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ORIGINAL ARTICLE

Structural and functional alteration of photosynthetic apparatus in rice under submergence

Debabrata Panda[1,2*] and R. K. Sarkar[1]

[1]*Division of Biochemistry, Plant Physiology and Environmental Sciences, Central Rice Research Institute, Cuttack-753 006, Orissa, India.*

[2]*Present address: Rubber Research Institute of India, Tura, Meghalaya-794 001, India.*

[*]*Tel: 91-3651-232413; Fax: 91-3651-232413*

[*]*E mail: dpanda80@gmail.com*

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Submergence induced alteration of photo-system II (PS II) structure and function was probed using fast O-J-I-P chlorophyll a fluorescence transient and CO_2 photo-assimilation rate. Submergence resulted in an inhibition of CO_2 photo-assimilation rate and reduction in leaf chlorophyll content in rice but the decrease was more in submergence susceptible (IR 42) and avoiding type (Sabita) cultivars compared to the tolerant (FR 13A) one. Quantification of the chlorophyll a fluorescence transients (JIP-test) revealed large cultivar differences in the response of PS II to submergence. The kinetics of chlorophyll a fluorescence transient showed complex changes in the magnitude and rise of O-J, J-I and I-P phases of fluorescence rise. Due to submergence both donor and acceptor sides of PS II were damaged and electron transport perturbed, which resulted in the fall of CO_2 photo-assimilation rate. Submergence also affected overall grouping probability (P_G) or energetic connectivity between PS II antennae. The fall of P_G was greater in submergence susceptible and avoiding types of cultivar compared to the tolerant one. These indicate that chlorophyll fluorescence can be used to know the sensitivity of rice to submergence stress.

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Key words: Chlorophyll fluorescence; Photo-system II; Rice; Submergence

Exposure to complete submergence by flash flooding is a major production constraint and affects 34 million hectares of rainfed lowland rice areas in South and South-East Asia (Sarkar et al., 2006). The adverse effect of submergence is results of various inter related factors such as limited gas diffusion, reduced irradiance and decrease in membrane barrier function (Drew, 1997), which are just few of the factors that slow down photosynthesis during submergence. The photosynthetic response of rice plant and its photosynthetic apparatus to submergence remains far from being understood so far. *In vivo* chlorophyll fluorescence has been used frequently in the past as a convenient and non-intrusive method to determine the tolerance of different species to different environmental condition including submergence (Dudeja and Chaudhary, 2005; Strauss et al., 2006; Pietrini et al., 2005). Fluorescence parameters derived by the theory of fluxes have been suggested to describe changes of absorbed, dissipative, trapping and electron transport fluxes (Lazar, 1999; Force et al., 2003). The analysis of these parameters named JIP test is

performed under typical fluorescence induction from the basal emission Fo (O) to a maximum emission Fm (P) through two intermediate steps i.e. J and I (Strasser et al., 1995). The fluorescence induction has a typical sigmoidal shape evidencing three main phases O-J, J-I and I-P. During this O to J phase mainly single turn over events with respect to Q_A reduction occurs (photochemical phase, light intensity dependent); J to I to P, reduction of PQ pool (non-photochemical phase). A satisfactory interpretation relating to all phases to specific photochemical event is not yet available especially under submergence stress in rice. Hence, the analysis of fluorescence induction could be considered as an interesting complement to the information obtainable by the various classical and well investigated fluorescence parameters like Fo, Fm, Fv/Fm, N, Vi, Vj etc (Force et al., 2003).

The main focus of the present study is to investigate the impact of submergence on the function of PS II in rice. In this study we have used fast chl a fluorescence transient, the polyphasic O-J-I-P rise to probe structural and functional alteration of PSII in rice under submergence to unravel the mechanism that leads to identify the possible selection criteria taking three rice cultivars, which exhibit varying degree of tolerance to submergence.

