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REGULAR PAPER

Mitochondrial electron transport protects floating leaves of long leaf pondweed (*Potamogeton nodosus* Poir) against photoinhibition: comparison with submerged leaves

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Abstract Investigations were carried to unravel mechanism(s) for higher tolerance of floating over submerged leaves of long leaf pondweed (*Potamogeton nodosus* Poir) against photoinhibition. Chloroplasts from floating leaves showed ~5- and ~6.4-fold higher Photosystem (PS) I (reduced dichlorophenol-indophenol → methyl viologen → O_2) and PS II (H_2O → parabenzoquine) activities over those from submerged leaves. The saturating rate (V_{max}) of PS II activity of chloroplasts from floating and submerged leaves reached at ~600 and ~230 μmol photons m^{-2} s^{-1}, respectively. Photosynthetic electron transport rate in floating leaves was over 5-fold higher than in submerged leaves. Further, floating leaves, as compared to submerged leaves, showed higher F_v/F_m (variable to maximum chlorophyll fluorescence, a reflection of PS II efficiency), as well as a higher potential to withstand photoinhibitory damage by high light (1,200 μmol photons m^{-2} s^{-1}). Cells of floating leaves had not only higher mitochondria to chloroplast ratio, but also showed many mitochondria in close vicinity of chloroplasts. Electron transport (NADH → O_2; succinate → O_2) in isolated mitochondria of floating leaves was sensitive to both cyanide (CN^-) and salicylhydroxamic acid (SHAM), whereas those in submerged leaves were sensitive to CN^-, but virtually insensitive to SHAM, revealing the presence of alternative oxidase in mitochondria of floating, but not of submerged, leaves. Further, the potential of floating leaves to withstand photoinhibitory damage was significantly reduced in the presence of CN^- and SHAM, individually and in combination. Our experimental results establish that floating leaves possess better photosynthetic efficiency and capacity to withstand photoinhibition compared to submerged leaves; and mitochondria play a pivotal role in protecting photosynthetic machinery of floating leaves against photoinhibition, most likely by oxidation of NAD(P)H and reduction of O_2.

Keywords Chlorophyll *a* fluorescence · CN-resistant alternative oxidase pathway · CN-sensitive cytochrome oxidase pathway · Chloroplast-mitochondria interaction · Photoinhibition · *Potamogeton nodosus*

Abbreviations

Chl	Chlorophyll
CN	Cyanide

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DCMU	3-(3,4-Dichlorophenyl)-1,1-Dimethylurea
DCPIP	Dichlorophenol indophenol
ETR	Electron transport rate
F_m	Maximum chlorophyll *a* fluorescence
F_o	Initial (minimum) chlorophyll *a* fluorescence
F_v	Variable (F_m–F_o) chlorophyll *a* fluorescence
MV	Methyl viologen
NAD(P)$^+$	Oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	Reduced nicotinamide adenine dinucleotide (phosphate)
pBQ	Parabenzoquinone
PFD	Photon flux density
PQ	Plastoquinone
PS I, PS II	Photosystem I, Photosystem II
Q_A	Oxidized primary plastoquinone electron acceptor of PS II
Q_{A^-}	Reduced primary plastoquinone electron acceptor of PS II
SHAM	Salicylhydroxamic acid

Introduction

Bioenergetics of plants involves chloroplasts, where photosynthesis takes place, as well as mitochondria, where respiration takes place (Cramer and Knaff 1991). These two organelles together maintain cellular redox status largely by regulating the balance of oxidized and reduced forms of nicotinamide dinucleotide (phosphate), NAD(P)$^+$, and NAD(P)H. Normally, the rate of light-driven NADP$^+$ reduction is balanced with simultaneous oxidation of NADPH through carbon assimilation (i.e., through Calvin–Benson cycle) and other assimilatory pathways such as that of nitrogen and sulfur (Foyer and Noctor 2002; Kramer et al. 2004; Hell et al. 2008; Scheibe and Dietz 2012; Taniguchi and Miyake 2012). However, whenever the rate of light-induced generation of NADPH and reduced ferredoxin exceeds the rate of their oxidation, reductants accumulate in thylakoid membranes, as well as in the stroma region. This results in cellular redox imbalance that favors an increase in one-electron reduction of molecular oxygen leading to the generation of reactive oxygen species (ROS) (Demmig-Adams and Adams 2006; Yoshida et al. 2006, 2008; Møller et al. 2007; Kangasjarvi et al. 2012; Schmitt et al. 2014). ROS, in turn, cause(s) photoinhibition by either damaging components of photosynthetic machinery, in particular Photosystem II (PS II), or inhibiting/inactivating PS II repair mechanisms through suppression of protein synthesis (Nishiyama et al. 2011).

In addition, ROS also interact(s) with and inactivate(s) several cellular macromolecules, which include proteins, nucleic acids, and lipids resulting in disruption of overall cellular metabolism (Demmig-Adams et al. 2006; Møller et al. 2007; Møller and Sweetlove 2010; Chen et al. 2012; Suzuki et al. 2012). Thus, cellular capacity to modulate the NAD(P)H/NAD(P)$^+$ ratio is critical not only for the redox control of metabolism, but also for restraining oxidative stress (Bartoli et al. 2005; Buchanan and Balmer 2005; Noctor 2006; Møller et al. 2007; Yoshida et al. 2006, 2008; Foyer and Noctor 2002, 2012).

Although photosynthesis is an autonomous metabolic process that takes place in chloroplasts, its efficiency is strongly linked to mitochondrial activity (Raghavendra and Padmasree 2003; Yoshida et al. 2006; 2008; Poolman et al. 2013; Araujo et al. 2014). It is known that mitochondrial electron transport, mediated by both cyanide (CN)-sensitive cytochrome c oxidase, and CN-resistant alternative oxidase pathways, optimizes photosynthetic activity at saturating light intensity by modulating reducing equivalents and ensuring the rapid availability of NADP$^+$ (terminal acceptor of photosynthetic electron transport) (Bartoli et al. 2005; Yoshida et al. 2006, 2011a, 2011b; Suzuki et al. 2012; Araujo et al. 2014). In fact, inhibition of either of the above-mentioned pathways leads to a decrease in the steady state level of photosynthetic O_2 evolution, as well as in the quantum yield of PS II (Raghavendra and Padmasree 2003; Yoshida et al. 2006; Møller et al. 2007; Zhang et al. 2012).

Potamogeton nodosus Poir (long leaf pondweed) is a heterophyllous monocotyledonous aquatic plant with two distinct types of leaves: floating and submerged (Ryan 1985; also see http://plants.usda.gov/core/profile?symbol=pono2). Submerged leaves are thin, translucent with little or no cuticle. In contrast, floating leaves are thick, opaque with shining thick cuticle. Ryan (1985) had recorded superior CO_2 fixation in cells isolated from floating leaves compared to those from submerged leaves of *P. nodosus*. Kordyum and Klimenko (2013), based on chlorophyll (Chl) *a* fluorescence data, have reported that submerged leaves of heterophyllous *Nuphar lutea* are susceptible to photoinhibition. Chlorophyll *a* fluorescence is widely used to probe various aspects of photosynthetic photochemical reactions (see e.g., Strasser and Strasser 1995; Papageorgiou and Govindjee 2004; Strauss et al. 2006). Here, in this paper, we show *for the first time* that floating leaves, as compared to submerged leaves, not only exhibit a superior photosynthetic capacity (measured in terms of PS I and PS II activities), but also show a superior potential to withstand photoinhibitory damage by high photon flux density. This potential of floating leaves to withstand photoinhibitory damage is linked not only to a large number of mitochondria in floating over submerged leaves, but it is also due to differences in CN-sensitive cytochrome oxidase and CN-resistant alternative oxidase pathways in mitochondria.

