference of protons ($\Delta\mu_{H+}$). The fast electric field generation was associated with charge separation at the level of photosynthetic reaction centre complexes of PS1 and PS2, and the slow component was associated with the vectorial proton transport to the lumen producing a ΔpH (Fiolet *et al.* 1975, Barber 1986, Mills 1986, Witt 1991, Strotmann and Shavit 1999).

Since nigericin promotes a strictly electroneutral exchange of protons for monovalent cations across the membrane, according to our results the application of this uncoupler to the excited thylakoids promotes proton gradient dissipation and a partial Δp dissipation, as can be verified by incomplete fluorescence recuperation (R) (Fig. 9). The proton gradient from chestnut was six times shorter than that from spinach, which might imply an unexpected ΔpH<1 (assuming a typical ΔpH 3–4 for thylakoids) with consequent limitations on ATP synthesis. The Δp dissipation provoked by the uncoupler

was equivalent to 80 % of ACMA fluorescence recuperation. This part can be related to $\Delta\mu_{H+}$ component; the remaining 20 % of Δp may be due to the residual charges due to the transmembrane potential as referred by Strotmann and Shavit (1999), and to the residual thylakoid membrane potential and the existence of microdomains in lumen which fix protons (Ewy and Dilley 2000). These findings are supported by the study of the ATP driven pumping activity (Fig. 10) which suggests an activity in chestnut thylakoids smaller than that observed in spinach. Both chestnut and spinach enzyme was not completely blocked by oligomycin suggesting the existence of other proton permeable regions than that from ATPase, namely the already referred pores in the membranes. Nevertheless, when spinach thylakoids were incubated in a low salt medium, the proton flow after oligomycin was completely stopped (data not presented). This finding is also supported by the half time of ACMA fluorescence recovery when incubated in a hypo-saline medium, which in both thylakoid species is relatively shorter than the value typical for spinach (Figs. 7 and 8). This indicates the presence of additional pores as a consequence of the imposed swelling (Gomes-Laranjo *et al*. 2002), these pores functioning as uncoupler agents.

An additional explanation for difference between the results on ΔpH generating measured by nigericin and pH electrode might be due to a possible high affinity of chestnut thylakoids to the uncoupler as a result of their architecture. Relative to spinach, chestnut thylakoids have a significantly different fatty acid composition, a high content of 16:0 (palmitic acid), a low content of 18:3 (α-linolenic acid), and an almost complete lack of 16:3 (hexadecatrienoic acid) components. These main three fatty acids represent about 68 and 80 % of the total amount and give an unsaturation index of 158 and 234 for chestnut and spinach, respectively (Gomes-Laranjo *et al*. 2002, 2004).

In conclusion, obtaining chestnut chloroplast extracts with good photosynthetic activity is now possible. The above mentioned assay may form a strong base for further investigations into the bioenergetic and economic importance of the chestnut tree.

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