MATERIALS AND METHODS

Plant material and growth conditions:

The experiment was conducted on three *Indica* rice [*Oryza sativa* (L.)] cultivars with different response to submergence namely, FR 13A (tolerant), IR 42 (susceptible) and Sabita (avoiding type). Seeds were sown directly in earthen pots containing two kg of farm soil and farmyard manure (3:1). Each pot was supplied with 80 mg urea, 192 mg single super phosphate (P_2O_5) and 70 mg murate of potash (K_2O). Ten days after germination, seedlings were thinned and five plants per pot were maintained. Twenty one-day-old seedlings were completely submerged for 10 days in a concrete tank under 110 cm depth of water. One more set kept out side under normal condition served as control. The pH of the floodwater was alkaline (7.2-7.5). The temperature varied between 25.2 to 31.4 ^oC during the study period. Light intensity at 60 cm depth of water was only 39.2 - 44.9 % of that of the surface. The oxygen concentrations were 2.32 - 3.45 mg L^{-1} at 06:30 h and 5.15 - 7.48 mg L^{-1} at 17:30 h.

Leaf CO_2 photo-assimilation rate and chlorophyll estimation:

Measurements of CO_2 photo-assimilation were made on the fully expanded leaves of five different plants within 30 minutes at the end of submergence treatment using an open system photosynthetic gas analyzer (PP Systems, USA) under normal ambient environmental conditions. The second and third leaf from the top was selected and kept inside the chamber under natural irradiance until stable reading was recorded. The chlorophyll was estimated following (Sarkar, 1998) as modified by (Porra, 2002).

Chlorophyll fluorescence measurements:

Chlorophyll fluorescence was measured using a Plant Efficiency Analyzer, Handy PEA (Hansatech Instruments Ltd., UK). The chlorophyll fluorescence transients were induced by a red light of 3000 µE m^{-2} s^{-1}, and recorded from 10 µs up to 1 s. All measurements were done on fully dark-adapted attached leaves. For each treatment, the Chlorophyll a fluorescence transients of 12 individual leaves were measured. Different chlorophyll fluorescence parameters were analyzed by the so-called JIP-test (Strasser et al., 1995). The

JIP- test represents translation of original data to biophysical parameters that quantify the energy flow through PS II (Table 1).

Statistical analysis:

Differences between various photosynthetic parameters were compared by ANOVA using IRRISTAT (International Rice Research Institute, Philippines) software's least significant difference (LSD<0.05), as this is a good test for determining whether means were significantly different.

Table 1. The chlorophyll fluorescence parameters using data extracted from the fast fluorescence O-J-I-P transient.

Fo = Minimal fluorescence- when all the reaction centers are open or in oxidized state

Fm = Maximal fluorescence- when all the reaction centers are closed or in reduced state

Fj = Fluorescence intensity at the J-step (at 2ms)

Fi = Fluorescence intensity at the I-step (at 30ms)

Mo = 4 .(F_{300} - Fo) / (Fm - Fo)

Sm = Area / (Fm - Fo)

Vj = (F_{2ms} - F_0) / (Fm - Fo)

Vi = (F_{30ms} – $F_{50\mu s}$)/(Fm-$F_{50\mu s}$)

N = Sm . Mo.(1/Vj), turn over number

Area = Area between fluorescence curve Fo and Fm

Fv/Fm =(1-Fo/Fm), Maximum photochemical activity of PS II

ETo/CSo = (Fv/Fm).(1-Vj).Fo

RC/CSo =(Fv/Fm).(Vj/Mo).Fo

DI_0 / CSo = (ABS / CS) - (TR_0 / CS)

Performance index (PI_{ABS})= (RC/ABS). [(Fv/Fm)/1-(Fv/Fm)]. [(1-Vj)/(1-(1-Vj)]

P_G = Grouping probability of PSII or energetic connectivity of PS II antenna

$$P_G = \frac{(W_{E,\,100\,\mu s} - W_{100\,\mu s})}{W_{100\,\mu s}\,(1 - W_{E,\,100\,\mu s}\,V_J)\,V_J} \cdot \frac{Fo}{(Fm - Fo)}$$

Where

$$W_{E,\,100\mu s} = 1 - (1 - W_{300\,\mu s})^{1/5}$$

$$W_{100\mu s} = \frac{F_{100\,\mu s} - F_{50\,\mu s}}{F_{2\,ms} - F_{50\,\mu s}} \quad \text{and} \quad W_{300\mu s} = \frac{F_{300\,\mu s} - F_{50\,\mu s}}{F_{2\,ms} - F_{50\,\mu s}}$$

Table 2. F value of different chlorophyll fluorescence parameters in rice under different days of submergence as calculated by ANOVA