Materials and methods

Plant material

Potamogeton nodosus Poir (Family: Potamogetonaceae) was obtained from Kapalmochan pond, Bilaspur (District Yamuna Nagar, Haryana, India). The temperature around the *Kapalmochan* pond varies between ~18 °C (in March) and ~40 °C (in May) and solar irradiance at the surface of the pond generally ranges to be from 800 to 2,400 μmol photons m^{-2} s^{-1} during the day. For the present investigation, *P. nodosus* plants were grown at the University of Delhi, India, in cement tubs of 87 cm (height), 60 cm (breadth), and 54 cm (width), with 12 cm soil (depth). Temperature at the water surface varied between ~18 °C (in March) and ~42 °C (in May), essentially as in *Kapalmochan* pond, whereas solar irradiance varied between 800 and 2400 μmol photons m^{-2} s^{-1}. In all our experiments, we used fully developed leaves and all the measurements were made during March to October.

Photosynthetic investigations with isolated chloroplasts

Isolation of chloroplasts

Chloroplasts were isolated following a protocol modified from that by Alia et al. (1992). Floating and submerged leaves of *P. nodosus* were rinsed in distilled water, chopped into small pieces, and then incubated in dark for 30 min in a chilled isolation buffer (20 mM tricine with 400 mM sucrose, 10 mM NaCl, 10 mM ascorbate and 4 % polyvinyl polypyrrolidone, pH 7.8). Subsequently, these leaf segments were homogenized in dark with the isolation buffer, using a chilled mortar and pestle. The resultant homogenate was filtered through 4 layers of Mira cloth and the filtrate was centrifuged for 10 min at 5,000 × *g* at 4 °C. The pellet was suspended in a small volume of suspension/reaction buffer (20 mM tricine with 100 mM sucrose, 10 mM NaCl and 2 mM $MgCl_2$, pH 7.8) and used for further investigations. Chlorophyll content was determined according to the method and the equations used by Arnon (1949).

Electron transport measurements

Photosystem (PS) I, PS II, and whole chain-dependent O_2 evolution/consumption were measured polarographically, using a Clark-type liquid phase O_2 electrode (Hansatech, UK). Light curves of PS II reaction (water to parabenzoquinone, pBQ), in chloroplasts isolated from both floating and submerged leaves, were plotted by measuring oxygen evolution, at various light intensities, obtained by using calibrated neutral density filters (Balzers, Neugrüt, Lichtenstein). For PS II electron transport, the reaction mixture was 20 mM tricine with 100 mM sucrose, 10 mM NaCl and 2 mM $MgCl_2$ (at pH 7.8), and 1 mM pBQ. Maximum light intensity (or photon flux density, PFD) used was 920 μmol photons m^{-2} s^{-1}. Based on the results of light saturation curve (Fig. 1), electron transfer activity for PS I ($DCPIPH_2 \rightarrow MV \rightarrow O_2$); for PS II ($H_2O \rightarrow$ pBQ); and for whole chain ($H_2O \rightarrow MV \rightarrow O_2$) of chloroplasts, isolated from floating and submerged leaves, were measured at a PFD of ~920 and ~230 μmol photons m^{-2} s^{-1}, respectively, All experiments were made at 25 °C. To assay PS I activity, we used 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 100 μM dichlorophenol indophenol (DCPIP) with 1 mM sodium ascorbate, 1 mM sodium azide, and 0.5 mM methyl viologen (MV); DCMU was used to block electron flow from PS II to PS I, reduced DCPIP as an electron donor, MV as an electron acceptor, and sodium azide as an inhibitor of catalase. For the assay of whole chain electron transport, reaction mixture consisted, in addition to the suspension buffer (20 mM tricine with 100 mM sucrose, 10 mM NaCl and 2 mM $MgCl_2$, pH 7.8), 0.5 mM MV, and 1 mM sodium azide. Chloroplasts equivalent to 20 μg of chlorophyll were used for all assays. Electron transport measurements were expressed as μmol O_2 evolved/consumed mg Chl^{-1} h^{-1} for PS II/PS I activities.

Photosynthetic investigations with leaves

Leaf level photosynthesis was measured using a portable photosynthesis system (LICOR-6400, LI-COR, Lincoln, Nebraska, USA) with an integrated fluorometer (LI-6400-40). After dark adaptation of leaves for 1 h, minimal chlorophyll (Chl) fluorescence (Fo) was measured with a weak modulated red (630 nm; half-band width, ~15 nm) light (< 0.1 μmol photons m^{-2} s^{-1}). Then a 600-ms red (630 nm) saturating flash (7,000 μmol photons m^{-2} s^{-1}) was applied to determine the maximum Chl fluorescence yield (Fm). Immediately, the leaf was illuminated continuously with actinic light (which had 90 % 630 nm red light; half-band width, ~15 nm, from LEDs, and 10 % 470 nm blue light from LEDs; half-band width, ~20 nm) of varying intensities for 2 min to determine the steady state fluorescence, F_s. Afterward, the same saturating red (630 nm) flash (7,000 μmol photons m^{-2} s^{-1}) was given to determine $F_{m'}$ (i.e., F_m in light). After the flash, the actinic light was removed and far-red light (740 nm, half band width, ~ 30 nm) was given to determine $F_{o'}$. The absolute rates of photosynthetic electron transport (ETR) in leaves at a given PFD were assessed by $\Phi_{PS\ II} \times I \times f \times a_{leaf}$, as described by Schreiber et al. (1998), where, I is incident PFD, f is the fraction of absorbed quanta that is used by PS II (assumed to be 0.5), and a_{leaf} is the leaf absorbance assumed to be 0.84. $\Phi_{PS\ II}$ was, however, determined using the equation $(F_{m'}-F_s)/F_{m'}$ (Genty et al. 1989).

Studies on tolerance of leaves to photoinhibition

To determine tolerance of floating and submerged leaves to photoinhibition, they were placed in borosil trays (5″ × 8″) containing distilled water; they were then exposed to high light from incandescent lamps (Powertone, Philips) at photosynthetically active radiation (PAR) of 1,200 µmol photons m^{-2} s^{-1}. A temperature of ~25 °C was maintained by blowing cool air through an air conditioner continuously over and sides of trays in which leaves were exposed to high light intensity. At intervals of 30 min, leaves were taken and dark-adapted for 15 min before Chl *a* fluorescence induction measurements were made. Fluorescence transient was measured at 25 °C, using plant efficiency analyzer (Handy PEA; Hansatech Ltd., UK), as described by Strasser et al. (1995). Fluorescence was excited by red light (peak at 650 nm) at an intensity of 3,500 µmol photons m^{-2} s^{-1}, as provided by an array of six light-emitting diodes. Fluorescence was recorded from 10 µs to 1 s; the 50 µs value was taken as the minimum fluorescence, F_o. Quantum yield of PS II activity was inferred from F_v/F_m, where F_v is variable fluorescence (F_m–F_o, F_m, being the maximum fluorescence, at the P level) (Strasser and Strasser 1995; for a review of earlier work, see: Govindjee 2004). Measurements were made on 8–10 different portions of each leaf, and at least 5 leaves were used for each experiment; all data were then averaged. A Biolyzer software HP 3 (Bioenergetics Laboratory, University of Geneva, Switzerland) was used to calculate several parameters (see "Results") from chlorophyll fluorescence measurements, usually referred to as the OJIPS transient, where O (= F_o) is the initial minimum fluorescence, P (= F_m), the peak, J and I are inflections between O and P, and S is the semi-steady state level (Stirbet and Govindjee 2011).

Testing role of mitochondrial electron transport in protecting photosynthetic machinery against photoinhibition

In order to test if CN-sensitive cytochrome oxidase pathway and CN-resistant alternative oxidase pathway of mitochondrial electron transport play any role in influencing PS II activity and Chl *a* fluorescence kinetics, floating and submerged leaves were exposed to high PFD in the presence of 1 mM NaCN, an inhibitor of CN-sensitive cytochrome oxidase pathway (Bartoli et al. 2000), and 2 mM SHAM (salicylhydroxamic acid), an inhibitor of CN-resistant alternative oxidase pathway (Elthon and Mcintosh 1987), independently and in combination, at 25 °C. Impact of CN^- and SHAM on high PFD-induced photoinhibtory damage to photosynthetic machinery was recorded at intervals of 30 min, as described above.