Source of variation	df	Vj	Vi	N	Area	Pi	ETo/CSo	RC/CSo	DIo/CSo
Replication(R)	2	<1	3.10ns	1.44ns	1.05ns	<1	1.34	<1	3.26ns
Variety(V)	2	5.25*	2.07ns	11.38**	52.15**	26.88**	20.09**	28.42**	94.01**
Treatment(T)	5	16.34**	10.78**	46.60**	97.38**	63.21**	132.84**	48.86**	58.57**
V*T	10	2.44*	2.63*	2.86*	2.61**	2.26*	7.45**	4.74**	15.63**
CV (%)		9.7	7.3	12.2	13.8	21.4	9.5	8.5	7.6

** = Significant at p<0.01 level; *= Significant at p<0.05 level; ns= Non significant; df= degrees of freedom

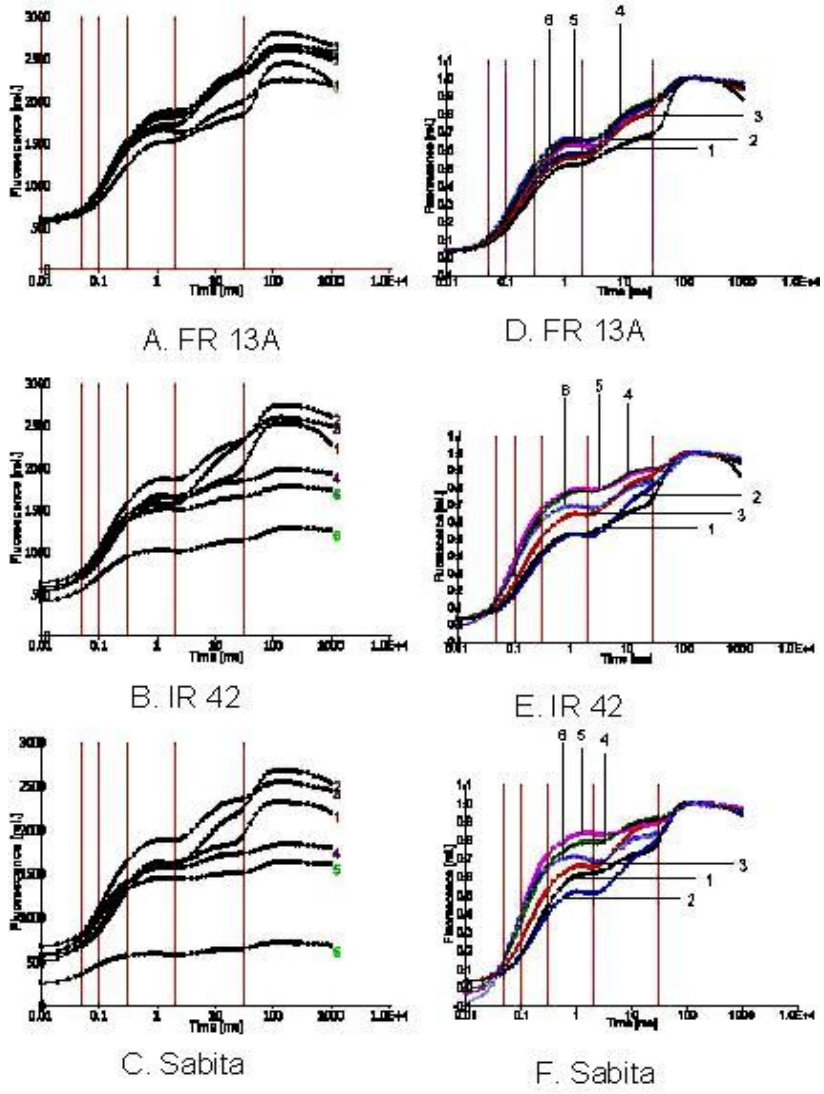

Figure 1. Polyphasic chlorophyll a fluorescence transients of FR 13A (tolerant), IR 42 (susceptible) and Sabita (submergence avoiding type) under control (non-submergence) and after different periods of submergence. The vertical lines represent the fluorescence intensity at a particular time spans. The first, second, third, fourth and fifth lines from left position demonstrate the fluorescence intensity at 50 µs, 100 µs, 300 µs, 2 ms, and 30 ms, respectively. The lines meet at the fluorescence curve at 50 µs, 2 ms and 30 ms are known as O-, J- and I-phase, respectively. The highest peak in the curve was designated as maximal fluorescence (P=Fm). 1, 2, 3, 4, 5 and 6 = control, 2, 4, 6, 8 and 10 d after complete submergence. A, B, C = normal Chl a fluorescence transients. D, E, F = Chl a fluorescence transients normalized at Fo and Fm levels.

Table 3. Changes of minimum (Fo), maximum (Fm) fluorescence and Potential photochemical activity of PS II (Fv/Fm) due to submergence; the unit of each parameter is arbitrary. DAS= days after submergence.

DAS	Fo			Fm			Fv/Fm		
	FR 13A	IR 42	Sabita	FR 13A	IR 42	Sabita	FR 13A	IR 42	Sabita
0	508	520	500	2440	2520	2310	0.791	0.792	0.788
2	493	510	502	2608	2732	2669	0.806	0.813	0.810
4	530	554	597	2799	2592	2544	0.810	0.786	0.763
6	514	489	594	2606	1978	1833	0.802	0.752	0.661
8	494	537	535	2644	1776	1632	0.813	0.695	0.691
10	501	451	457	2245	1292	1038	0.775	0.620	0.524
LSD *p<0.05		74			378			0.0638	

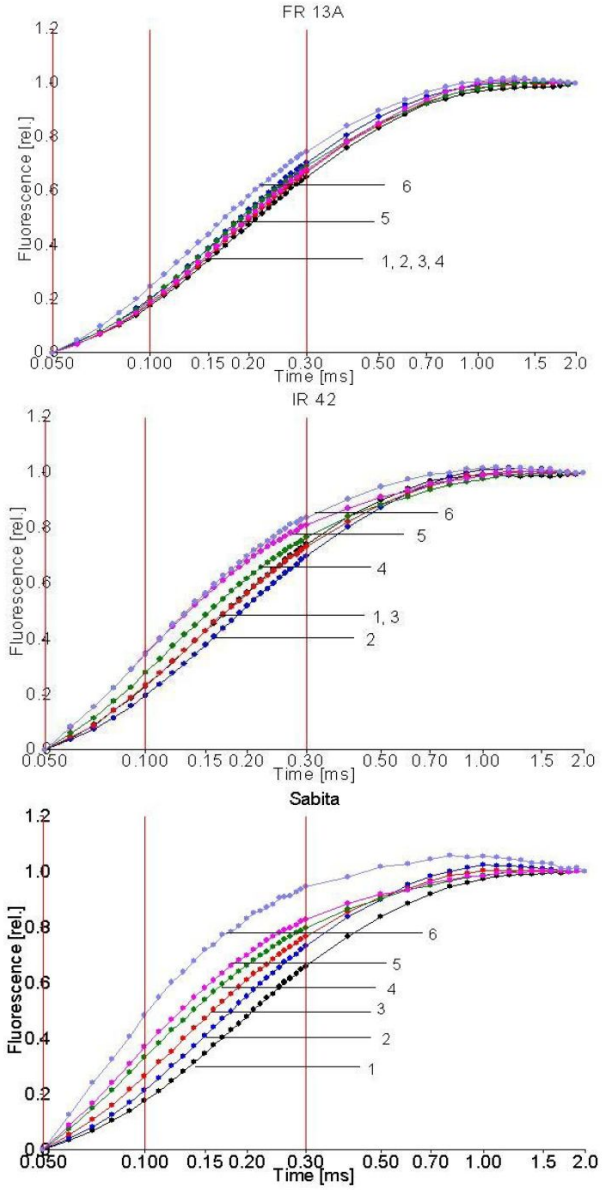

Figure 2. Changes in the shape of the fluorescence transients normalized at Fo and Fj designated as K-step in three rice cultivars under different days of submergence. 1, 2, 3, 4, 5 and 6 = control, 2, 4, 6, 8 and 10 d after complete submergence.

Table 4. Changes of leaf chlorophyll content (mg g-1 fresh wt.) and CO_2 photo-assimilation rate (µmol CO_2 m^{-2} s^{-1}) in rice under different days of submergence. DAS= days after submergence.