Isolation and characterization of mitochondria

Mitochondria were isolated using a protocol modified from Kolloffel (1967). Leaves were homogenized in chilled isolation buffer (50 mM phosphate buffer (pH 7.2) with 400 mM sucrose, 1 mM EDTA (ethylene diamine tetra acetic acid), and 4 % polyvinyl pyrrolidine) in prechilled mortar and pestle, and the resultant homogenate was filtered through four layers of mira cloth. The filtrate was centrifuged at 1,500 × *g* for 10 min. The supernatant was centrifuged at 20,000 × *g* for 15 min and the resultant pellet was suspended in a small volume of suspension/reaction buffer (50 mM phosphate buffer (pH 7.6) with 200 mM sucrose). This suspension was used as isolated mitochondria for further experiments.

Mitochondrial electron transport activities were determined using NADH (2 mM) and succinate (2 mM), independently, in the presence and the absence of 1 mM NaCN (which inhibits CN-sensitive cytochrome oxidase pathway) (Bartoli et al. 2000), and 2 mM SHAM (salicylhydroxamic acid, which inhibits CN-resistant alternative oxidase pathway) (Elthon and Mcintosh 1987). Mitochondrial suspension equivalent to 250 µg protein was used for the assays. Protein content was determined using Bradford reagent (Bradford 1976).

Ultrastructural studies

Leaves of *P. nodosus* were fixed and processed according to David et al. (1973). Ultrathin sections (60–80 nm thick) were cut using a Leica Ultracut UCT ultramicrotome, and were stained with uranyl acetate for 10 min followed by lead citrate for 10 min. The stained grids were observed by Morgagni 268 D, Fei Transmission Electron Microscope at 80 kV. Photographs were taken through Megaview III, CCD (Charge-Coupled Device) camera.

All experiments were carried out independently at least six times and Duncan's multiple range test was used to determine the level of significance (Duncan 1955).

Results

Electron transport in isolated chloroplasts of floating and submerged leaves

As compared to those from submerged leaves, isolated chloroplasts from floating leaves of *P. nodosus* showed significantly higher (i) PS II electron transfer from water to pBQ, measured as oxygen evolution; (ii) PS I electron transfer from reduced dichlorophenol-indophenol (DCP-IPH_2) to methyl viologen (MV), measured as oxygen

Table 1 Rates of photosystem I (PS I), photosystem II (PS II), and whole chain electron transport in chloroplasts, isolated from floating and submerged leaves of *P. nodosus*

Assay	μmol O_2 evolved/consumed (mg Chl^{-1} h^{-1})		μmol O_2 evolved/consumed (cm^{-2} h^{-1})	
	Floating leaf	Submerged leaf	Floating leaf	Submerged leaf
PS I reaction ($DCPIPH_2$ → MV → O_2)	635.0 ± 42.7	127.0 ± 15.3	12.3 ± 0.82	1.82 ± 0.21
PS II reaction (H_2O → pBQ)	232.5 ± 27.4	36.5 ± 2.6	4.5 ± 0.53	0.52 ± 0.04
Whole chain reaction (H_2O → MV → O_2)	486.0 ± 52.3	98.5 ± 11.2	9.41 ± 1.01	1.41 ± 0.16

Data represent mean ± SE of at least six independent measurements. $DCPIPH_2$ (reduced dichlorophenol-indophenol) was used as an electron donor; and MV: (methyl viologen) and pBQ (parabenzoquinone) were used as electron acceptors. For details, see "Materials and methods"

uptake; as well as (iii) whole chain electron transport from water to methyl viologen, measured as oxygen uptake (see Table 1). Chloroplasts of floating leaves showed ~5- and ~6.4-fold higher PS I and PS II activities (per mg chlorophyll) compared to those from submerged leaves. Photochemical activities measured in isolated chloroplasts, on the basis of chlorophyll (Chl) concentration, were then extrapolated to determine their activities per unit leaf area (Table 1; see last two columns). On leaf area basis, floating leaves had ~6.8- and 8.7-fold higher PS I and PS II mediated photochemical reactions, compared to the submerged leaves.

Figure 1 shows light curves of PS II activity (water to parabenzoquinone), for chloroplasts from floating and submerged leaves. Chloroplasts of floating leaves show that the maximum rate (V_{max}) is reached at a photon flux density (PFD) of ~600 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (PAR), whereas PS II activity in submerged leaves was saturated at ~200 μmol photons m^{-2} s^{-1}, and that too at a very low rate (Fig. 1). V_{max} of PS II activity of chloroplasts of floating leaves was ~8 times higher than that of submerged leaves. Unlike chloroplasts of floating leaves

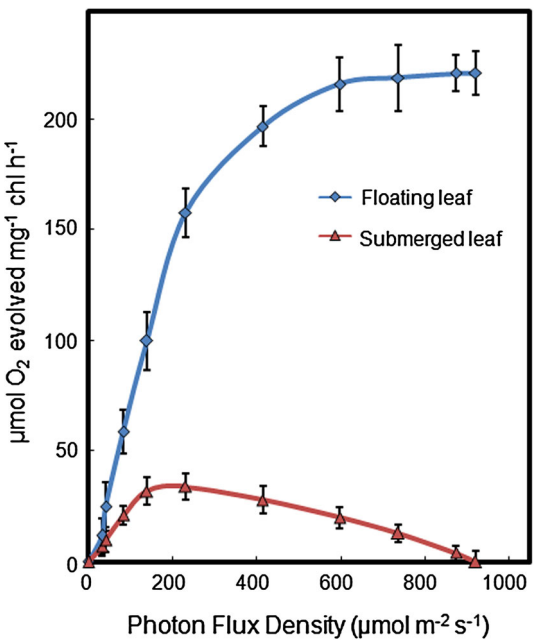

Fig. 1 Rate of oxygen evolution as a function of light intensity for Photosystem II (H_2O → pBQ) in chloroplasts isolated from floating and submerged leaves of *P. nodosus*. One mM parabenzoquinone (pBQ) was used as an electron acceptor (for details, see "Materials and methods"). Data represent mean of recordings from six independent experiments. *Vertical lines* on data points represent standard errors. Data was subjected to Duncan's multiple range test. Data points of chloroplast from floating leaves differed significantly at $P \leq 0.05$ from that of submerged leaves at all points with the exception of data points at 0, 32, and 41 μmol photons m^{-2} s^{-1}

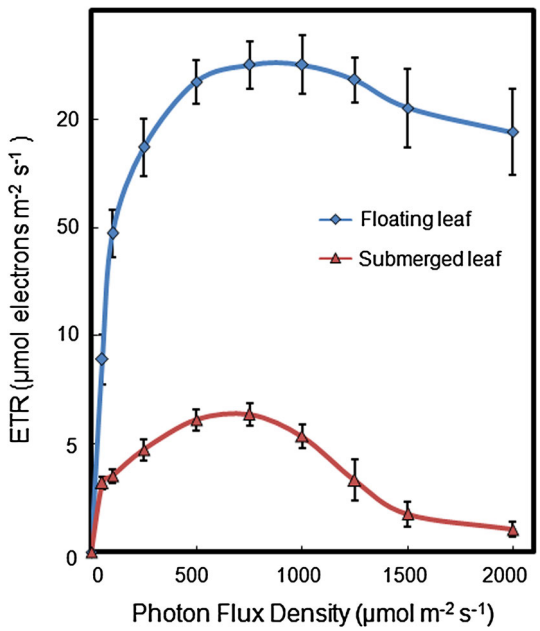

Fig. 2 Light intensity dependence of rate of electron transport rate (ETR), measured using LICOR-6400 portable photosynthesis system in floating and submerged leaves of *P. nodosus*. ETR was calculated using the formula [$ETR = \Phi_{PS\ II} \times I \times f \times a_{leaf}$], as described by Schreiber et al. (1998), where, I is incident PFD, *f* is the fraction of absorbed quanta that is used by PS II (assumed to be 0.5), and a_{leaf} is the leaf absorbance which was assumed to be 0.84. Data represent mean of recordings from six independent experiments. *Vertical lines* on data points represent standard error. Data was subjected to Duncan's multiple range test. Data points of floating leaves differed significantly at $P \leq 0.05$ from that of submerged leaves at all points

that retained maximal PS II activity even at 920 µmol photons m^{-2} s^{-1}, those of submerged leaves showed a sharp decline above ~230 µmol photons m^{-2} s^{-1}, with a complete loss in PS II activity at ~920 µmol photons m^{-2} s^{-1} (Fig. 1). These results clearly show that chloroplasts of submerged leaves have higher susceptibility to photoinhibition than those of floating leaves.