DAS	Chlorophyll			CO_2 photo-assimilation rate		
	FR13A	IR42	Sabita	FR13A	IR42	Sabita
0	1.6	1.40	1.37	21.7	19.3	18.8
2	1.61	1.39	1.37	9.0	4.5	4.3
4	1.48	1.11	1.18	5.3	4.3	3.0
6	1.34	0.99	1.02	6.2	3.5	3.4
8	1.14	0.58	0.86	3.5	0.7	0.6
10	1.07	0.36	0.36	3.1	0.6	0.4
LSD *p<0.05		0.95			1.18	

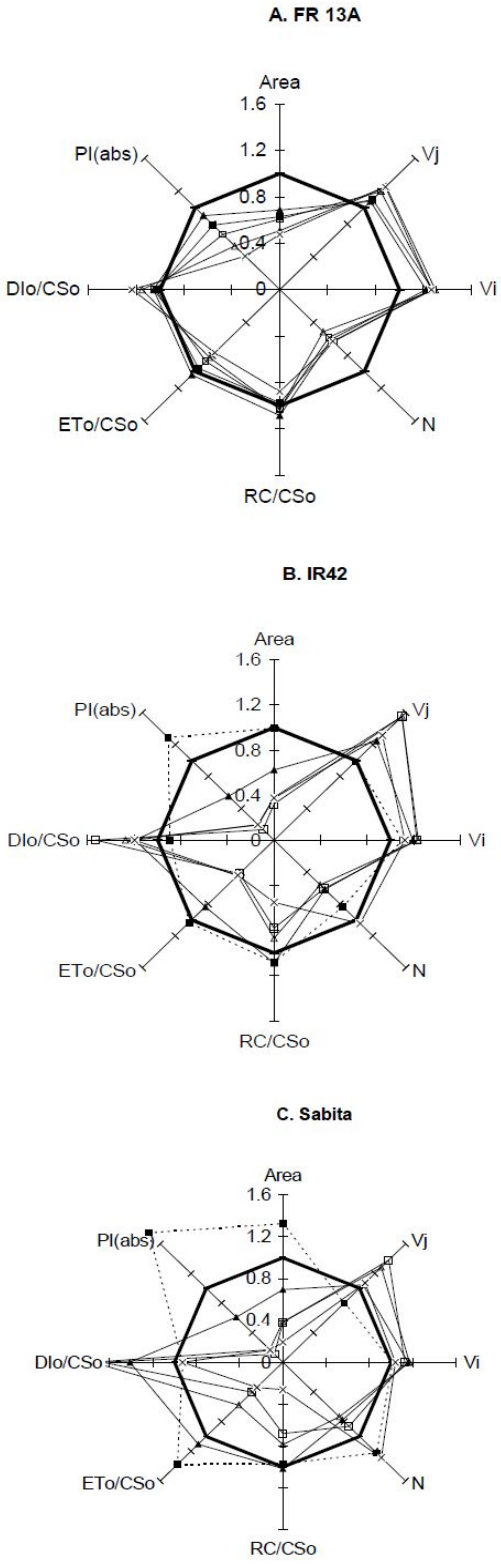

Figure 3. Spider type visual plot showing quantitative extend of changes in various fluorescence parameter during different days of submergence. The black circle with radius 1 represents non-submerged control condition. ....■..., –▲–, –Δ–, –□–, –x– = 2, 4, 6, 8, and 10 d after submergence, respectively. A =FR 13A, B = IR 42, C = Sabita.