Electron transport rate in floating and submerged leaves

Chl fluorescence measurements, made using LI-6400-40 leaf chamber fluorometer, revealed that the calculated overall photosynthetic electron transport rate (ETR; for details see "Materials and methods") in floating leaves was over 5-fold higher than that of submerged leaves. Light response curves of electron transport rate in floating and submerged leaves are shown in Fig. 2.

Further, the maximum electron transport rate (V_{max}), for both floating as well as submerged leaves, was attained at PFD of 750 µmol photons m^{-2} s^{-1}. However, V_{max} of ETR in floating leaves was ~5-fold higher than in submerged leaves. Submerged leaves showed a sharp decline in ETR beyond 750 µmol photons m^{-2} s^{-1}, while floating leaves showed only a marginal decline beyond 1,250 µmol photons m^{-2} s^{-1} (Fig. 2). In contrast to ~85 % decline shown by submerged leaves, floating leaves showed only ~13 % decline when they were exposed to 2,000 µmol photons m^{-2} s^{-1}. Light curves of floating and submerged leaves clearly show that floating leaves possess better tolerance capacity to photoinhibition than the submerged leaves.

Tolerance capacity of floating and submerged leaves to photoinhibition

The ratio of variable ($F_v = F_m - F_o$) to maximum (F_m) Chl *a* fluorescence of floating leaves was ~0.8, while that of submerged leaves varied between 0.6 and 0.7, irrespective of whether they were from natural habitat or grown in a cement basin (as detailed under "Materials and methods"). Since these values represent maximum quantum yield of PS II (Adams et al. 1990; Govindjee 2004; Baker 2008; Wientjes et al. 2013), it is clear that, in submerged leaves, this yield is ~20 % less than that in floating leaves. Although, both floating and submerged leaves of *P. nodosus* showed a decline in PS II activity on exposure to high PFD (1,200 µmol photons m^{-2} s^{-1},

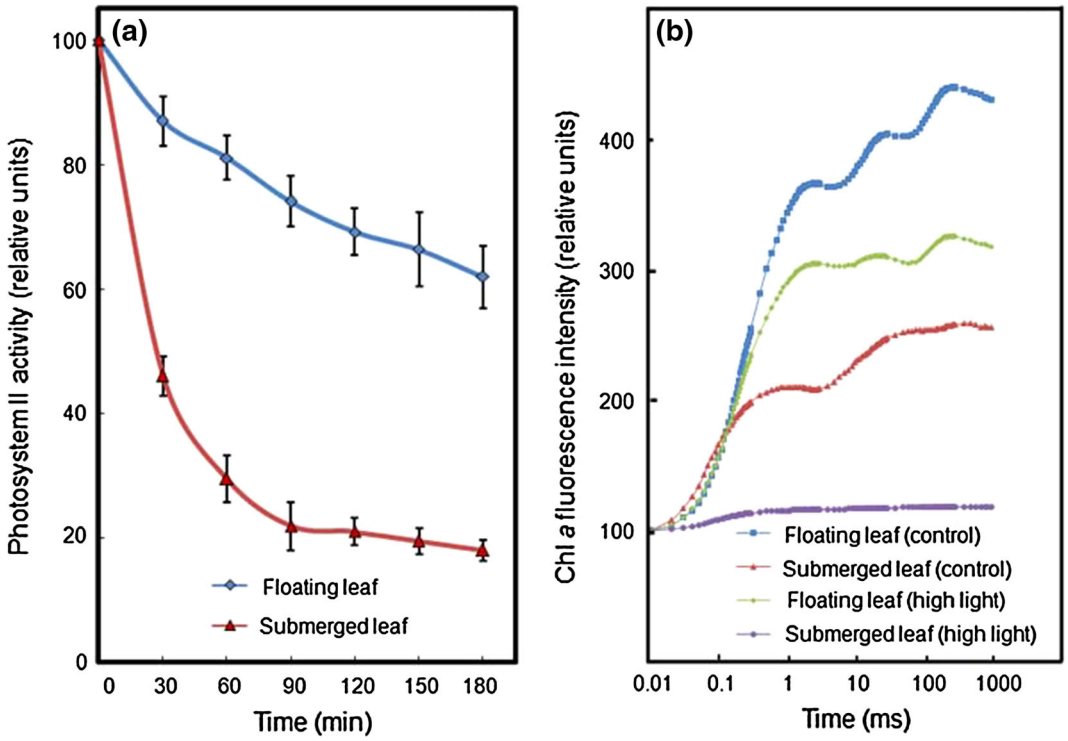

Fig. 3 Effect of high photon flux density (PFD, 1,200 µmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation, PAR) (High Light) on Photosystem II in floating and submerged leaves of *P. nodosus*. **a** Time dependence of PS II activity (as calculated from the ratio of variable to maximum fluorescence, F_v/F_m) in leaves kept under high light, up to 3 h. Initial F_v/F_m values of floating and submerged leaves were 0.835 ± 0.015 and 0.675 ± 0.035, respectively; **b** Chlorophyll (Chl) *a* fluorescence transients plotted by normalizing data at F_o values (301 ± 45 for floating, and 491 ± 24 for submerged leaves). Data are a mean of recordings from six independent experiments. Vertical lines on data points in **a** represent standard errors. Data represented in panel **a** were subjected to Duncan's multiple range test. Data points of floating leaves differed significantly at $P \leq 0.05$ from that of submerged leaves at all points in **a**

Table 2 Photosynthetic pigment content in floating and submerged leaves of *P. nodosus*

	Pigment level (μg g^{-1} fresh weight)		Pigment level (μg cm^{-2})	
	Floating leaf	Submerged leaf	Floating leaf	Submerged leaf
Chl *a*	727.7 ± 59.3	641.7 ± 60.5	13.7 ± 1.5	8.1 ± 0.8
Chl *b*	310.8 ± 37.8	332.5 ± 19.2	5.9 ± 0.7	4.2 ± 0.4
Chl *a* + *b*	1036.7 ± 83.7	972.5 ± 81.8	19.5 ± 1.7	12.3 ± 1.3
Carotenoids	158.8 ± 22.2	112.4 ± 19.7	2.99 ± 0.33	1.21 ± 0.21
Chl *a*/*b*	2.34 ± 0.28	1.93 ± 0.13	2.34 ± 0.28	1.93 ± 0.13

Data represent mean ± SE ($n = 10$). Abbreviation used is Chl: chlorophyll

Fig. 3a), the degree of decline recorded in submerged leaves was sharper (~54 % decline within 30 min) than that recorded in floating leaves which showed only ~38 % decline, even after 180 min exposure to high PFD.

Both floating and submerged leaves of *P. nodosus* exhibited typical polyphasic Chl *a* fluorescence induction curves (Fig. 3b). Chl *a* fluorescence intensity, plotted on a logarithm time scale, rose from the initial fluorescence level (F_o), at the O level, to a maximum fluorescence level (Fm) at the P level, with intermediate fluorescence (F_j) at J and (F_i) at I levels, showing a typical OJIP fluorescence transient (Strasser et al. 1995; Stirbet and Govindjee 2011, 2012). Although, Fo was ~21 % higher, Fv was ~50 % lower in submerged leaves compared to those in floating leaves. Low Chl *a*/*b* ratio in submerged leaves, compared to that in floating leaves (Table 2), indicates a larger antenna size and accordingly a higher F_o.

The O–J rise, which represents reduction of Q_A to Q_A^- (Stirbet and Govindjee 2011), and the I–P rise, which is due to further increased concentration of Q_A^- because of a "traffic jam" of electrons on the electron acceptor side of PS I (Munday and Govindjee 1969a, b; Toth et al. 2007; Stirbet and Govindjee 2012), were ~3 and ~67 % lower in submerged leaves compared to floating leaves, respectively. In contrast, the J–I rise, which implies further increased concentration of Q_A^- due to the reduction of the PQ pool (Toth et al. 2007), was ~21 % higher in submerged leaves compared to floating leaves. Although Chl *a* fluorescence induction curve of both floating and submerged leaves exposed to high PFD for 60 min showed significant decline in the amplitude of fluorescence, Chl *a* fluorescence transient of floating leaves retained its polyphasic nature and showed distinct O, J, I, and P levels, while that of the submerged leaves lost its polyphasic nature (Fig. 3b).