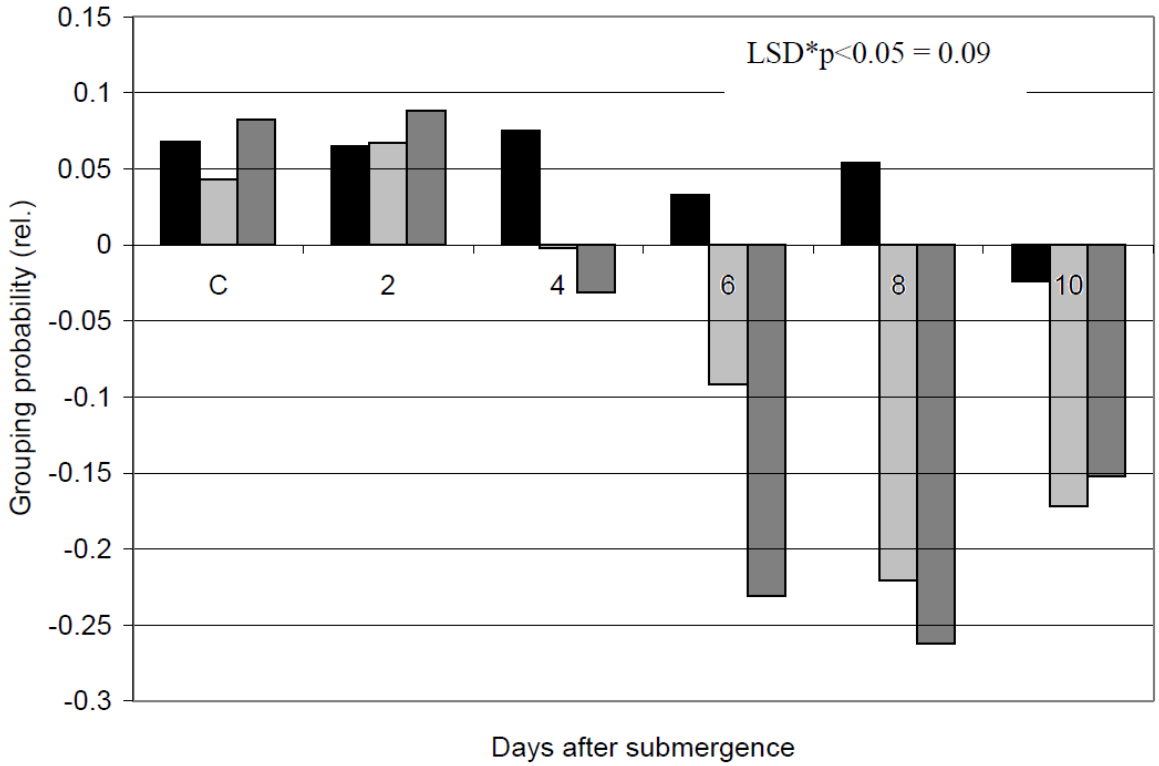

Figure 4. Changes of grouping probability (P_G) or energetic connectivity in rice leaves under different days of submergence. C = non-submerged control condition. ■ = FR 13A, ▒ = IR 42, ▓ = Sabita.

RESULTS AND DISCUSSION

Survival percentage:

The extent of visible injuries caused due to submergence is commonly used as an indicator their sensitivity. In this experiment three, cultivars gave distinctly different response to submergence in terms of visible symptoms and survival. Tolerant cv. FR 13A showed 100 % survival after 10 d of submergence whereas it was less than 15 % in IR 42 and Sabita. There was no mortality even in susceptible cultivar after 6d of submergence (data not shown).

Test of significance:

The test of significance of different chlorophyll fluorescence parameters, chlorophyll content and CO_2 photo-assimilation rate are presented in table 2. The differences were non- significant in the case of replication whereas in most of the cases the differences in the case of variety, treatment and variety x treatment interactions were highly significant.

Analysis of chlorophyll a fluorescence O-J-I-P transients:

The present investigation characterizes the main effect of submergence on the function of PS II in tolerant and susceptible cultivars of rice as observed by the chl a fluorescence induction kinetics (Fig. 1). All oxygenic photosynthetic organisms investigated so far using this method showed the polyphasic rise with the basic step O-J-I-P and minor differences among the different phenotypes (Strasser et al., 2000). The shape of the O-J-I-P transient has been found to be very sensitive to stress caused by changes in different environmental conditions, e. g. light intensity, temperature, drought, ozone elevation and chemical influences (Zhang and Gao, 1999;

Prakash et al., 2003; Govindachary et al., 2004). Under complete submergence the shape of the O-J-I-P transient also changed in rice leaves with decrease in maximal fluorescence (P=Fm) intensity that resulted in lowering of variable fluorescence levels. The decrease was more pronounced in susceptible (IR 42) as well as submergence avoiding (Sabita) types of cultivar compared to the tolerant (FR 13A) cultivar. The partial loosening of sigmoidal shape of O-J phase and the large suppression of P step during submergence in IR 42 and Sabita was attributed to some difference in the composition and organization of PS II antenna and reaction center induced by submergence; they most likely reflected the changes in PS II grouping (Strasser and Stirbet, 2001). Complete suppression of P step especially after 6, 8 and 10 d of submergence indicated that the slower electron donation from PS II together with the higher unbalance between a stable PS I activity and a damage of PS II occurred in IR 42 and Sabita (Srivastava et al., 1997; Stribet et al., 1998).