Ultrastructural investigations

In comparison with submerged leaves, floating leaves had not only significantly higher efficiency of PS II-mediated electron transport reactions, but their potential to withstand high PFD-induced photodamage was greater. Thus, ultrastructural investigations were made to probe if there is any variation in the structure of chloroplasts and other components in their cells. These investigations revealed the presence of a large number of chloroplasts intermingled with other organelles such as mitochondria, endoplasmic reticulum, peroxisomes, nucleus, and vacuole in cells of both floating (Fig. 4a) and submerged leaves (Fig. 4b).

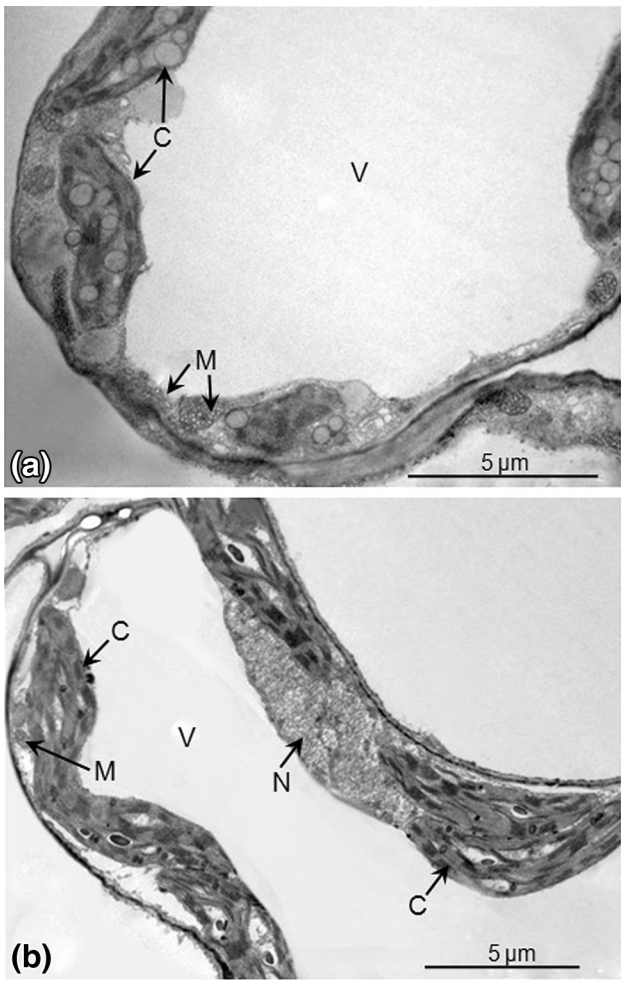

Fig. 4 Transmission electron micrographs (TEM) of a portion of a cell from floating (**a**) and submerged (**b**) leaves of *P. nodosus*. Note numerous mitochondria in **a** and few mitochondria in **b**. Also note distinct variations between the chloroplasts in **a** and **b**; and the presence of mitochondria in close vicinity of chloroplasts in **a**. *C* chloroplasts; *M* mitochondria; *V* vacuole. See figure for magnification scale

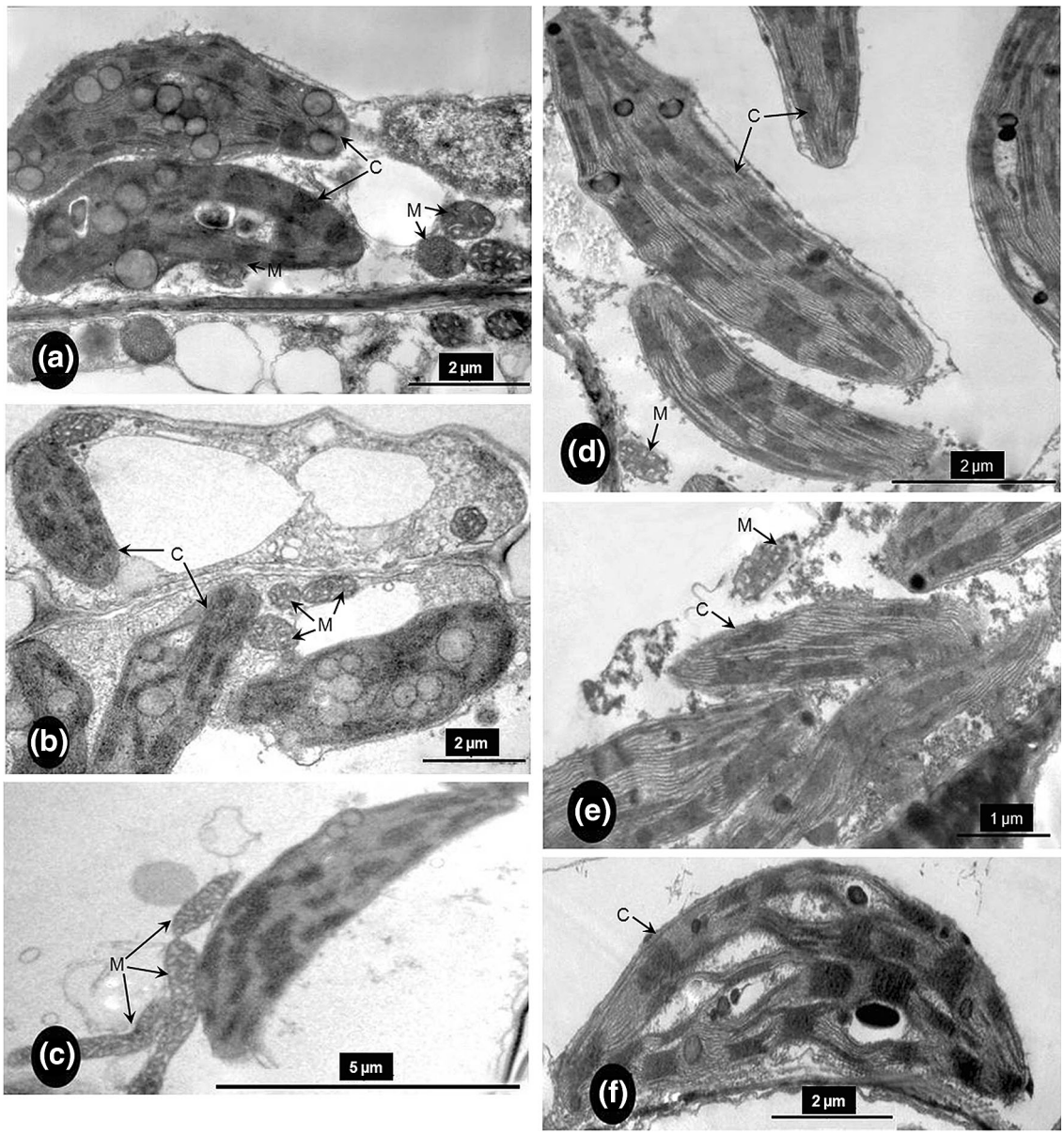

Fig. 5 Transmission electron micrographs (TEM) of parts of cells of floating (**a**–**c**) and submerged (**d**–**f**) leaves of *P. nodosus*. Note numerous mitochondria in close association with chloroplasts in **a** and **c**, large number of starch grains in **a** and **b**, and more distinct grana and plastoglobuli in **d** and **f**. *C* chloroplasts; *M* mitochondria; *V* vacuole. Note different magnification scales in different subfigures

In general, chloroplasts of both floating (Fig. 5a–c) and submerged (Fig. 5d–f) leaves had typical well-developed grana and intergranal thylakoids. However, chloroplasts of submerged leaves had significantly higher number of grana and larger number of thylakoids per granum compared to floating leaves. Unlike chloroplasts of submerged leaves that contained distinct plastoglobuli and fewer starch grains, those of floating leaves had numerous large starch grains (Fig. 5a,b). Further, cells of floating leaves had significantly higher number of mitochondria than submerged leaves (Fig. 5). In general, the ratio of mitochondria to chloroplasts was 3:1 in floating leaves, whereas it was 1:3 in submerged leaves. Further, majority of mitochondria in cells of floating leaves were seen in close vicinity of chloroplasts (Fig. 5a–c). This suggests that mitochondria, which are present in large numbers and in close vicinity to chloroplasts, may have a role in providing tolerance to floating leaves against high PFD-induced damage to PS II-mediated photochemical reactions, compared to that in submerged leaves.

Potential of isolated mitochondria to oxidize reductants

As shown in Table 3, mitochondria, isolated from both floating and submerged leaves of *P. nodosus*, effectively

Table 3 Mitochondrial electron transport activity (*n*moles of O_2 consumed mg^{-1} protein min^{-1}) in mitochondria isolated from floating and submerged leaves of *P. nodosus*

	Electron Donor	Leaf type	Control	CN^-	SHAM
1	NADH	Floating	142.5 ± 6.7	68.2 ± 11.8 (52.1)	71.6 ± 9.9 (49.7)
2		Submerged	28.9 ± 3.1	2.7 ± 0.9 (90.7)	27.7 ± 1.7 (4.1)
3	Succinate	Floating	58.5 ± 7.3	29.0 ± 4.1 (50.4)	32.1 ± 4.3 (45.1)
4		Submerged	15.2 ± 1.9	0.9 ± 0.4 (94.1)	14.7 ± 1.8 (3.3)

Two electron donors NADH (reduced form of nicotinamide adenine dinucleotide; 2 mM) and succinate (5 mM) were used for determining electron transport activity. Electron transport activities were determined in dark in the absence (control) and presence of CN^- (cyanide, 1 mM)/SHAM (Salicylhydroxamic acid; 2 mM). For further details, see "Materials and methods". Data represent mean ± SE of at least five independent measurements. The figures in parentheses indicate percentage inhibition compared to their controls

oxidized NADH and succinate. It is well known that NADH donates electrons to complex-I (NADH-UQ oxidoreductase), and succinate feeds electrons to complex–II (succinate-UQ oxidoreductase) (Millar et al. 2011). Mitochondria isolated from floating leaves had ~3- and 5-fold higher potential to oxidize succinate and NADH, respectively, compared to those from the submerged leaves.

In order to evaluate if mitochondria of floating and submerged leaves possess alternative oxidase-mediated CN-resistant pathway, along with CN-sensitive cytochrome c oxidase pathway, mitochondrial electron transport activities were measured in the absence and the presence of inhibitors of (i) CN-sensitive cytochrome oxidase pathway, i.e., CN^-, which inhibits cytochrome c oxidase of complex IV; and (ii) CN-resistant alternative oxidase pathway, i.e., salicylhydroxamic acid (SHAM), which inhibits alternative oxidase. Even though potential of isolated mitochondria of both floating and submerged leaves to oxidize NADH and succinate was sensitive to CN^-, the CN^--induced reduction in electron transport activities (NADH → O_2, Succinate → O_2) in mitochondria of submerged leaves was ~ 91–94 %, while that in floating leaves, it was only ~ 50–52 %. In contrast, although mitochondria isolated from floating leaves showed ~45–50 % reduction,

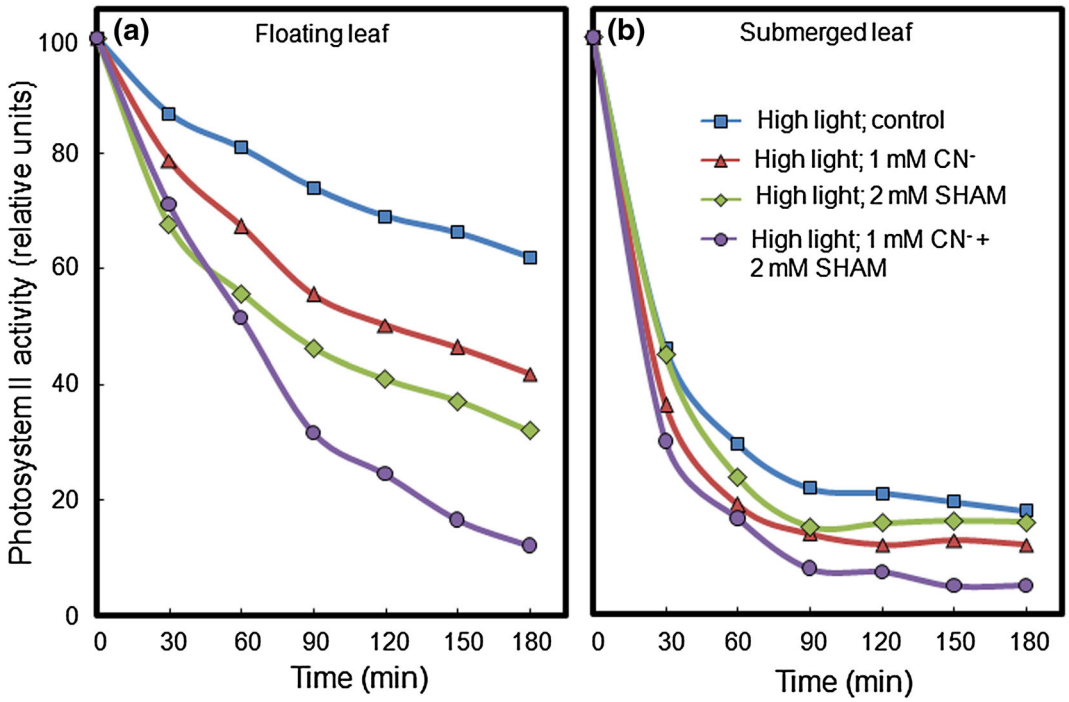

Fig. 6 Time dependence of Photosystem II activity (as measured in Fig. 3) of floating (**a**) and submerged (**b**) leaves of *P. nodosus* exposed to high photon flux density (PFD, 1,200 μmol photons m^{-2} - s^{-1} of photosynthetically active radiation, PAR) (High light). Data are for control leaves as well as for those treated with 1 mM cyanide, CN^- (an inhibitor of CN-sensitive cytochrome oxidase pathway) and 2 mM salicylhydroxamic acid, SHAM (an inhibitor of CN-resistant alternative oxidase pathway), and both CN^- and SHAM together. Data represent mean of recordings from six independent experiments

those from submerged leaves showed only marginal (3–4 %) reduction in electron transport activities in the presence of SHAM. These results imply that while mitochondria of floating leaves possess both CN-sensitive cytochrome c oxidase and CN-resistant alternative oxidase pathways, that of submerged leaves show largely CN-sensitive cytochrome c oxidase pathway.

Is there a role of mitochondria in protecting photosynthetic machinery against photoinhibition?

In order to probe if mitochondria have a role in protecting the machinery responsible for photochemical reactions against photoinhibition, both floating and submerged leaves were exposed to high PFD in the absence and the presence of (i) CN^- and (ii) SHAM. We note that these inhibitors of CN-sensitive cytochrome oxidase pathway and CN-resistant alternative oxidase pathway accelerated high PFD-induced damage to PS II activity in both floating and submerged leaves (Fig. 6). However, the degree of acceleration in loss in PS II activity by these inhibitors was significantly more in floating leaves, when compared to that in submerged leaves (Fig. 6).

Further, the OJIP fluorescence transient of floating leaves exposed to high PFD for 60 min was severely affected in the presence of these inhibitors, independently as well as in combination. However, submerged leaves exposed to high PFD for 60 min, both in the presence and the absence of these inhibitors, failed to exhibit polyphasic Chl *a* fluorescence transient (Fig. 7). The potential of SHAM to accelerate suppression in PS II activity of floating leaves under high PFD was significantly higher than that with CN^- (Fig. 6). High PFD-induced suppression in PS II activity of floating leaves by SHAM and CN^- in combination was significantly higher than that recorded when they were used independently.