All transients are normalized at the O and P step in order to reveal changes between these two extrema more clearly (Fig.1D, E, F). The O-J-I-P fluorescence transient reflects the filling of the electrons acceptor side of PS II (Q_A, Q_B, and PQ pool) with electron from the donor side (Strasser and Govindjee, 1992). Submergence stress resulted in a much larger increase in the fluorescence intensity at 2 ms (J-peak) in IR 42 and Sabita than in FR 13A. An increase of fluorescence rise at J step usually interpreted as the indication in the accumulation of reduced Q_A^- pool (Strasser et al., 1995), possibly due to the decrease of electron transport beyond Q_A- or acceptor side of PS II (Haldimann and Strasser, 1999). These findings suggest that PS II electron transport was inhibited to a lesser extent in FR 13A than IR 42 and Sabita. The rapid chlorophyll rise from the O to J step is photo-chemically controlled and J-I rise is restricted by thermal reactions (Govindachary et al., 2004). The donor side reactions of PS II control the fluorescence quenching during J- I phase. Any abiotic stress that perturbs the structure-function relations of the oxygen evolving centre (OEC), influences the rates of oxygen evolution which increases the quenching of fluorescence rise at J- or I- steps. Therefore, the fluorescence rise at J- and I-steps envisages structural and functional integrity of OEC and is a useful indicator of water splitting activity (Govindachary et al., 2004). The increase of fluorescence rise between J and I steps under submergence was negligible in FR 13A compared to the IR 42 and Sabita (Fig. 1D, E, F).

Under various stress conditions, an early fast step K was found at 200 to 300 µs leading to a polyphasic transient of the type O-K-J-I-P. Generally, the K-step is usually 'hidden' in the O-J rise (Prakash et al., 2003; Guisse et al., 1995; Strasser et al., 2004). Apparently, the K-step was not noticed visualizing the normal raw fluorescence curves under different days of submergence (Fig. 1). In order to compare the amplitude of the K-step during complete submergence, the fluorescence curves were normalized between Fo and Fj (Jiang et al., 2006). It showed that the K-step appeared under submergence and it was more prominent especially in submergence susceptible cultivars (Fig. 2). The appearance of K-step may be influenced by factors such as S-state of OEC, the acceptor side of PS II, the connectivity among PS II units, etc (Jiang et al., 2006). Normalization at Fo and Fj excluded the effect of acceptor side of PS II. Thus, the appearance of K-step might be attributed to the inactivation of OEC (Lazar, 1999) and loss of connectivity among PS II. An increase in J-step (Fig. 1) and decrease of

grouping probability (Fig. 4) observed in this investigation also supported this contention.

Changes in the parameters of JIP test:

The maximal photochemical efficiency (Fv/Fm) ratio did not significantly decrease even after 10 days of submergence in tolerant cultivar whereas the differences were significantly lower after 6 days of submergence in susceptible and avoiding types of cultivars (Table 3). The change in Fo values was significant only after 10 days of submergence especially in susceptible cultivar. The values of Fm decreased significantly after 10 days of submergence in tolerant cultivar FR 13A, whereas the values of it started to decline after 6 days of submergence in other two cultivars. The quenching of Fv/Fm indicates more extensive damage to reaction center so that charge recombination is prevented or may be decrease in the activity of water splitting enzymes (Waldhoff et al., 2002; Lazar, 2006).

No significant increase in Vi and Vj was observed during submergence in FR 13A but in IR 42 and Sabita the values of Vj and Vi started to increase after 4 days of submergence (Fig. 3). The increase of Vj and Vi were used as a probe for the inhibition of the electron transport at the acceptor side of the PS II (Lu and Zhang, 1999; Chen et al., 2004). On the basis of this parameter significant increase of Vj and Vi in IR 42 and Sabita indicated that submergence strongly reduce the electron transport at the acceptor side of PS II. The Area above the fluorescence curve between Fo and Fm is proportional to the pool size of electron acceptor on the reducing side of the PS II, which includes Q_A, Q_B, and PQ (Joliot and Joliot, 2002) is highly sensitive to submergence (Fig. 3). Submergence significantly decreased the plastoquinone pool and number of times of Q_A- reduction or turn over number (N) in all the genotypes even after 2 d of submergence (Fig. 3). The decrease was more in the susceptible and avoiding type cultivars than the tolerant one. The phenomenological energy fluxes like electron transport per cross section (ETo/CSo) represents the re-oxidation of reduced Q_A via electron transport over a cross-section of active and inactive reaction center whereas RC/CSo represents the active PS II reaction center per exited cross-section. The greater values of ETo/CSo and RC/CSo designate better structural and functional integrity of PS II. Both ETo/CSo and RC/CSo decreased due to submergence. The decrease of electron transport might be due to the inactivation of acceptor side of the PS II and the decrease in number of active reaction centre in a PS II cross-section (RC/CSo) was due to the damage of donor side of PS II. The initial stage of photosynthetic activity of a reaction center complex is regulated by three functional steps namely absorption of light energy, trapping of the excitation energy and the conversion of excitation energy to electron transport. Combining these three steps a multi parametric expression of over all performance index (PI) of PS II was calculated. Submergence significantly decreased the PI in all the cultivars. However, tolerant cultivar always maintained higher values of this parameter compared to susceptible and avoiding cultivars. Under submergence both donor and acceptor sides got damaged. Tolerant cultivar to some extent prevented the damage as evident by higher area, ETo/CSo, RC/CSo and performance index.

Grouping probability:

The JIP test is meant to calculate the over all P_G or the energetic connectivity between the PS II antennae. Grouping probability accounts for all possible way of energetic communication of neighboring PS II core antenna. The connectivity between the PS II core antennae influences the

function of photosynthetic apparatus both quantitatively enhancing the electron transport and qualitatively enhancing the stability (Strasser and Stirbet, 2001). In IR 42 and Sabita the value of grouping was negative after 4 days of submergence whereas FR 13 maintained their connectivity up to 8 days of submergence (Fig. 4). Thus, the tolerant variety maintains its energetic connectivity between the PS II antennae more than the susceptible cultivar.

CO_2 photo-assimilation rate:

Submergence significantly decreased the leaf chlorophyll content after 4 days of submergence in IR 42 and Sabita whereas in FR 13 A the decrease was significant only after 6 day (Table 4). Reduction of chlorophyll during submergence is common in rice (Sarkar et al., 1996; Das et al., 2001). Significant decrease of CO_2 photo-assimilation rate was observed in all the genotypes after 2 d of submergence compared to the control plant. The decrease was less in FR 13A compared to IR 42 and Sabita. The change in CO_2 assimilation under flooding was attributed to the stomatal and non stomatal limitation (Pezeshki, 2001), which include reduction of RuBP generation, down regulation of RuBP carboxylase and ethylene mediated chlorosis (Jackson and Ram, 2003; Ella et al., 2003). The present study envisages that the decrease in CO_2 photo-assimilation rate under complete submergence is a complex phenomenon. When the submergence stress was not so severe i.e. after 2 days of submergence, the decrease in CO_2 photo-assimilation rate could be attributed to the stomatal limitation. With progression of submergence stress, the functional and structural damage suffered by the photosynthetic apparatus might be responsible for the fall in CO_2 photo-assimilation rate as evident from the different chlorophyll fluorescence characteristics (Table 2; Fig. 1-3). Dysfunctions at donor and acceptor sides of PS II were greater in susceptible cultivars and hence, the fall of CO_2 photo-assimilation was also greater in these types of cultivars (Table 4).

These results showed that chlorophyll a fluorescence parameters provide a non-invasive and rapid method for investigation of structural and functional alteration of PS II. Due to submergence both donor and acceptor sides of PS II were damaged, electron transport perturbed, connectivity between the antennae of PS II lost which resulted in the fall of CO_2 photo-assimilation rate. The structural and functional damage of PS II was more prominent in susceptible cultivars. Fluorescence parameters differentially changed in the three contrasting rice cultivars during the progression of submergence treatment and hence, can be used as a rapid screening technique to identify submergence tolerant rice cultivar in the field or in green house.

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