In agreement with the above results, polyphasic nature of Chl *a* fluorescence transient of floating leaves was completely lost in the presence of SHAM alone or in

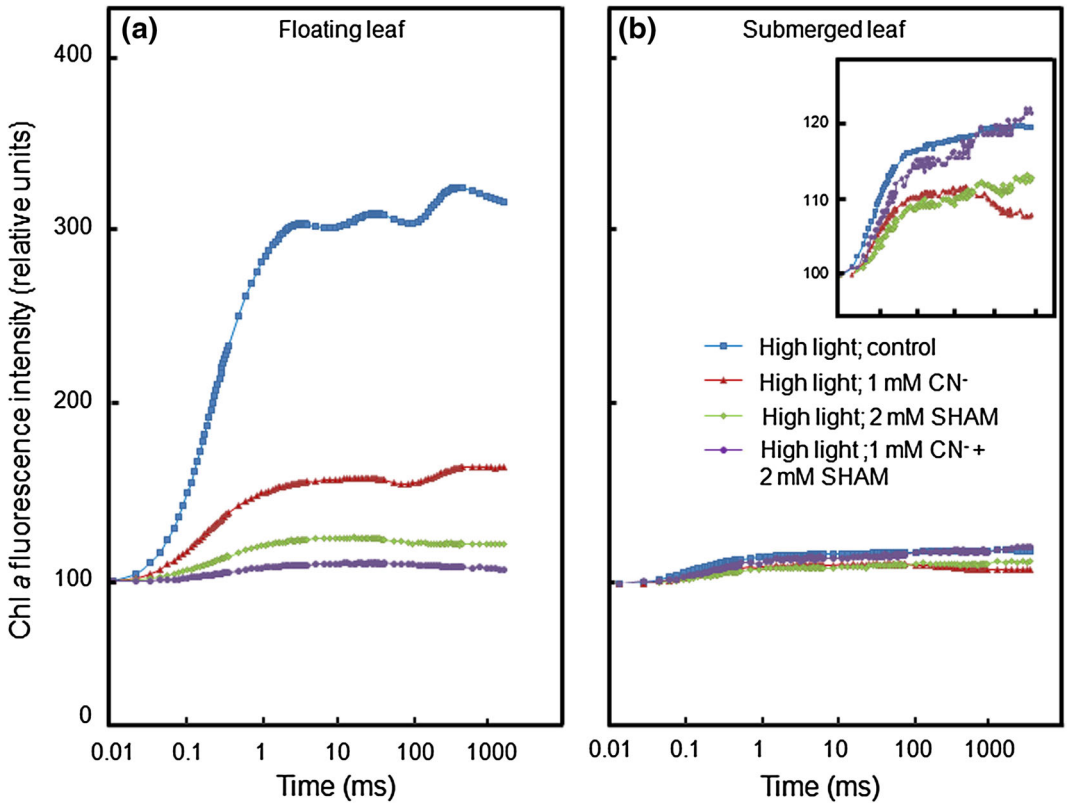

Fig. 7 Chlorophyll *a* fluorescence transients of floating (**a**) and submerged (**b**) leaves of *P. nodosus* after 60 min exposure to high photon flux density (PFD, 1,200 µmol photons m^{-2} s^{-1} of photosynthetically active radiation, PAR) (High light) in the absence and the presence of 1 mM CN^- (an inhibitor of CN-sensitive cytochrome oxidase pathway) and 2 mM SHAM (inhibitor of CN-resistant alternative oxidase pathway). Transients were plotted by normalizing data at F_o values; these values for high light exposed floating leaves (**a**) in the absence (high light control) and the presence of CN^- (high light; CN^-), SHAM (high light; SHAM), and CN^- + SHAM (high light; CN^- + SHAM) were 299 ± 25, 302 ± 28, 411 ± 45, and 419 ± 37, respectively. Similarly, F_o values of high light exposed submerged leaves (**b**) in the absence (high light; control) and the presence of CN^- (high light; CN^-), SHAM (high light; SHAM), and CN^- + SHAM (high light; CN^- + SHAM) were 460 ± 32, 580 ± 45, 426 ± 28, and 519 ± 36, respectively. *Inset* shows Chl *a* fluorescence transients in **b** on a magnified *y* axis

combination with CN^- (Fig. 7). Although, Chl *a* fluorescence transient of floating leaves exposed to high PFD for 60 min in the presence of CN^- was significantly affected, it retained the polyphasic nature of Chl *a* fluorescence (Fig. 7).

Discussion

Superior (or better) photosynthetic efficiency of floating leaves over submerged leaves

We have established in this paper that floating leaves of long leaf pondweed, and their isolated chloroplasts, have higher photosynthetic electron transport efficiency, compared to that in the submerged leaves and their isolated chloroplasts. Photosynthetic electron transport rate of floating leaves was ~5 times higher than that of submerged leaves. Similarly, V_{max} of PS II activity of isolated chloroplasts from floating leaves was ~8 times higher than that in chloroplasts of submerged leaves. Further, isolated chloroplasts of submerged leaves were highly sensitive to PFD above ~230 µmol photons m^{-2} s^{-1}, whereas that of floating leaves retained maximal activity even at PFD of 920 µmol photons m^{-2} s^{-1}. Similarly, while floating leaves retained maximal photosynthetic electron transport rate at a PFD of 1,250 µmol photons m^{-2} s^{-1}, that of submerged leaves declined sharply at PFD beyond 750 µmol photons m^{-2} s^{-1}.

These results indicate distinct and large variations in the photosynthetic machinery involved in harnessing light energy and in electron transport between floating and submerged leaves of *P. nodosus*. Earlier, Jana and Choudhary (1980) had recorded maximal PS II activity in chloroplasts of *P. pectinatus* (which is a submerged species of *Potamogeton* and bears only submerged leaves) at significantly lower light intensity than in spinach chloroplasts.

In agreement with findings with isolated chloroplasts, photosynthetic efficiency, measured indirectly from the ratio of variable to maximum Chl *a* fluorescence (F_v/F_m), was also higher in floating than in submerged leaves. Wientjes et al. (2013) explicitly demonstrated that low light-acclimated *Arabidopsis thaliana* plants show low PS II efficiency inferred from F_v/F_m ratio. Higher photosynthetic efficiency (measured in terms of PS II activity) in chloroplasts of floating leaves compared to submerged leaves also clearly depicts prevalence of efficient biochemical reactions mediated by enzymes that oxidize NAD(P)H and make $NAD(P)^+$ available for photosynthetic electron transport. Both floating and submerged leaves showed normal Chl *a* fluorescence transient—the so-called OJIP transient, where the O level is F_o and the P level is F_m (Fig. 3b). F_o of submerged leaves was ~21 % higher than of floating leaves. A higher F_o value recorded in submerged leaves could be due to (i) low Chl a/b ratio (Table 2), which is indicative of larger antenna size; and/or (ii) large number of inactive reaction centers. Large antenna size and low Chl *a*/*b* ratio of submerged leaves corresponds to shade adapted feature of these leaves (Lichtenthaler and Burkart 1999; Puthur and Pardha-Saradhi 2004; Puthur et al. 2013). Light intensity, during growth of plants, has a large effect on the antenna size of PS II. In low light, the antenna size increases to promote increase in absorption cross section, at the cost of a lower PS II efficiency and, in contrast, high light-acclimated plants show smaller PS II antenna size with highly efficient PS II operation (Ballottari et al. 2007; Wientjes et al. 2013; Croce and Amerongen 2014). Accordingly, we suggest that lower PS II efficiency (inferred from Fv/Fm) together with larger antenna size and low Chl *a*/*b* ratio is an ecophysiological adaptation exhibited by submerged leaves under low light environment. It is known that shade adapted leaves are highly sensitive to photoinhibition (Oquist et al. 1992; Puthur and Pardha-Saradhi 2004; Puthur et al. 2013). We note that all Chl *a* fluorescence transient data (e.g., higher O–J and I–P phases and the F_v/F_m ratio) and their analyses (Fig. 3b) agree with the conclusion that photosynthetic efficiency of floating leaves is significantly higher over that of the submerged leaves. However, the J–I rise in Chl *a* fluorescence transient of submerged leaves was significantly higher than that of the floating leaves suggesting substantial variation in structural/functional aspects of components/complexes involved in the flow of electrons from the plastoquinone pool to the PS I acceptor side. Higher photochemical and electron transport activities in floating leaves and their isolated chloroplasts over that of submerged leaves of *P. nodosus* are in agreement with the earlier findings of Ryan (1985) where cells isolated from floating leaves of *P. nodosus* showed superior CO_2 fixation potential than the cells from submerged leaves.

Potential of floating leaves to withstand photoinhibitory damage

Although both floating and submerged leaves showed decline in PS II activity on exposure to high PFD, the degree of loss was sharp in submerged leaves (~54 % decline within 30 min) than in the floating leaves (~13 and 38 % decline after 30 and 180 min, respectively) (Fig. 3a). Likewise, while chloroplasts from submerged leaves showed decline in PS II activity at PFD > 230 µmol photons m^{-2} s^{-1}, that of floating leaves retained V_{max} even at PFD of 920 µmol photons m^{-2} s^{-1} (Fig. 1). Sensitivity of PS II-mediated photoreactions in terrestrial/aquatic plant species to high PFD is well known (Demmig and Björkman 1987; Pardha-Saradhi et al. 2000; Sharmila et al. 2009; Shabnam and Pardha-Saradhi 2013). The potential of

floating leaves to withstand photodamage is also evident from data on Chl *a* fluorescence transients. Upon exposure to high light (for 60 min), submerged leaves failed to exhibit OJIP fluorescence transient, while floating leaves retained polyphasic nature of Chl *a* fluorescence transient (Fig. 3b). Susceptibility of submerged leaves to photoinhibition has been earlier reported in submerged hydrophytes (Hussner et al. 2010) as well as in heterophyllous hydrophytes (Kordyum and Klimenko 2013).

Using paramagnetic fusinate particles for sensing O_2, Ligeza et al. (1997) found differences in oxygenation of leaves placed in water-filled cell (analogous to submerged leaves) and leaves exposed to air (partly analogous to floating leaves). According to their findings, neither illumination nor dark respiration caused any apparent change in oxygen concentration in the interior of leaves exposed to air. This was reported to be due to relatively high permeability of thylakoid membranes for O_2, (Ligeza et al. 1998; also see Ligeza et al. 1994). Higher sensitivity of submerged leaves of *P. nodosus* to photoinhibition compared to floating leaves might be due to elevated levels of oxygen, albeit to a very small extent, due to restricted diffusion of O_2 from submerged leaves in water compared to floating leaves. Elevated levels of O_2 in chloroplasts can stimulate formation of ROS, i.e., superoxide anion radical and hydrogen peroxide (Ligeza et al. 1998).

Variation in ultrastructure of chloroplasts of floating and submerged leaves

Ultrastructural investigations revealed distinct variation in chloroplasts and other cell components between floating and submerged leaves. Chloroplasts of submerged leaves were distinct from those of floating leaves; they had (i) larger number of grana with higher number of thylakoids per granum; (ii) lesser and smaller starch grains; and (iii) a number of distinct plastoglobuli (Figs. 4, 5). The presence of larger number of grana and/or higher number of thylakoids per granum is a well-known shade adaptive feature (Puthur and Pardha-Saradhi 2004; Anderson et al. 2008; Puthur et al. 2013). In contrast, chloroplasts of floating leaves had numerous large starch grains indicative of proficient photosynthetic efficiency.

Further, ultrastructural investigations revealed the presence of (i) a larger number of mitochondria and a higher ratio of mitochondria to chloroplast; and (ii) mitochondria in close vicinity of chloroplasts in floating leaves compared to that in the submerged leaves (Figs. 4, 5), indicating a possible "cross talk" between chloroplasts and mitochondria. It is likely that the higher mitochondria to chloroplasts ratio and cross talk/coordination between these organelles may have an important role in imparting tolerance to photoinhibition in floating leaves over the submerged leaves.

Potential of mitochondria to impart tolerance against photoinhibition

It is well established that plants exposed to abiotic stress including high PFD-induced stress show significant impact on their reducing equivalents, in particular a distinct decrease in $NAD(P)^+$/NAD(P)H ratio (Raghavendra and Padmasree 2003; Prasad and Pardha-Saradhi 2004; Sharmila et al. 2008), which invariably promotes generation of reactive oxygen species (Sharmila et al. 2008). Plants have evolved various mechanisms to tackle excess redox-equivalents, in particular NAD(P)H (Raghavendra and Padmasree 2003; Sharmila et al. 2008; Yoshida et al. 2008; Biel and Nishio 2010; Potters et al. 2010). One such important strategy is the potential of mitochondria to appropriately regulate redox-equivalents, especially, NAD(P)H/$NAD(P)^+$ (see Araujo et al. 2014; Taniguchi and Miyake 2012). Appropriate modulation of redox-equivalents is known to significantly curtail photoinhibitory damage (Yoshida et al. 2008, 2011a, b; Foyer and Noctor 2012).

Electron transport activities (NADH → O_2; Succinate → O_2) in isolated mitochondria of floating and submerged leaves were ~50 and 94 % sensitive to CN^-, respectively. However, electron transport activities of isolated mitochondria of floating leaves were ~45–50 % sensitive to SHAM, which is a known inhibitor of CN-sensitive alternative oxidase pathway (Elthon and Mcintosh 1987), while that of submerged leaves were virtually insensitive to SHAM. These results have convincingly established the prevalence of alternative oxidase-mediated CN-resistant pathway in the mitochondria of floating leaves.

In contrast to ~90 % decline in PS II activity in submerged leaves, floating leaves showed only ~35 % decline upon 180 min exposure to high PFD (Fig. 6). Inhibitors of CN-sensitive cytochrome oxidase pathway (CN^-) and CN-resistant alternative oxidase pathway (SHAM) accelerated photodamage (decline in PS II activity) drastically in floating leaves but only marginally in submerged leaves clearly establishing the significance of these mitochondrial electron transport pathways in imparting tolerance to floating leaves against photoinhibition (Fig. 6). It is well established that electron transport pathways modulate NAD(P)H/$NAD(P)^+$ ratio. Although, it has been established that CN^- inhibits photosynthetic electron transport, by inhibiting plastocynanin and therefore blocking flow of electrons between cytochrome bf complex and PS I (Berg and Krogmann 1975), the concentration of CN^- that is required to bring about inhibition of plastocyanin is as high as 30 mM (Ort et al. 1973); this is much higher than that required for inhibiting cytochrome c oxidase which is as low as 1 mM (Bartoli et al. 2000).

These findings and conclusions are consistent with the ultrastructural findings that have revealed the presence of not only a higher ratio of mitochondria to chloroplast, but also the presence of mitochondria in close vicinity of chloroplasts in the cells of floating leaves compared to that of submerged leaves. However, the potential of SHAM to accelerate photoinhibitory damage in floating leaves was significantly higher than that caused by CN^- (Fig. 6), indicating that alternative oxidase pathway plays a vital role in indirectly modulating the level of redox-equivalents, thus ensuring ready recycling of $NADP^+$ for effective photosynthetic electron transport. These results further establish important role of mitochondria in imparting tolerance to floating leaves against photoinhibition.

A comparison of the findings on (i) photosynthetic electron transport and fluorescence measurements; (ii) ultrastructural investigations; and (iii) photoinhibitory studies in the presence of mitochondrial electron transport inhibitors, presented in this paper, has unequivocally revealed the pivotal role of mitochondria in protecting photosynthetic machinery of floating leaves against photoinhibition.

Conclusions

Our findings have clearly established that (i) floating leaves of *P. nodosus* exhibit higher photosynthetic efficiency and higher potential to withstand photoinhibitory damage compared to the submerged leaves; (ii) floating leaves possess large number of mitochondria, especially in close vicinity of chloroplasts, than submerged leaves; (iii) mitochondria of floating leaves possess alternative oxidase pathway while that of submerged leaves lack the same; (iv) mitochondrial cytochrome c oxidase and alternative oxidase pathway play an important role in modulating redox-equivalents and indirectly ensuring desired levels of $NADP^+$ for effective photosynthetic electron transport; and (v) mitochondria play an important role in protecting photosynthetic machinery of floating leaves against high PFD-induced photoinhibitory damage. We conclude that the cross talk between chloroplasts and mitochondria play a pivotal role in apt modulation of redox-equivalents for protecting photosynthetic machinery in chloroplasts of floating leaves of *P. nodosus* against photodamage.

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